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Noura El Jamal

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par

Noura EL-JAMAL

Le 20 Décembre 2012

Titre:

**HOMEOSTASIE DE L'INTESTIN ET DE LA PEAU:
CIBLES ET MODELES POUR ETUDIER L'INFLAMMATION ET LA CARCINOGENESE.**

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No one achieves success without acknowledging the help of others

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LIST OF ABBREVIATIONS

15d-PGJ2	15-deoxy-D12,14-prostaglandin J2
16:0/18:1GPC	1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine
2Br-C16	2-bromopalmitate
5-ASA	5-aminosalicylic acid
5-HT	5-hydroxytryptamine
ACTH	adrenocorticotrophic hormone
AD	atopic dermatitis
ADMET	absorption, distribution, metabolism, excretion and toxicology
ADRP	adipose differentiation related protein
AIDS	acquired immunodeficiency syndrome
AP-1	activator protein-1
APC	adenomatous polyposis coli
APCs	antigen presenting cells
ATG16L1	autophagy-related protein 16-1
ATP	adenosine triphosphate
BADGE	bisphenol A diglycidyl ether
BOLD	blood oxygenation level dependent
Bp	base pair
CAC	colitis-associated cancer
cAMP	cyclin adenosine monophosphate
CBF	changes in cerebral blood flow
CBV	cerebral blood volume
CCD	charged coupled device
CD	Crohn's disease
CDC	cell division cycle
CDDO	2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid
CDDO-Me	2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid methyl ester
CDK	cyclin-dependent kinase
CHK	serine/threonine-protein kinase

CIN	chromosomal instability
COX-2	cyclooxygenase-2
CRC	colorectal cancer
CREB	cAMP response element-binding protein
CYP	cytochrome P450
CYP7b1	oxysterol 7 α -hydroxylase cytochrome P450-b1
DC(s)	dendritic cell(s)
DHA	docosaheanoic acid
DKK	dickkopf
DMBA	7,12-dimethylbenz[α]anthracene
DPP-IV	dipeptidylpeptidase-IV
DR	direct repeat
DRG-1	developmentally regulated GTP-binding protein 1
Dsh	disheveled
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
EphB	B-type ephrin receptors
ER	estrogen receptor
ERE-luc	estrogen receptor element-Luciferase
ERK	extracellular signal-regulated kinase
ETYA	eicosatetraenoic acid
FA(s)	fatty acid(s)
FABP	fatty acid binding protein
FDA	Food and Drug Administration
FFA	free fatty acids
FIAF	fasting-induced adipose factor
FLuc	Photinus pyralis
fMRI	functional magnetic resonance imaging

FXR	farnesoid X receptor
Fzd/LRP	frizzled/low-density lipoprotein receptor-related protein receptors
GABP α	GA-binding protein α
GATA-3	guanosine adenosine thymidine adenosine 3
GFP	green fluorescent protein
GI	gastrointestinal tract
GIP	glucose-dependent insulinotropic peptide
GLP	glucagon-like peptide
GLP-2R	glucagon-like Peptide-2 Receptor
GLuc	Gaussia luciferase
GLUT	glucose transporter
GPCR	G protein-coupled receptor
GPX	glutathione peroxidases
GREs	glucocorticoid-responsive elements
GRPP	glicentin-related pancreatic polypeptide
GSK-3	glycogen synthase kinase-3
GW1929	(2S)-((2-benzoylphenyl)amino-3-[4-[2-(methylpyridin-2-ylamino)ethoxy]phenyl)-propionic acid
GW501516	2-methyl-4(((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)methyl)sulfanyl)phenoxy)acetic acid
GW7647	2-([4-{2-({cyclohexylamino} carbonyl)[4-cyclohexylbutyl]amino)ethyl}-phenyl]thio)-2-methylpropanoic acid
GW9662	2-chloro-5-nitro-N-phenylbenzamide
HAT	histone acetyl transferase
HB-EGF	heparin binding-epidermal growth factor
HDAC(s)	histone deacetylase(s)
HEET	hydroxyepoxyeicosatrienoic acid
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
HF	hair follicle

HODE	hydroxyoctadecadienoic acid
HpODE	hydroperoxyoctadecadienoic acid
hTERT	human telomerase reverse transcriptase
IAP	inhibitor of apoptosis protein
IBD	inflammatory bowel disease
IE	interfollicular epidermis
IECs	intestinal epithelial cells
IFN	interferon gamma
Ig	immunoglobulin
IGF(s)	insulin-like growth factor(s)
IGRM	immunity-related GTPase family M
IHC	immunohistochemistry
IL	interleukin
ILK	integrin-linked kinase
iNOS	inducible nitric oxide synthase
IP	intervening peptide
IRGM	immunity-related GTPase family M protein
ISCs	intestinal stem cells
ISH	in situ hybridization
iTreg	inducible regulatory T cells
K20	keratin 20
KGF	keratinocyte growth factor
KLF4	krüppel-Like Factor 4
L165041	(4-[3-{4-acetyl-3-hydroxy-2-propylphenoxy}propoxyl]phenoxy) acetic acid
LCM	laser-capture microdissection
LDL	low density lipoprotein
LTB	leukotriene
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MDSC	myeloid-derived suppressor cells

miRNA	micro ribonucleic acid
MK886	3-(1-[p-chlorobenzyl]-5-[isopropyl]-3-tert-butylthioindol-2-yl)-2,2-dimethylpropanoic acid methyl ester
MLN	mesenteric lymph nodes
MMP(s)	matrix metalloproteinase(s)
MPGF	major proglucagon fragment
MRI	magnetic resonance imaging
MSI	microsatellite instability
MyD88	myeloid differentiation 88
NAC	N-acetylcysteine
NAG1	non-steroidal anti-inflammatory drug activated gene 1
NEC	necrotizing enterocolitis
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-kappa B
NIRF	near-infrared fluorescence imaging
NK	natural killer
NO	nitric oxide
NOD	nucleotide oligomerization domain
NOS	nitric oxide synthase
nTreg	natural regulatory T cells
OR	odd ratio
oxoODE	oxidized octadecadienoic acid
p21waf1/cip1	cyclin-dependent kinase inhibitor 1A
PC	prohormone convertase
PCR	polymerase chain reaction
PCS	primary sclerosing cholangitis
PDK1	3-phosphoinositide-dependent kinase-1
PET	positron emission tomography
PG	prostaglandin
PI3K	phosphatidylinositol-3 kinase
PKA	protein kinase A

PKB α /Akt1	Protein kinase B alpha/protein kinase B
PKC α	Protein Kinase C alpha
Pmol/l	picomol per liter
PN	parenteral nutrition
POI	postoperative ileus
PP	protein phosphatase
PPAR(s)	peroxisome proliferator-activated receptor(s)
PPRE	Peroxisome proliferator responsive element
PPRE-luc	Peroxisome proliferator responsive element-luciferase
PRRs	pattern recognition receptors
PTEN	phosphatase and tensin homolog
PTP(s)	protein tyrosine phosphatase(s)
PUFA	polyunsaturated fatty acids
RA	retinoic acid
RLU	relative luciferase units
RLuc	Renilla luciferase
RNI	reactive nitrogen intermediates
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
RXR	retinoid X receptor
SB-219994	3-[4-[2-[N-(2-Benzoxazolyl)-N-methylamino]ethoxy]phenyl]-2(S)-(2,2-trifluoroethoxy)propionic acid
SBS	short bowel syndrome
SCC	squamous cell carcinoma
SCG	sodium cromoglycate
SEMFs	subepithelial myofibroblasts
SG	sebaceous gland
SGLT-1	sodium/glucose cotransporter-1
sIL-1ra	secreted IL-1 receptor antagonist
SPECT	single photon emission computed tomography

SPF	specific pathogen free
STAT3	signal Transducer and Activator of Transcription 3
T0070907	2-chloro-5-nitro-N-(4-ylidyl)benzamide
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
T-bet	T-box expressed in T cells
TG	triglycerides
TGF- β	transforming growth factor- β
Th	helper T cells
TLRs	toll like receptors
TNBS	trinitrobenzene sulfonic-acid
TNF	tumor necrosis factor
TNF-R	TNF-receptor
TPA	tetradecanoylphorbol acetate
TPN	total parenteral nutrition
Treg	regulatory T cells
TZD	thiazolidinedione
UC	ulcerative colitis
UV	ultraviolet
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein
WB	western blot
Wif1	wnt inhibitory factor
WY14643	4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid
XIAP	X-Linked Inhibitor of Apoptosis
α	alpha
β	beta
δ	delta
κ	Kappa
γ	gamma

AT A GLANCE

This work has been performed in the Inserm unit 995 “**Inflammation: Regulatory Mechanisms and Interactions with Nutrition and Candidosis**”, particularly in team 1 “**Digestive Inflammatory Diseases: Pathophysiology and Development of Therapeutic Targets**”. Different themes and approaches have been the subject of intense research in the 995 unit. In particular, this work addresses multiple axes related to the homeostasis of the intestine and the skin. Intestinal and skin physiologies are quite similar as far as the homeostasis in both depends on complex interactions between the microbiota, the epithelium and the host immune system. Diverse regulatory mechanisms cooperate to maintain the equilibrium, and a breakdown in these pathways may precipitate pathological conditions. Hence the title of this thesis: **INTESTINAL AND SKIN HOMEOSTASIS: TARGETING INFLAMMATION AND CARCINOGENESIS**.

First, we addressed the expression pattern of “Glucagon-like Peptide 2 Receptor” (GLP-2R). Glucagon-like peptide 2 (GLP-2) has been recently demonstrated as an intestinal hormone displaying intestinotrophic cytoprotective and neuroprotective properties. The apparent intestinal specificity of GLP-2 properties, unlike other growth factors, renders this peptide attractive for clinical use in conditions of intestinal dysfunction. Notable intestinal actions of GLP-2 analogs have been demonstrated in animal models and they are currently considered in clinical trials for intestinal diseases. Despite the advanced stages of clinical studies evaluating GLP-2 analogs, our knowledge concerning the exact distribution of GLP-2R is for far incomplete. According to our knowledge, no study has ever investigated the expression pattern of GLP-2R receptor along the intestinal tract. Moreover, nothing so far is known about the potential regulation of GLP-2R expression during pathological and inflammatory conditions. Hence, to enhance our knowledge about the potential functions of GLP-2 analogs, a better understanding for GLP-2R expression is considered necessary. We therefore realized a panel of GLP-2R expression in mice tissues and in several human, murine and rat cells lines. Given the therapeutic utility of GLP-2 analogs in intestinal disorders, we investigated the intestinal expression of GLP-2R in inflammatory bowel disease patients and in mice models of chemically-induced colitis.

The second research axis within the context of intestinal homeostasis, deals with the anti-neoplastic role of 5-aminosalicylic acid (5-ASA) and its signaling pathway through peroxisome proliferator-activated receptor gamma (PPAR γ). In fact, epidemiological reports and case control studies have shown that the chronic use of 5-ASA has chemopreventive effects on the development of colorectal cancer (CRC). Furthermore, several studies have demonstrated a potent anti-tumoral role for 5-ASA in animal models of colon cancer. However, the mechanisms sustaining the putative anti-neoplastic property of 5-ASA are still under investigation. On the other hand, PPAR γ recently characterized as 5-ASA receptor, is a nuclear receptor with anti-inflammatory and anti-neoplastic functions. Recently, our laboratory has demonstrated that PPAR γ mediates 5-ASA-induced anti-inflammatory effects in the colon epithelium in mice and human cultures colonic biopsies. In this study, we hypothesized that the anti-neoplastic effects of 5-ASA are as well mediated via PPAR γ . We have employed several *in vitro* and *in vivo* models to test the effect of 5-ASA on cell growth, proliferation and apoptosis. We have also examined the anti-neoplastic role of 5-ASA in three different animal models. The involvement of PPAR γ was regularly controlled by blocking this receptor.

The biological function of PPAR γ is not confined to the gastrointestinal tract; it rather extends to several physiological and pathophysiological conditions, notably in the skin. PPAR γ is a subtype of PPARs (peroxisome proliferator-activated receptors) which play a relevant role in mammalian physiology and are currently believed to represent a major therapeutic target in metabolic, inflammatory, and skin diseases. Of note, PPARs have been shown to be involved in key physiological functions in the skin such as cell proliferation and differentiation, and inflammatory responses; and their malfunctioning has been associated with major disorders. These new functions identify PPARs and corresponding ligands as potential targets for the treatment of various skin diseases. Because of that, several pharmaceutical companies have engaged in research into the identification of selective ligands for each PPAR subtype. However, new drug approval is a lengthy process employing a time frame of several years and spans a vast array of disciplines. Minor delays within individual disciplines may easily add to the cost of drug development and widen the gap

between the discovery and the request of valuable patent exclusivity. Pharmaceutical companies work hard to improve the decision making process and to make project phases more efficient by applying novel technologies. In this study, we have used a combined application of imaging and genetic engineering technology to generate Peroxisome Proliferator-Activated Receptor Responsive Element-Luciferase (PPRE-luc) Reporter Mice. In this mice model, luciferase expression is under the control of a PPAR-inducible promoter in all organs. Using PPRE-luc mice, we have performed *in vivo* selective activation of PPAR subtypes using specific ligands and showed that these reporter mice could provide a powerful tool to verify the effects of drug candidates and to streamline the process of drug development from its preclinical to clinical phases.

This work addressed multiple axes related to the homeostasis of the intestine and the skin with the aim of elucidating the roles of endogenous factors displaying important role in inflammation and carcinogenesis. This study provides as well the basis for pharmacological profiling of drugs targeting skin disorders using a novel reporter mouse.

INTESTINAL HOMEOSTASIS – SECTION 1

Glucagon-Like Peptide 2 Receptor Expression is Not Restricted to the Gastrointestinal Tract and is Modulated by Inflammation

PREFACE

In the gastrointestinal tract, cellular and acellular components such as immune cells, epithelial cells, endothelial cells, extracellular matrices, cytokines, growth factors, adhesion molecules...etc interact with each others in a dynamic continuous fashion. When these interactions occur properly, the result is intestinal homeostasis. Each of these components would represent alone a subject of intense research and investigation. We are particularly concerned by the intestinal hormone Glucagon-like peptide 2 (GLP-2).

GLP-2 is a 33 amino acid peptide, secreted from intestinal endocrine cells in the small and large intestine. The function of GLP-2 was unrecognized for many years. The story starts with the observation that several human subjects with glucagonomas exhibit small bowel growth. In 1996, GLP-2 administration in mice was shown to markedly increase small bowel growth in only a few days, mainly due to hyperplasia of the villus epithelium. Later, the potential therapeutic role of GLP-2 and its complex interplay has begun to be elucidated, particularly in terms of proliferative actions on the gut. Peripheral GLP-2 action is essential for maintaining intestinal homeostasis, improving absorption efficiency and blood flow, and promoting immune defense; and producing efficacy in treatment of gastrointestinal diseases. Thus, GLP-2 is so far characterized as growth factor and anti-inflammatory agent. This has stimulated a considerable interest in the development of GLP-2 analogs. Practically, current clinical studies are revealing promising beneficial effects for GLP-2 analogs in the treatment and/or prevention of an expanding number of intestinal diseases. GLP-2 is therapeutically attractive in diseases to enhance absorptive capacity, restore mucosal health and reduce inflammation. On the other hand, the potential GLP-2 side effects following long-term treatment, notably enhanced risk of carcinogenesis, should not be underestimated. Functional studies have shown that GLP-2 analogs will likely require chronic administration given that their growth-promoting effects in humans and in mice are

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reversed upon withdrawal of treatment. Hence, the therapeutic benefit of GLP-2 analogs is limited by concern that it predisposes patients to gastrointestinal cancers, or their re-occurrence in cancer patients.

Although the development of *in vitro* systems that recapitulate the *in vivo* setting would allow for accelerated discoveries; to date, exhaustive information about the localization of Glucagon-like peptide-2 receptor (GLP-2R) is still lacking. There is general agreement that GLP-2R is predominantly expressed in the gastrointestinal tract, particularly in the stomach and proximal small intestine. However, some studies have reported extra-intestinal expression of GLP-2R with unknown biological significance. The cellular distribution of GLP-2R expression remains controversial, and studies using different techniques have detected GLP-2R-immunoreactive protein or GLP-2R transcript in different cell types in and outside the intestinal compartment. The lack of conclusive studies describing the exact localization of is probably due from one side to the characteristic cell-specific location of this receptor and from the other side to the lack of specific tools to assess this particular expression.

Despite the advanced stages of clinical studies evaluating GLP-2 analogs in digestive disorders, the therapeutic utility of GLP-2 is limited by various concerns, notably potential extra-intestinal GLP-2 actions. Therefore, to ensure that GLP-2 analogs could be characterized as 'first-in-class' treatment for intestinal disorders, studies involving GLP-2 necessitate elucidation of the exact signaling pathways by which GLP-2 acts, the localization, and of the regulatory mechanisms of GLP-2R expression. In this study, we evoked the importance of the expression pattern of GLP-2R, not extensively explored so far. Our aim is to better understand the *in vitro* expression pattern and the *in vivo* tissue localization of GLP-2R, a safety endpoint for further evaluation of GLP-2 trophic actions in the bowel.

(A) ABSTRACT

Background: The dual function of glucagon like peptide 2 (GLP-2) as growth factor and anti-inflammatory agent, has led to its consideration as a therapeutic potential in various digestive diseases. However, the localization of GLP-2 receptor (GLP-2R) remains controversial. **Experimental methods:** Here, we examined the expression of GLP-2R in various primary and transformed cell lines. We realized a panel of GLP-2R mRNA expression in murine tissues and we assessed its expression in human and murine intestinal tissues in inflammatory conditions. **Results:** The expression of GLP-2R was detected in human colonic myofibroblasts (CCD-18Co) but not in intestinal epithelial cell lines, lymphocytes, monocytes, and umbilical vein endothelial cells. GLP-2R was expressed in murine (GLUTag), but not human (NCI-H716) enteroendocrine cells. We also detected GLP-2R expression in primary culture of rat enteric nervous system but not in enteric glial cells. The screening of GLP-2R mRNA in mice organs revealed an increasing gradient of GLP-2R toward the distal gut and a detectable expression in the central nervous system, mesenteric adipose tissue, mesenteric lymph nodes, bladder, spleen, and in the liver, particularly in hepatocytes. Using two experimental mice models of colitis, we showed that expression of GLP-2R mRNA was decreased in the colon of mice with TNBS- and DSS-induced colitis. Also, GLP-2R mRNA was significantly downregulated in intestinal tissues of IBD patients, notably in inflamed areas. **Conclusion:** Previous hypotheses limiting GLP-2R expression and function to proximal bowel needs to be revisited, and further studies should address the extra-intestinal biological function of GLP-2 as safety endpoints.

(B) BACKGROUND

I- Glucagon-Like Peptide 2 (GLP-2), a Multifaceted Intestinal Growth Factor

I-A. Synthesis and Secretion

The intestinal epithelium is a highly dynamic tissue that serves an important role both as a barrier to the external environment and as the ultimate site for nutrient digestion and absorption. These critical functions are maintained through constant tissue renewal, driven by a high rate of crypt cell proliferation that is carefully balanced against apoptosis and exfoliation, thus maintaining the size and integrity of the epithelium. This process is highly regulated; the tropic status, or growth, of the epithelium remains relatively constant in the healthy adult but is altered by developmental stage, enteral nutrition, injury, and disease. The mechanisms regulating these processes are not wholly understood; however, nutrients, humoral factors, intrinsic factors, and pathogenic/commensal organisms are clearly involved in determining crypt cell proliferation rates and the tropic status of the epithelium. The role of humoral factors is particularly interesting, as the intestine both produces and is responsive to a wide variety of regulatory peptide hormones that function through endocrine and paracrine pathways to affect intestinal growth. Of particular interest is the hormone glucagon-like peptide-2 (GLP-2) and its multifaceted role in the regulation of intestinal growth and function in physiological and pathophysiological states [18, 24].

Glucagon-like peptide-2 is a 33-amino acid member of the pituitary adenylate cyclase-activating peptide/glucagon superfamily [29]. GLP-2 is encoded within the proglucagon gene, which in mammals, gives rise to a single mRNA transcript that is expressed in the alpha (α) cells of the endocrine pancreas, in the enteroendocrine L cells of the intestine and in the hypothalamus and brainstem in the central nervous system (CNS). Proglucagon mRNA is translated into a single 160 amino acid precursor protein, producing several biologically active proglucagon-derived peptides via tissue-specific posttranslational processing. In pancreatic α cells, proglucagon is cleaved by prohormone convertase (PC)-2 to form glucagon, the major glucagon fragment and intervening peptide (IP)-1. In the GI tract and in the brain, the processing of proglucagon, which is operated by PC1/3, results in GLP-1, GLP-2, IP-2, oxyntomodulin and glicentin formation [30].

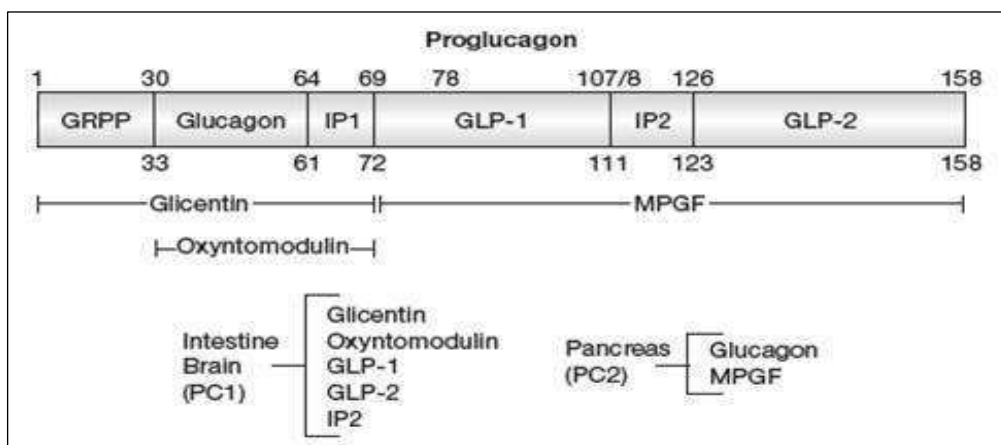


Figure 1. Structure of proglucagon and the proglucagon-derived peptides. The numbers refer to the amino acid position within proglucagon, starting at the N-terminal amino acid. Differences between the numerals in the top and bottom rows reflect processing and removal of spacer amino acids between the peptides. GLP, glucagon-like peptide; GRPP, glicentin-related pancreatic polypeptide; IP, intervening peptide; MPGF, major proglucagon fragment. PC, prohormone convertase [18].

GLP-2 is secreted by enteroendocrine L cells, most of which are located in the distal ileum and colon. Nutrient intake is the primary stimulus for GLP-2 secretion. *In vivo* animal studies and human data have determined dietary fibers and short chain fatty acids, carbohydrates and fats to be potent stimulators of GLP-2 secretion, whilst protein has almost no effect [31]. GLP-2 is secreted in a biphasic pattern, with an early peak followed by a longer second phase after ingestion of nutrients [32]. It is likely that the early phase of GLP-2 secretion is due to the stimulation of L cells by various neural and endocrine factors, in contrast with the second or late phase, which is caused by direct stimulation of intestinal L cells by digested nutrients [31]. After ingestion of nutrients, plasma levels of GLP-2 increase 2- to 5-fold, depending on the size and nutrient composition of the meal [31]. GLP-2 diffuse across the subepithelial lamina propria to activate afferent nerves and/or enter the circulation; thus it may act as paracrine agent as well as endocrine hormone. The mechanisms by which nutrients induce the release of GLP-2 from the enteroendocrine cells are not yet elucidated.

GLP-2¹⁻³³ (33 amino acids) is secreted from enteroendocrine L cells into circulation, after which GLP-2 is degraded by ubiquitously expressed proteolytic enzyme

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dipeptidylpeptidase (DPP)-IV by cleavage of N-terminal histidine and alanine to the inactive metabolite GLP-2³⁻³³ (31 amino acids) (Figure 2) [31, 33, 34]. Once in the plasma, the kidney provides the major route of clearance for GLP-2 [35]. To date, there are two different successful strategies in mitigating the issue with the short half-life of GLP-2. The first is the use of mimetics of GLP-2 that are resistant to inactivation by DPP-IV, thus prolonging and enhancing the effect of the hormone. The most prominent example is teduglutide (GATTEX; NPS Pharmaceuticals, Bedminster, NJ, USA). Teduglutide was developed by replacing alanine with glycine in position 2 of GLP-2, providing a molecule with a half-life of 3–4 h [36, 37]. The second strategy involves inhibition of DPP-IV, prolonging the effect of endogenously secreted GLP-2 with drugs like vildagliptin (Galvus; Novartis Pharmaceuticals, East Hanover, NJ, USA) and sitagliptin (Januvia; Merck & Co., Whitehouse Station, NJ, USA).



Figure 2. N-terminal post-translational processing of GLP-2¹⁻³³ to the biologically inactive GLP-2³⁻³³ [24].

I-B. Physiological Role and Biological Function

GLP-2 is a pleiotropic hormone, affecting multiple facets of intestinal physiology. It would be important to state that several studies demonstrated that exogenously administered GLP-2 is trophic for the small intestine and, to a lesser extent, the colon, implying that most of the proliferative and cytoprotective properties of GLP-2 were detected in the small intestine [5, 38]. Generally, GLP-2 acts by suppressing gastric secretion [39] and gastric motility [40], stimulating intestinal nutrient transport [41, 42], intestinal blood flow [43, 44] and crypt cell proliferation [45, 46], whilst enhancing gut barrier function

[47], and inhibiting crypt cell apoptosis [48, 49] (Figure 3). These effects will lead to enlarged crypts and villi and, hence, an enhanced absorptive surface area, digestive enzyme activity, and nutrient absorption [41, 50, 51]. Moreover, a physiological role for GLP-2 appears to be the restoration of epithelial growth following periods of fasting [52].

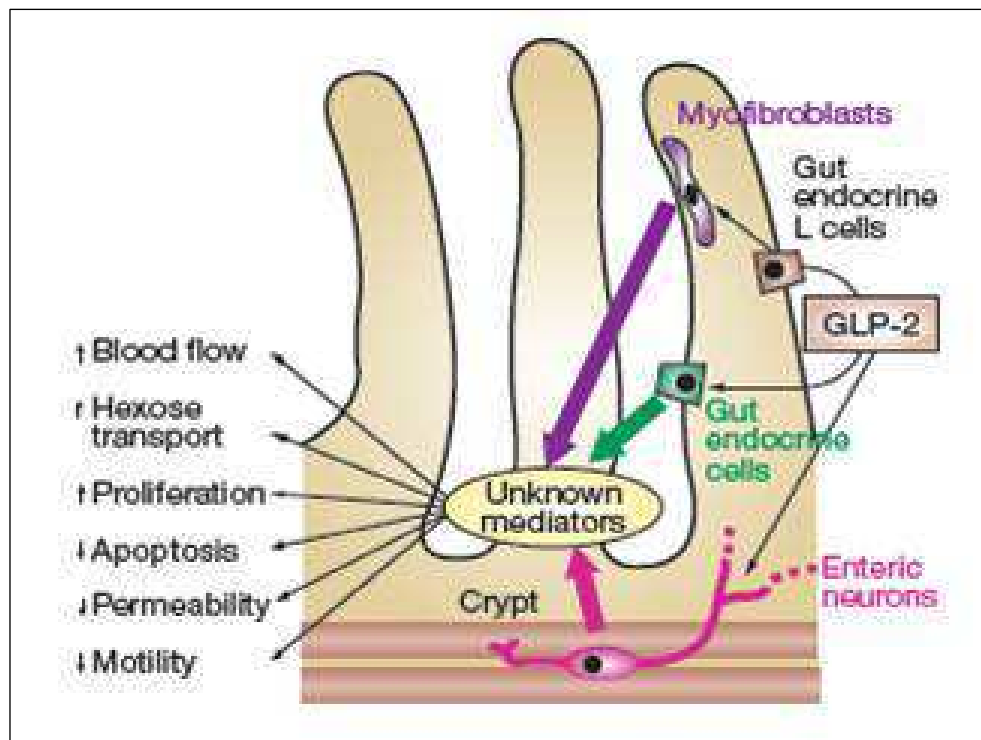


Figure 3. The actions of glucagon-like peptide 2 in the gastrointestinal mucosa [18].

II-B.1 GLP-2 in Animal Studies

Normal mice and rats treated with GLP-2 demonstrate increased small intestinal weight in association with enhanced mucosal thickness, mucosal surface area, crypt-depth, villus height and crypt cell proliferation (Figure 4). These effects are not limited to the small intestine, as the colon also responds weakly to GLP-2 with increased crypt depth and proliferation [5, 53]. Importantly, GLP-2-induced growth appears to be relatively specific for the intestinal epithelium, as there have been only limited reports of growth in the intestinal muscularis [54] and no indication of growth in any other organs [5]. Furthermore, GLP-2 enhances intestinal function in parallel with this growth, such that nutrient digestion and

absorption, as well as barrier function are all increased in normal mice following chronic administration of GLP-2 [41, 50, 51]. The trophic effect of GLP-2 treatment is also due to inhibitory effects on apoptosis. Studies in rodents and pigs have shown that GLP-2 decreases the percentage of apoptotic cells in both the crypt and villus tips of the small intestine [46, 48, 52, 55].



Figure 4. Histological appearance of small intestine epithelium from control and GLP-2-injected (10 days) mice [5].

Studies in pigs and rodents have also demonstrated that GLP-2 increases mesenteric blood flow and inhibits gastrointestinal motility, thus providing another mechanism to facilitate digestion and absorption of nutrients [43, 44, 56]. Within the context of nutrient absorption, treatment of mice and hamsters with GLP-2 has been linked to increased plasma triglyceride and cholesterol levels, through a CD36-dependent mechanism following an oral fat load [57]. Besides, GLP-2 was shown to enhance epithelial barrier capacity through decreasing transcellular and paracellular permeability, as well as accelerating wound closure following injury. The effects of GLP-2 to increase barrier function have been confirmed in murine models of both type 1 and type 2 diabetes, and the mechanism whereby these effects are transduced, has begun to be elucidated [58, 59]. Administration of a prebiotic to *ob/ob* mice not only promoted GLP-2 synthesis, but also resulted in GLP-2-

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dependent upregulation of the tight junction proteins, zonula occludens-1 and occludin [58]. Furthermore, blocking the GLP-2 receptor signaling completely abolished the major features of the prebiotic treatment [58].

Excitingly, GLP-2 has recently been shown to produce anti-inflammatory effects, independent of its proliferative actions [55]. In two models of trinitrobenzene sulfonic acid (TNBS)-induced ileitis and dextran sulfate sodium (DSS)-induced colitis, GLP-2 treatment, whether given immediately or delayed until inflammation was established, resulted in significant improvements in animal weights, mucosal inflammation indices (myeloperoxidase levels, histological mucosal scores), and reduced levels of inflammatory cytokines [55].

Beside its effect on epithelial cells, several studies have suggested that GLP-2 may also regulate intestinal stem cells (ISCs). For example, an instrumental study examining the effects of GLP-2 on ISCs revealed that pretreatment of mice with GLP-2 protects progenitor/ISCs from radiation damage [60]. GLP-2 administration for 10 days also increases the number of putative ISCs and absorptive progenitor cells, as determined by a somatic mutation-progenitor assay [61]. Chronic administration of GLP-2 to mice also increases the number of musashi-1-positive cells within the crypt base and the enriched stem cell zone [53]. Although musashi-1 is not a definitive ISC marker, these results collectively imply that GLP-2 exerts a positive effect on early progenitor cells. Furthermore, GLP-2 activates Wnt signaling in intestinal crypt cells, a pathway known to be critical in the regulation of ISC proliferation [62]. Together, these findings suggest that GLP-2 positively regulates the proliferation of both ISC and transit-amplifying cells, although it remains unknown whether these effects are context specific and/or are different in healthy gut compared with an injury model. Although it has been suggested that GLP-2 preferentially increases the number of absorptive enterocytes rather than goblet cells, others have reported an increase in the number of mucin-positive cells following chronic GLP-2 administration [54]. Thus, in addition to lengthening of the villi and crypts through stimulation of progenitor and/or stem cells in the crypt, GLP-2 treatment may alter the distribution of differentiated epithelial cells, although this effect may be model specific.

Few studies have been conducted to elucidate the roles of GLP-2 in the central nervous system (CNS), and an in-depth understanding of the complex neurobiology of preproglucagon-derived peptides in general is lacking [63]. Consistent with a general cytoprotective effect of GLP-2 within the GI mucosa, few studies have suggested that activation of GLP-2Rs can protect neurons from excitotoxic damage [64-66]. More specifically, GLP-2 has been reported to reduce glutamate-induced cell death in cultured hippocampal cells [64], enhance survival of primary rat enteric neurons, and to stimulate the proliferation of rat astrocytes [65-67]. Antidepressant-like effects of GLP-2 that occur via monoamine pathways have also been noted in mice, but this has yet to be confirmed [68].

II-B.2 GLP-2 in Human Studies

Since GLP-2 has a relatively short half-life (7 min), there has been a growing interest in the development of protease-resistant analogs such as Gly²-GLP-2 and teduglutide (GATTEX) which would extend the half-life of GLP-2 and improve its efficacy in the context of pharmacological delivery of GLP-2. Notable intestinal actions of GLP-2 have also been demonstrated in humans. Chronic administration of either GLP-2 for 6 wk or teduglutide for 3 wk (0.03 – 0.15 mg/kg/day) to subjects with short bowel syndrome (SBS) increases crypt-villus height and mitotic index and reduces fecal output in association with increased absorption of enteral nutrients [38, 69, 70]. A pilot study investigating the effects of teduglutide (0.05 – 0.2 mg/kg·day) in patients with Crohn's disease is also suggestive of beneficial effects to increase intestinal mucosal mass and/or mucosal healing, as assessed through measurements of plasma citrulline concentration [71]. Furthermore, the findings suggested that both the low (0.05 mg/kg/day) and the high (0.2 mg/kg/day) dose of teduglutide were more effective than the intermediate dose (0.1 mg/kg/day). Although the reasons for the apparent discrepancies in teduglutide effectiveness are not clear, previous studies in normal mice have indicated that the intestinotropic effects of GLP-2 are dose dependent [46]. Hence, further studies in humans determining the exact relationship between the dose of teduglutide and clinical effectiveness are required. On the other hand, the growth-promoting effects of GLP-2 or teduglutide and their effects on nutrient absorption in patients with SBS are reversed upon withdrawal of treatment [36, 46]. Thus teduglutide will likely require chronic administration to maintain the beneficial effect.

Finally, Because of the beneficial effects of GLP-2 on the gastrointestinal growth and function, teduglutide (GATTEX) has recently completed clinical trials (phase 3) for the treatment of short bowel syndrome (SBS), is currently under investigation for Crohn's disease (phase 2), and is also in preclinical development for gastrointestinal mucositis and pediatric indications [38, 69, 71] (discussed in details later). Indeed, unlike other mitogenic factors, the apparent intestinal specificity of GLP-2 renders this peptide attractive for use in conditions of intestinal dysfunction.

II-B.3 A Modest Physiological Role for Endogenous GLP-2

Interestingly, although exogenously administered GLP-2 produces a robust effect on gut growth and function, the tropic actions of the endogenously produced peptide appear to be relatively more modest but sufficient. It is important to state that the doses administered in experimental studies in animals were largely supraphysiological, often being approximately 10^3 -fold greater than physiological concentrations. The importance of endogenous GLP-2 has largely been elucidated through the use of a GLP-2R antagonist (e.g., the GLP-2 metabolite, GLP-2³⁻³³), the GLP-2R knockout mouse model, or immunoneutralization techniques. Of note, although the GLP-2 metabolite, GLP-2³⁻³³ acts as an antagonist at lower concentrations, it also functions as a partial agonist at increased doses, with significant tropic effects observed in both the small and large bowel following administration of higher amounts of GLP-2³⁻³³ [72]. Hence, caution must be taken in interpreting the results of studies using the GLP-2R antagonist, and appropriate controls must always be included. Nonetheless, administration of GLP-2³⁻³³ for either 24 h or 4 wk demonstrated a physiological role for GLP-2 in basal intestinal growth, reducing small bowel weight and decreasing crypt-villus height; these changes occurred in the absence of any detectable changes in proliferation but, at least in the 24-h model, were associated with increased intestinal epithelial cells apoptosis [52]. Although these findings stand in contrast to the report of normal intestinal weight, crypt-villus height, and proliferative index in the GLP-2R null mouse, it is possible that chronic adaptation occurs in these animals to maintain basal intestinal growth [73]. Furthermore, endogenous GLP-2 has been implicated in intestinal adaptation in rodent models of resection. Hence, studies by *Dahly et al.* and *Martin et al.* demonstrated that intestinal resection is followed by a marked increase in

intestinal proliferation, an adaptation that is strongly associated with elevated plasma levels of GLP-2 [74, 75]. Also, endogenous GLP-2 also plays a role in the adaptive intestinal growth that occurs in mice and rats in response to oral refeeding after a period of nutrient deprivation, as demonstrated with use of both the GLP-2 antagonist and GLP-2R null mice [52, 76, 77]. Finally, immunoneutralization of GLP-2 reduces the adaptive intestinal growth that is associated with experimental type 1 diabetes in rats, although these findings were not recapitulated in streptozotocin-diabetic GLP-2R knockout mice [73, 78]. Hence, the tropic effects of endogenous GLP-2 appear to be related to adaptation of the intestine in response to varying nutrient intake, although the relative importance of this role appears to vary with the species and/or the model utilized.

II- Glucagon-Like Peptide-2 Receptor (GLP-2R), a Curious Receptor for GLP-2

The integrative gastrointestinal responses to GLP-2 are mediated via the GLP-2 receptor, which is a G protein-coupled receptor (GPCR) belonging to the class B glucagon-secretin receptor family [79].

II-A. Arguable Expression Pattern

The localization of GLP-2R has been described as curious and raises important questions regarding the mechanisms underlying GLP-2-induced intestinal growth. GLP-2R expression is restricted to the gastrointestinal tract and central nervous system, with limited expression in lung, cervix, and vagal afferents [43, 80-82]. However, the exact cellular localization of the GLP-2R, particularly in the intestine, has been a source of some controversy. The vast majority of studies pretended that the location of the receptor in the bowel is region-specific, where expression appears to be the highest in the stomach and ileum. Variable reports have demonstrated GLP-2R expression in enteroendocrine L cells, enteric neurons, and/or subepithelial myofibroblasts (SEMFs) of mice gastrointestinal tract. A summary of cellular GLP-2R expression in the intestine is summarized in Table 1. Similar findings have been reported for humans, where GLP-2 receptor expression is localized to enteroendocrine cells of the stomach, small bowel and colon; to subepithelial myofibroblasts, and to enteric neurons [82]. Also, extra-intestinal expression of GLP-2R has

been reported notably in central nervous system, lung, and pancreas in rodents and humans, notably in pancreatic alpha cells and in astrocytes [66, 80, 82, 83].

Table1. Reported cellular localization of the GLP-2R in the intestine. IHC, immunohistochemistry; ISH, in situ hybridization; LCM, laser-capture microdissection; RT-PCR, reverse transcriptase-polymerase chain reaction; WB, Western blot.

Cell Type	Species	Method(s) Used	References
Enteroendocrine cells	Human	IHC, ISH	[43, 82]
	Pig	IHC, ISH, LCM/RT-PCR	[43]
	Rat	IHC, WB	[81]
	Human	IHC, ISH	[43]
Enteric neurons	Pig	IHC, ISH, LCM/RT-PCR	[43]
	Rat	ISH	[81, 84]
	Mouse	ISH, RT-PCR	[61]
Subepithelial myofibroblasts	Human, rat, mouse, marmoset	IHC, ISH	[42, 84]

The expression pattern of GLP-2R continues to the subject of ongoing debate and disagreement in the literature. GLP-2R was shown by *Lovshin et al.* to be expressed in hypothalamic and extrahypothalamic regions in mice but was completely absent from the central nervous system in the work of Drucker D.J. [80, 82]. In addition, *de Heer et al.* have localized GLP-2R on alpha cells of both human and rat pancreas but *Yusta et al.* failed to detect GLP-2R transcript in pancreatic mouse tissue and rat insulinoma cell lines [82, 83]. The latter group has also reported GLP-2R expression in rat but not in mouse lungs. While an explanation for the discrepancies between the studies remains elusive, it is nonetheless evident that crypt epithelial cells do not express the GLP-2R. This has led to the hypothesis that the actions of GLP-2 are thought to be mediated indirectly through the cells that express the receptor through a paracrine mechanism, to induce its effects on intestinal growth.

II-B. Downstream Signaling Pathways

Mechanistic studies have shown that signaling through GLP-2R activates several downstream signaling pathways such as mitogen-activated protein kinase (MAPK), cyclin

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adenosine monophosphate (cAMP), protein kinase A (PKA), cAMP response element-binding protein (CREB), and activator protein-1 (AP-1) [85, 86]. The GLP-2R can initiate anti-apoptotic responses through the cAMP pathway involving both PKA-dependent and -independent mechanisms [87]. This anti-apoptotic effect appears to involve inhibition of glycogen synthase kinase-3 (GSK-3). A distinct feature of this receptor is the mechanisms by which it undergoes desensitization.

A major obstacle in the study of the GLP-2R has been the lack of cell lines that demonstrate endogenous GLP-2R signaling. It is therefore notable that the majority of studies addressing the mechanisms of GLP-2R signaling have utilized cell lines transfected with the GLP-2R, including 293-EBNA [79], COS [43], BHK [87], fibroblasts, DLD-1 colon cancer cells [88, 89], and HeLa cervical cancer cells [88]. Only a few cell lines have been identified that endogenously express the GLP-2R, including CCD-18Co intestinal myofibroblasts and FHC fetal colon cells [90]. In CCD-18Co cells the endogenous GLP-2R shows limited activity with slight activation of MAPK. This paucity of robust cell models is relevant, as there may be significant differences between the signaling activities of the transfected and endogenous GLP-2R. For instance, the endogenous GLP-2R from hypothalamus [91], pituitary [91], intestinal mucosa [92], intestinal muscularis [52], and fetal intestine [53] produces a reproducible GLP-2 dose response curve, in which peak cAMP accumulation occurs at moderate concentrations (10^{-10} to 10^{-9} M) but is reduced with higher levels of GLP-2, reminiscent of an “inverted U-shaped” dose-response. The mechanistic explanations for such a phenomenon are unknown but may potentially result from the unique nature of GLP-2R desensitization [88] or from dose-dependent coupling to alternate G proteins [86]. Nevertheless, this characteristic dose-response is not observed in cells transfected with the GLP-2R, which demonstrate a maximum “plateau” of cAMP accumulation and only a slight reduction at concentrations of 10^{-6} M or greater, suggesting that these cells may not appropriately reflect the true nature of GLP-2R signaling *in situ*. Therefore, one major issue in the field is to determine which signaling mechanisms are relevant to the endogenous GLP-2R, especially as they relate to the intestinal growth effects of GLP-2, and to determine the intracellular pathways underlying the biological actions of GLP-2 with the use of relevant cell types.

II-C. Downstream Mediators

Although there is a wealth of information about the ultimate effects of GLP-2 on intestinal physiology and on the signaling mechanisms initiated by the GLP-2R, there are relatively few studies addressing how these two events are functionally connected. Unraveling the mechanisms behind this has been a difficult task, which is understandable given the multiple interrelated actions of GLP-2 and the complex nature of the GLP-2R. It is clear that the diverse effects of GLP-2 require an indirect mechanism, likely involving not one but multiple indirect signals, interacting in a complementary fashion resulting in different intestinal responses (i.e., proliferation, apoptosis, absorption, barrier function, blood flow, anti-inflammation). Several studies have now provided important mechanistic data illustrating several of the indirect pathways of GLP-2 action as shown in Figure 5 and discussed in detail below.

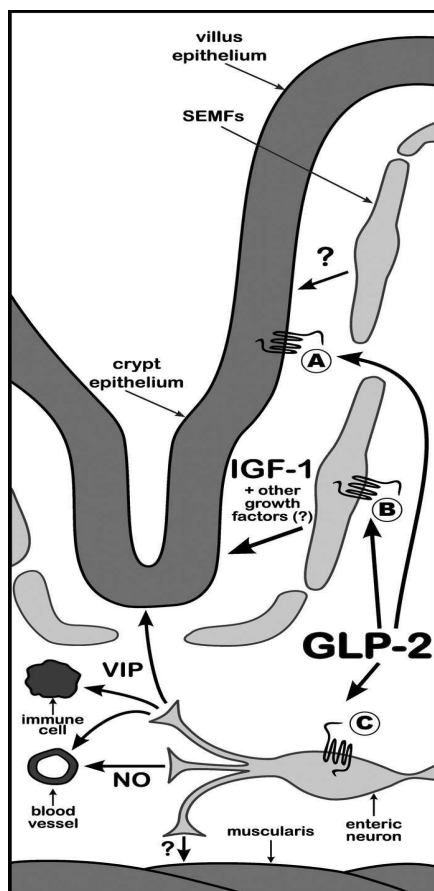


Figure 5. Proposed model for the indirect mechanisms of glucagon-like peptide-2 (GLP-2) action in the intestine. Expression of the GLP-2R in intestinal endocrine cells (A), intestinal subepithelial myofibroblasts (B) and enteric neurons (C), suggests that GLP-2 acts indirectly to produce its diverse actions in the intestine. Intermediate mediators include: IGF-1, KGF, NO, and VIP...and potentially undiscovered yet agents, which would be responsible for cytoprotective, proliferative and anti-inflammatory effects of GLP-2 in the intestinal epithelium. [20].

II-C.1 Insulin Growth factors

The insulin-like growth factors (IGFs) have recently been implicated in the intestinal growth effects of GLP-2. The role of insulin growth factor-1 (IGF-1) in GLP-2 action was first hypothesized following the observation that GLP-2 increases the expression and secretion of intestinal IGF-I [53]. Later, IGF-1 has been characterized as a major mediator released from SEMFs and through which GLP-2 increases intestinal growth, specifically by inducing small intestinal crypt cell proliferation. This observation was supported primarily by studies of IGF-1 knockout mice, which demonstrate marked impairments in small and large intestinal growth in response to GLP-2, compared with wild-type littermates, despite normal responses to other intestinal growth factors [53]. Indeed, the effect of GLP-2 on crypt cell proliferation is completely lost in the absence of IGF-1. In normal mice, GLP-2 increases the rate of proliferation in the upper half of the intestinal crypts, thus expanding the number of cells responsible for populating the epithelium. This occurs in concert with a potential effect on the epithelial stem cells, as GLP-2 also increases the number of cells expressing musashi-1, a putative stem cell marker [53]. Furthermore, GLP-2 was shown to act as a novel activator of Wnt/ β -catenin signaling in the intestinal crypt, through a mechanism requiring IGF-1 signaling through the IGF-1R [62]. The Wnt/ β -catenin signaling system represent of the most important mediators regulating crypt cell proliferation (Figure 6). The activation of β -catenin transcriptional signaling by IGF-1 occurs through a phosphatidylinositol-3 kinase (PI3K)-dependent pathway, involving Ras activation and GSK-3 inhibition [93]. One role of β -catenin in the intestine is to drive the transcription of genes required for proliferation, such as c-myc [94] while also inhibiting genes involved in terminal cell differentiation [95]. This maintains cells within the active cell cycle, thereby increasing the overall numbers of proliferating cells. These observations provide a mechanistic basis for IGF-1 as a mediator for GLP-2-induced proliferation and serves to link the GLP-2-IGF-1 signaling system to other regulators of intestinal crypt cell fate, through common effects on β -catenin.

Although intestinally derived IGF-1 is the likely mediator of GLP-2-induced proliferation in a paracrine fashion, it is unclear whether GLP-2 might also depend upon non-intestinal, endocrine IGF-1. This is an important issue as the GLP-2R is expressed in both hypothalamus and pituitary [80]. As studies in whole body IGF-1 knockout mice do not

differentiate between local and endocrine IGF-1, a model of tissue-specific IGF-1 ablation would be needed to isolate a specific paracrine role for SEMF derived IGF-1.

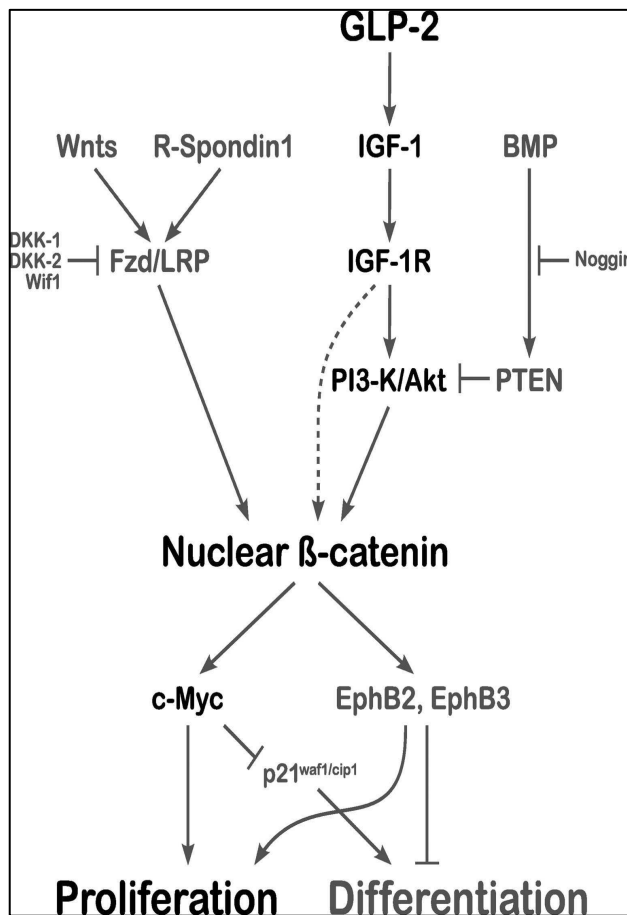


Figure 6. Integrated model for control of crypt cell proliferation and differentiation. GLP-2 acts through an IGF-1-dependent pathway to activate intestinal crypt β -catenin, which integrates signals from multiple pathways to regulate the balance between crypt cell proliferation and differentiation. Activators of β -catenin signaling include Wnt proteins, R-Spondin1, IGF-1, GLP-2, and Noggin. Negative regulation of β -catenin occurs through Wnt antagonists (DKK-1, DKK-2, Wif1) and BMP/PTEN. B-type ephrin receptors (EphB2, EphB3), cyclin-dependent kinase inhibitor 1A (p21waf1/cip1), Dickkopf (DKK-1, DKK-2), Frizzled/low-density lipoprotein receptor-related protein receptors (Fzd/LRP), phosphatase and tensin homolog (PTEN), and Wnt inhibitory factor (Wif1) [27].

The essential role of IGF-1 in GLP-2-induced crypt cell proliferation is contrasted by the limited impact of the loss of IGF-2, in which the enhancement of intestinal growth by GLP-2 is only partially blunted [53]. IGF-2 signals through several receptors, including the IGF-1R and isoform A of the insulin receptor, offering a potential explanation as to why the effects of IGF-2 and IGF-1 might differ. Indeed, the role of endogenous IGF-2 in the regulation of intestinal growth is unique; IGF-2 initiates a process known as crypt fission, in which whole crypts divide, resulting in an increase in the overall number of crypts [96]. These additional crypts therefore become available to respond to other growth factors. This raises the possibility that, when faced with the absence of IGF-2, the intestine is unable to

fully respond to GLP-2 due to the resulting impairment in crypt fission. This hypothesis would represent yet another level of complexity in the actions of GLP-2.

II-C.2 Keratinocyte Growth Factor

Keratinocyte growth factor (KGF) has also been implicated in GLP-2-induced colonic growth. KGF is a member of the fibroblast growth factor family, expressed in SEMFs throughout the gastrointestinal tract, and is a tropic factor for epithelial cells [97]. KGF colocalizes with the GLP-2R in SEMFs, and immunoneutralization of KGF in mice prevents the effect of GLP-2 on colonic weight and mucosal area without affecting colonic crypt depth or small intestinal growth [84]. This is in contrast to the more marked effect of IGF-1 ablation on both small and large intestinal growth responses to GLP-2. Moreover, unlike IGF-1, KGF treatment produces differential effects to those of GLP-2, affecting mainly colonic growth and the differentiation of goblet cells [98, 99]. Indeed, *Ørskov et al.* found that the GLP-2- induced increase in mucin expression was blocked by KGF antibodies, suggesting that the specific role of KGF may be to promote colonic goblet cell differentiation in response to GLP-2 [84]. Nonetheless, it is possible that immunoneutralization may be insufficient to block small intestinal KGF, and therefore additional studies, utilizing a knockout model, would be helpful to determine whether this effect of KGF is truly restricted to the colon. Furthermore, a more detailed study of the role for KGF in crypt cell proliferative responses would help to determine whether the KGF effect extends beyond that on the goblet cells and whether KGF may interact with the proliferative actions of IGF-1.

II-C.3 ErbB Ligands

In addition to the IGFs, ErbB ligands have recently generated interest as downstream mediators of GLP-2-induced intestinotropic actions. The ErbB network is a potent proliferative system that contributes to the maintenance of intestinal mucosal growth and function [100]. The ErbB ligands epiregulin and neuregulin were found to be upregulated in the murine small intestine 1 and 4 h after GLP-2 treatment [101]. Similarly, administration of GLP-2 or epidermal growth factor (EGF) increased mRNA expression of the ErbB ligands,

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amphiregulin, epiregulin and Heparin binding-epidermal growth factor (HB-EGF), as well of the immediate early genes, c-fos, egr-1, and phlda-1 within full-thickness intestinal sections. Furthermore, the ErbB receptors were shown to be required for the chronic intestinal proliferative and growth effects of GLP-2, as determined through the use of a pan-ErbB inhibitor. More recently, EGF administration was also found to rescue the lack of adaptive regrowth in the refed GLP-2R knockout mouse [76]. In contrast, using the ErbB receptor inhibitor gefitinib, others have reported that the positive effects of GLP-2 on intestinal weight and crypt-villus length are independent of ErbB signaling [102]. Nonetheless, collectively, the data suggest the involvement of an ErbB ligand-ErbB signaling pathway in the proliferative actions of GLP-2.

II-C.4 Nitric oxide & Vasoactive Peptide

Several studies have uncovered an exciting role for GLP-2R signaling in submucosal enteric neurons. Functioning through a nitric oxide (NO)-dependent mechanism, GLP-2 acutely and dose-dependently increases intestinal blood flow by activating endothelial nitric oxide synthase (eNOS) [43, 44]. GLP-2 has been also shown to reduce intestinal damage and the levels of inflammatory cytokines in a rat model of IBD through a mechanism requiring vasoactive intestinal polypeptide (VIP)-expressing submucosal enteric neurons [55]. One of the curious findings of this report was that, in a counterintuitive manner, GLP-2 administration reduced the rate of epithelial proliferation in this setting; given that inflammation itself induces crypt cell proliferation, it would seem that this effect of GLP-2 may be an indirect result of a VIP-dependent anti-inflammatory action [55, 103]. It is thus possible that NO or VIP signaling may affect GLP-2-induced intestinal growth through several indirect mechanisms.

II-C.5 The List Keeps Growing

These studies have only begun to uncover the mechanisms through which GLP-2 induces growth in the normal intestine. Although proliferative responses in the small intestinal crypt appear to require IGF-1, it is unknown how GLP-2 alters apoptosis, permeability, or nutrient digestion or absorption in the villus epithelium. Cheeseman

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reported that GLP-2-mediated epithelial glucose uptake, through the sodium-dependent glucose transporter, occurred through a PI3K-dependent mechanism [41]. Furthermore, the activation of Akt in the intestinal mucosa has been implicated in the antiapoptotic actions of GLP-2 [104]. The PI3K-Akt pathway is a common signaling effector for multiple growth factors and cytokines, including IGF-1. However, although IGF-1 activates Akt signaling in the intestinal mucosa, it appears that, unlike the response observed with β -catenin in the crypt, IGF-1 signaling is not strictly required for the activation of Akt by GLP-2 [62]. This suggests that, in addition to the requirement of IGF-1 for proliferation, other factors exist and mediate the diverse biological actions of GLP-2. The SEMFs, for example, express and secrete a wide range of different growth factors and cytokines in addition to the IGFs and KGF that may participate in some of the effects of GLP-2 [97].

III- Clinical Indications: lessons from animal models

The spectrum of GLP-2 actions in the intestine suggests that GLP-2-based therapy may be promising in multiple clinical settings, including short bowel syndrome (SBS), inflammatory bowel disease (IBD), and intestinal injury. In fact, the clinical evidence for benefit in conditions requiring longer term treatment with GLP-2 receptor agonists, for example short bowel syndrome and inflammatory bowel disease. Benefits of short-term GLP-2 treatment are emerging in pre-clinical models, such as post-operative ileus, gastrointestinal mucositis and conditions of altered intestinal permeability. The therapeutic utility of GLP-2 receptor agonists is limited by concern that it predisposes patients to gastrointestinal cancers, or their re-occurrence in cancer patients. This affects the types of diseases treated and, possibly, the duration of dosing.

III-A. Short Bowel Syndrome

Short bowel syndrome (SBS) typically arises from multiple or extensive bowel resections that can involve the small or large intestines, where the remaining bowel is insufficient to meet the digestive and absorptive needs of the body. During the first 12 - 24 months following resection, an intestinal adaptive response to the loss of mucosal surface area occurs. This adaptive response increases absorptive capacity by structural hyperplasia,

absorptive cell hyperplasia, increased transporter expression, and the slowing of GI transit, and is thought to be regulated by nutrients and growth factors [105]. In many cases, parenteral nutrition is required to supplement enteral nutrition. Thus, a major goal in the management of SBS is to improve patient nutritional status and quality of life by eliminating or reducing dependence on parenteral nutrition. With this in mind, the effects of several growth hormones as well as GLP-2 on intestinal adaptive responses have been studied extensively in preclinical models [106]. While many of these factors have shown efficacy in animals, to date human growth hormone (Zorbtive; somatotropin) is the only treatment approved for SBS in patients requiring specialized nutritional support.

In SBS patients the maximum increase in serum GLP-2 levels following a meal is much lower than in healthy volunteers [107]. Administration of native GLP-2 or GLP-2 analogs has been shown to be effective in improving clinical, laboratory and histological outcomes in SBS. An additional benefit in humans is that GLP-2 increases mesenteric blood flow which enhances nutrient uptake [20, 56]. A synthetic recombinant human GLP-2 analog is being developed for the treatment of SBS. Teduglutide (GATTEXTM, ALX-0600; NPS Allelix Corp.) has increased resistance to enzymatic degradation by dipeptidyl peptidase-IV compared with native GLP-2 due to an N-terminal substitution at the second position of glycine for alanine and the engineered variant has an increased half life of hours, rather than minutes. The actual elimination half life of teduglutide in patients is influenced by body weight and is approximately 3 h in 90 kg male patients [108]. The clinical efficacy of teduglutide in SBS was evaluated in an open-label, Phase II, pilot study published in 2005 and in a multicenter, multinational, randomized, placebo-controlled, Phase III study published in 2011 [36, 69]. In the Phase II, metabolic balance study, 16 SBS-patients received three different doses (0.03mg/kg/day, 0.10 mg/kg/day, and 0.10 mg/kg/day) of teduglutide for 21 days. Compared with baseline, 21 days of treatment with teduglutide increased absolute intestinal wet weight absorption in 15 out of 16 SBS patients. The average increase in wet weight absorption was 743 ± 477 g/day and the overall increase in the relative wet weight absorption was $22 \pm 16\%$. The fecal wet weight decreased significantly by 711 ± 734 g/day and the urine weight increased by 555 ± 485 g/day compared with baseline. In addition to the metabolic balance studies, the study also examined the possible histological changes in bowel biopsies obtained from the patients. In

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the jejunum biopsies an increase in villus height ($38 \pm 45\%$), an increase in crypt depth ($22 \pm 18\%$), and an increase in mitotic index ($115 \pm 108\%$) were demonstrated. The general observation at follow-up 3 weeks after termination of the treatment was that teduglutide showed intestinotrophic properties in humans, possibly as a result of increasing the absorptive surface area of the intestine. However, most changes in intestinal absorption and histology related to teduglutide treatment had reversed at follow-up. This indicates that long-term treatment with teduglutide will be necessary, but also that treatment does not lead to irreversible structural changes [36]. Later, a Phase III multicenter, doubleblind, international clinical trial has been completed with 83 SBS patients on total parenteral nutrition (TPN) receiving daily subcutaneous injections of teduglutide (0.05 or 0.10 mg/kg/day) or placebo for a period of six months. Positive results were reported from this study where the clinical efficacy end point of the study was a reduction in TPN of 20% compared with baseline to weeks 16 - 24. Surprisingly, patients receiving the lower, but not the higher, dose responded with a statistically significant difference compared with placebo[109]. The urine volume increased by ~ 350 ml/day and the parenteral volume was decreased by ~ 350 ml/day in the 0.05-mg/kg/day teduglutide dose group, whereas the oral fluid intake decreased by ~ 350 ml/day and the parenteral volume decreased by ~ 350 ml/day in the 0.10-mg/kg/day teduglutide dose group. In fact, these parameters reflect the fluid composite effect which is the sum of increase in urine production, decrease in parenteral support, and reduction in oral fluid intake. The fluid composite effect is an indication of the increase in intestinal fluid absorption and the concomitant reduction in diarrhea. Analysis also showed a significant increase in small bowel villus height, plasma citrulline (a biomarker of enterocyte mass in SBS patients) concentration and lean body mass in both teduglutide dose groups compared with placebo. Of note, three teduglutide-treated patients were completely weaned off parenteral support.

Regarding the safety and tolerability, teduglutide was well-tolerated by patients with SBS. No withdrawals of participants were related to adverse events. Four patients reported serious adverse events (dehydration, sepsis, catheter-related infection), none of which were linked to the study drug as judged by the investigators. Common adverse effects included lower extremity edema (44%), localized swelling of the jejunostomy nipple (70%), headache (25%) and abdominal pain and symptoms of bowel obstruction (19%). Three patients had

minor injection site reactions. No clinically significant abnormal laboratory values were identified in relation to teduglutide treatment, and no antibodies to teduglutide were detected. Data on kinetics and metabolism of teduglutide are not yet available in the public domain. So far, no metabolites of teduglutide have been identified. Bioavailability after subcutaneous administration is $87 \pm 14\%$. It is taken into systemic circulation with a median T_{max} of 4 h, and the mean terminal elimination half-life is 3.2 h (based on the dose 0.12 mg/kg). It has a distribution similar to blood volume. Overall, Teduglutide was shown safe, well tolerated, intestinotrophic and suggested pro-absorptive effects facilitating reductions in parenteral support in patients with SBS with intestinal failure [20, 69].

In pediatric patients, necrotizing enterocolitis (NEC) is a common neonatal GI disease that affects approximately 10% of premature infants with 20 - 40% mortality. Bowel resection is often required, and survivors may have malabsorption and failure to thrive. In preterm infants that required bowel resection, endogenous circulating GLP-2 levels were correlated with tolerance of enteral feeding. After a feed, infants with GLP-2 levels greater than 15 pmol/l were able to be weaned from TPN, whereas infants who had GLP-2 levels less than 15 pmol/l failed to survive or remained on TPN for at least one year [110]. Thus, in preterm infants endogenous GLP-2 levels may have a predictive value on chance of/ability to be weaned off TPN, with a lower level (< 15 pmol/l) having a negative prognostic value. In a preclinical model of necrotizing enterocolitis in neonatal pigs fed a formula that induced mucosal dysfunction and bacterial overgrowth, administration of trophic factors, including GLP-2 had limited effects [111]. Although further studies are necessary to confirm whether GLP-2 supplementation is of benefit in infants with SBS, it is encouraging that teduglutide was recently evaluated in a Phase I clinical study for safety and pharmacokinetic profile in pediatric patients with SBS who have undergone resection for NEC, volvulus, or intestinal atresia [112]. In a rodent model of Roux-en-Y gastric bypass, the surgically treated animals had elevated systemic levels of GLP-2 in the days following surgery [113]. This early increase of endogenous GLP-2 appears to act as a compensatory mechanism that helps to drive intestinal adaptation after intestinal resection. Furthermore, in a rat model of intestinal resection with TPN, GLP-2 administration during the first week after surgery resulted in greater bowel weight crypt cell proliferation, and nutrient transporter expression (sodium/glucose cotransporter 1 (SGLT-1), glucose transporter (GLUT)-2, GLUT-5) [114].

Therefore, timing the administration of a GLP-2 receptor agonist to a period soon after intestinal resection may improve outcome.

III-B. Inflammatory Bowel Disease

Both cytoprotective and neuroprotective properties have been attributed to GLP-2 suggesting that it may have an important role in intestinal adaptation and repair during inflammatory events. In animal models, mice subjected to Dextran Sulfate Sodium (DSS)-induced colitis for 10 days in drinking water were given twice daily injections of 750 ng or 350 ng Teduglutide in 0.5 ml saline [27]. The weights and lengths of the small bowel and colon were significantly greater in mice receiving Teduglutide and DSS compared to mice receiving DSS alone. Improvements were also noted in histological morphology and crypt cell proliferation. In the HLA-B27 rat model of colitis, a 14-day systemic infusion of Teduglutide at a dose of 50 mg/kg/day decreased histological lesions by 90% in both the small and large intestine in rats treated with GLP-2 [115]. Furthermore, expression of the inflammatory cytokines, interferon (IFN)- γ and tumor necrosis factor (TNF)- α was decreased by 100% in GLP-2 treated rats. In an almost identical study by *Arthur et al.*, HLA-B27 rats received 50 mg/kg/day of the GLP-2 analog for 14 days via an intraluminal pump or an intravenous injection [116]. In rats treated with GLP-2, there were significant improvements in stool consistency as well as histological inflammation scores compared to control groups. *Sigalet et al.* have as well investigated the therapeutic potential of GLP-2 in mice with TNBS-induced ileitis or DSS-induced colitis [55].

The protective effects of GLP-2 in both models of intestinal inflammation and damage were indicated by increased in crypt depth, villus height and proliferative activity of crypt cells. Consistent with the findings of *Alavi et al.*, there was a significant reduction in inflammatory markers such as interleukin (IL)-1 β , IFN- γ and TNF- α as well as a reduction in neutrophil activity [115]. The authors proposed that GLP-2 may have exerted its anti-inflammatory action via the GLP-2R expressed on enteric neurons. By using an antagonist of vasoactive intestinal polypeptide (VIP), they suggested that VIP mediated the anti-inflammatory properties of GLP-2 [55]. Specifically, GLP-2 in the mucosa may contribute to an endogenous beneficial response since there are increased numbers of GLP-2-positive

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enteroendocrine cells during ileitis and after resolution of TNBS-induced colitis [117]. Interestingly, increases in epithelial 5-hydroxytryptamine (5-HT) were noted in colon when inflammation was present in ileum, suggesting that GLP-2 can mediate a pan-enteric response to inflammation and release mediators controlling vascular perfusion and motility at site(s) distant from the insult [118]. In a study by *Ivory et al.*, using the IL-10 knock-out (IL-10^{-/-}) mouse model of spontaneous colitis, it was demonstrated that the anti-inflammatory actions of GLP-2 may have been IL-10 independent [119].

Overall, studies in rodent models of intestinal injury have shown that teduglutide ameliorates mucosal damage, intestinal lesions, and inflammation and, in patients with inflammatory bowel diseases. [27, 116, 120]. As an alternative to Teduglutide, the capacity for inhibitors of DPPIV activity to increase physiological concentrations of biologically active GLP-2 was investigated. There was no observed difference in the development and resolution of DSS-induced colitis between wild-type and DPPIV gene knock-out mice [121]. However, DPPIV inhibitors were significantly able to reduce disease activity in wildtype mice and crypt cell proliferation was greater in mice treated with DPPIV inhibitors during the recovery phase, indicating an increased bio-activity of GLP-2 [122].

As for human studies, analysis of circulating active and non-active GLP-2 in human subjects with either CD or UC revealed that patients with active disease had elevated levels of bio-active GLP-2 compared to healthy controls [123]. The authors suggested that this may have been an innate compensatory mechanism to the intestinal injury associated with IBD. In contrast, *Schmidt et al.* reported no difference in either tissue or plasma concentrations of meal stimulated GLP-2 between patients with IBD and non-IBD controls [124]. Further studies are required to characterize the innate adaptive GLP-2 response during human intestinal inflammation and damage. Concerning exogenous GLP-2 analogs administration, in an eight week dose-ranging study of teduglutide (0.05, 0.10, or 0.20 mg/kg daily) in 71 patients with moderate-to-severe Cohn's Disease, remission was induced after 8 weeks of treatment in over 55% of study participants at the highest dose, compared to only 33% of those receiving placebo [71]. These results provide compelling evidence for the use of Teduglutide in the treatment of intestinal diseases associated with compromised repair of the intestinal mucosa. However, further studies are required to fully investigate

the effects of exogenously administered GLP-2 into the gastrointestinal environment. However, the authors suggested that teduglutide could be successful in Phase III clinical trials in Crohn's disease. If these are completed successfully, teduglutide could be considered a 'first-in-class' because, unlike the approved therapies, it is not expected to be directly immunosuppressive or immunomodulatory.

III-C. Diabetes

The GI tract contributes to glucose homeostasis of the organism primarily by the release of GLP-1 and glucose-dependent insulintropic peptide (GIP), both of which stimulate insulin secretion. The endogenous release of GLP-1 may be insufficient in type 2 diabetic (T2DM) patients and GLP-1 receptor agonists are approved for use in T2DM [125]. Increasing the endogenous level of GLP-1 via inhibition of dipeptidyl peptidase-4 has also been found to be effective in patients with T2DM [126]. In contrast to the GLP-1, GLP-2 does not modulate insulin secretion and little attention was paid to its potential effects on other metabolic hormones [127]. However, GLP-2 receptor immunoreactivity and mRNA transcripts were detected in rat (but not mouse) pancreatic alpha cells [73, 83]. In mice null for GLP-2 receptor, Bahrami and colleagues could not find evidence for a role for endogenous GLP-2 in the control of glucose homeostasis or islet function under normal or diabetic conditions [73]. However, GLP-2 stimulated glucagon secretion in rats, which is consistent with data in healthy human volunteers, where native GLP-2 increased circulating glucagon levels, although plasma glucose levels were unchanged [83, 128]. The possibility remains that in diabetic patients, pancreatic α -cells exhibit inappropriate glucagon responses to GLP-2 receptor activation. In type 1 diabetes mellitus (T1DM) patients, GLP-2 stimulated the release of glucagon without endogenous insulin production during maintenance of stable glucose concentrations [129]. Thus, GLP-2 could contribute to excessive postprandial glucagon levels in T1DM patients. It is therefore of concern that GLP-2 agonists in development for treatment of SBS could have potential deleterious effects on glucose tolerance via increased glucagon-induced hepatic glucose production.

Obese and diabetic mice display enhanced intestinal permeability and metabolic endotoxemia. GLP-2 peptide treatment reduces mucosal bacteria adherence and

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translocation and enhances intestinal epithelial barrier function potentially by altering the expression of tight junctional proteins [58, 130]. Thus, GLP-2 treatment would limit the increased gut permeability that promotes bacterial translocation, specifically the endotoxemia and inflammation associated with metabolic dysfunction. Indeed, GLP-2 treatment reduced inflammation in ob/ob mice fed a high-fat diet, whereas a GLP-2 receptor antagonist reversed many of the beneficial metabolic actions of a prebiotic [58, 79]. Further support for this notion is evidence that microbiota modulate gut permeability through a GLP-2 mechanism [131]. Acute pancreatitis is also associated with increased intestinal permeability, and teduglutide treatment significantly decreased intestinal permeability associated with taurocholate and trypsin induced pancreatitis in rats [47]. Overall, the inter-relationship between GLP-2 the intestinal microbiome with respect to bacterial translocation and inflammatory conditions including metabolic syndrome warrants further attention.

III-D. Post-operative Ileus

Postoperative ileus (POI) continues to be a significant clinical problem following open abdominal surgery, extending the length of hospital stay and contributing to postoperative morbidity and complications. Disturbance to the bowel during abdominal surgery elicits multifactorial responses within the bowel wall that inhibit intestinal smooth muscle contractility. In POI a cellular inflammatory response is initiated via the activation of resident macrophages and the production of chemokines that recruit neutrophils, monocytes and mast cells to the intestinal muscularis and mucosa. The onset of cellular inflammation correlates temporally with the decline in intestinal smooth muscle contractility, with both inflammatory cell numbers and the degree of bowel stasis reaching their peak 24 h postoperatively. The dysmotility associated with inflammation is thought to be due to the release of cytokines (IL-6, IL-1 β), NO and prostaglandins. These mediators impair neuromuscular communication, inhibit smooth muscle contractility, and enhance central inhibitory neural reflexes. Intestinal dysmotility is exacerbated by post-operative opioid analgesic use, which has a direct inhibitory effect of neuromuscular communication [132, 133].

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Alvimopan, a mu-opioid receptor antagonist that acts locally within the GI tract (Entereg, Adalor Corp., Exton, PA) is the only FDA-approved treatment for accelerating recovery time following partial large or small bowel resection surgery. The mechanism for this enhanced recovery is the blockade of opioid receptors, primarily on neural reflexes that are inhibited by administration of opioid analgesics and by local release of endogenous opioids [134]. While alvimopan addresses the opioid-induced inhibitory effects on GI motility, it does not alter the mechanisms by which inflammation suppresses gastrointestinal motility. The administration of GLP-2 peptide is expected to delay gastric emptying and slow intestinal transit [135]. Surprisingly, when a GLP-2 peptide analog was administered chronically for 10 days in mice, upper gastrointestinal transit was enhanced [136]. This finding, plus the fact that the initiation and maintenance of POI involves immune cell and neural interactions raised the question whether GLP-2 receptor agonism could be beneficial in POI.

A peptide analog of GLP-2 linked to the Fc portion of an immunoglobulin (GLP-2/IgG) was constructed, which dramatically increased the half life of native GLP-2 from minutes to days. In a mouse model of POI, the prophylactic administration of GLP-2/IgG 24 h before intestinal surgery attenuated inflammation associated with POI and accelerated recovery from bowel stasis [136]. GLP-2 receptor agonism mediated these beneficial effects shortly after intestinal manipulation by decreasing the expression of pro-inflammatory genes and enhancing the expression of genes involved in maintaining mucosal integrity and repair. Surgical manipulation of the bowel adequate to induce POI has also been shown to impair mucosal barrier function [137]. Although transient in duration, this increase in mucosal permeability is sufficient to allow passage of gut derived bacterial cell wall components, enterotoxin and intact bacteria, contributing to the postoperative inflammatory response. Mediators that enhance mucosal barrier function have the potential to modify inflammation. GLP-2/IgG treatment resulted in a modest increase in occludin gene expression involved in the enhancement of the trans-epithelial resistance [136]. Thus the protective effect of GLP-2 receptor agonism may in part be due to an effect on mucosal integrity.

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Interestingly, induction of GLP-2 receptor gene expression is also observed in the mucosa following abdominal surgery, with levels peaking six hours after bowel manipulation in the same mouse POI model [136]. Similar observations were made in a rat model of 90% intestinal resection, where increased GLP-2 receptor mRNA was noted in full thickness ileal segments 3 days after sham surgery or bowel resection, with the greatest increase seen in the resected animals [114]. This indicates that relatively minor disturbances to the bowel can lead to a rapid increase in GLP-2 receptor gene expression, and that the magnitude of expression is dependent on the degree of disturbance to the bowel. Systemic GLP-2 protein is also elevated in the hours and days following surgery, thus GLP-2 is positioned to affect GI function via upregulation of its receptor [113]. Although subpopulations of mucosal epithelial cells and neuronal elements within the GI are known to express the GLP-2 receptor, it is not known whether these or other cell types are the site of receptor upregulation. Both exogenous GLP-2 and abdominal surgery leads to activation of a subpopulation of neurons of the submucosal plexus, and it is well established that neural reflexes within the submucosal plexus play an important role in regulating mucosal blood flow as well as secretory and absorptive function [114]. How GLP-2 modulates the function of these various cell types to generate its various protective and adaptive effects is yet to be elucidated.

III-E. Chemo and Radiation Induced Gastrointestinal Mucositis

GI mucositis is a frequent complication of cytoreductive cancer chemotherapy and radiotherapy. Patients have difficulty digesting and absorbing nutrients from an oral diet which contributes to the malnutrition or cachexia associated with cancer chemotherapy. Symptoms can be so severe as to prevent patients from completing their treatment schedule. GI mucositis is most prominent in the small intestine, but also occurs in the esophagus, stomach, and large intestine [138]. Approximately 10 - 30% of patients who receive multi-cycle standard dose chemotherapy undergo severe GI mucositis [139]. To our knowledge, there are no currently approved treatments for GI mucositis. A recent review of intestinal growth factors has highlighted the role of GLP-2 in its capacity to reduce chemotherapy-induced intestinal mucositis in preclinical models, leading to reduced mortality and weight loss, increased crypt survival, crypt death, and villus height [140]. In

addition, we found that GLP-2 receptor agonism suppressed the increased mucosal mRNA expression of early growth response gene (EGR)-1 which has been linked to increased intestinal epithelial cell injury and reduced barrier function [141]. Mucositis increases the risk of infection in myelosuppressed patients and the risk of infection-related death. Therefore improving GI barrier function and nutrient absorption would be beneficial for these patients.

III-F. Wound Healing

Hormones such as GLP-2 that increase crypt cell proliferation may have a beneficial effect on recovery from intestinal resection. Changes in cytokine or growth factor expression can affect anastomotic healing, which is a complicated series of events which proceeds similarly to skin wound healing [142]. Growth hormone and IGF-1 have been shown to have positive effects in rodent models [143, 144]. The question of whether GLP-2 treatment would affect anastomotic strength and wound healing was recently addressed in a rat model of colonic healing. Neither GLP-2 peptide nor the long acting analog GLP-2/IgG affected the anastomotic bursting pressure under normoxic, and hypoxic conditions [145]. These data combined with the effects on crypt cell proliferation and cytokine levels suggest that GLP-2 analogs used perioperatively in humans with intestinal resection would not hinder intestinal anastomotic and wound healing.

III-G. Therapeutic Potential

The therapeutic potential of modulating GLP-2 receptor signaling in GI diseases is based on the multiplicity of indirect effects in the gut of locally released GLP-2 from intestinal enteroendocrine L cells. The resulting paracrine and endocrine responses of GLP-2 combine to meet the nutrient absorptive requirements of the individual by enhanced mesenteric blood flow, enhanced mucosal absorption, enhanced barrier function and coordination of GI propulsion. Thus, it is an attractive therapeutic option in conditions where in patients cannot meet their nutrient requirements without TPN which may be further compounded by perturbed inflammatory responses. This would include conditions such as SBS and NEC in infants, as well as damage to mucosal epithelial cells resulting from

chemotherapy or radiation-induced mucositis. So far, the real promising effects of GLP-2 analogs, especially teduglutide, was revealed in patients with SBS after phase III clinical trials was completed. However, the optimal duration and concentration requirements for GLP-2 or teduglutide to induce beneficial effects on intestinal secretion, motility, morphology, and, most importantly, absorption, are not known. Future studies have to show if a more frequent administration, higher dose, or longer duration of treatment will further improve intestinal function. It is clear that optimal dosage and administration to SBS patients may eventually result in long-term improvements in nutritional status and independence of parenteral nutrition (PN) in a fraction of SBS patients. However, because the effects of GLP-2 and teduglutide are reversed following withdrawal, lifelong administration is likely required to maintain sustained response.

IV- Proliferation of Normal and Neoplastic Tissue?

The therapeutic advantage of the intestinal-specific actions of GLP-2 makes this hormone an attractive candidate over other gut growth factors that also exhibit extraintestinal tropic actions, such as IGF-1. However, as with any growth factor, the potential for carcinogenic effects of chronic GLP-2 treatment must not be overlooked. Specifically, in chemotherapy-induced mucositis the proliferative and cytoprotective actions of GLP-2 are of concern because of the potential to support the growth and survival of tumor cells. Also, GLP-2 growth-promoting effects could be deleterious in IBD patients, who already possess an increased risk of gastrointestinal cancer due to chronic inflammation.

The role of GLP-2 in carcinogenesis is controversial. GLP-2 did not stimulate cell growth or attenuate cycloheximide-, LY294002-, indomethacin- or chemotherapy-induced cytotoxicity in several intestinal cancer cell lines and had no effect on the growth of human colon cancer cell xenografts in nude mice [146]. Moreover, GLP-2 did not modulate tumor growth in APC^{min+/-} (adenomatous polyposis coli; multiple intestinal neoplasia) mice, which have an activating mutation in APC causing increased cWnt signaling and are, thus, a model of familial colon cancer [146]. However, after azoxymethane treatment, aberrant mucin-depleted crypts and adenocarcinomas developed in GLP-2-treated mice suggesting that chronic treatment with GLP-2 may enhance colon carcinogenesis. Furthermore, the

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promoting-effect on ACF was prevented by treatment with GLP-2³⁻³³, implicating endogenous GLP-2 in the development of colonic dysplasia [147]. Moreover, methylating carcinogen (dimethylhydrazine)-induced colonic tumors were increased in mice treated with long-acting Gly²-GLP-2 compared with untreated control groups. Interestingly, Gly²-GLP-2 specifically increased the number of small, medium, and large polyps in these animals, with medium and large polyps characterized as pedunculated nonmalignant adenomas, whereas native GLP-2 only increased the number of small polyps, none of which were malignant [148]. Together, these findings suggest that GLP-2 increases tumor initiation as well as progression in the dimethylhydrazine and azoxymethane models of sporadic murine colon cancer. Although the mechanism of this action has not been established, one possibility is the regulatory relationship between the cWnt and PI3K/Akt signaling pathways, both of which have been implicated in the pathogenesis of colorectal cancer [149]. In addition, the GLP-2 receptor was found to be expressed at low levels on some cervical cancer cell lines as well as a subset of epithelial cells in normal cervix and cervical cancer. Exogenous GLP-2 also inhibited apoptosis in a cervical cancer cell line transfected with the GLP-2 receptor [86]. Thus, concerns remain that GLP-2 receptor signaling engages pathways that could promote tumor cell growth or survival in the intestine or other organs.

The risk of enhanced growth and survival of intestinal tumor cells, due to proliferative and cytoprotective actions of GLP-2 might be mitigated by short-term treatment for acute disorders, such as POI. But whether patients with colon cancer requiring intestinal resection may be predisposed to relapse for colon carcinogenesis due to a short term prophylactic dose of GLP-2 agonists is unknown. If exogenous GLP-2 in pediatric SBS patients is sufficient to wean them from TPN, then it is of special concern in this patient population whether this could predispose infants to intestinal carcinoma.

Overall, the greatest challenge for the field is the satisfactory demonstration of a lack of GLP-2-enhanced risk of carcinogenesis after chronic or short-term treatment with longer-acting analogs. Thus, despite the beneficial effects of GLP-2 and its long-acting derivative, teduglutide, in the treatment of gastrointestinal disease, the potential for GLP-2 to induce carcinogenesis, albeit controversial, is an important issue that warrants further long-term investigation. Until sufficient long-term surveillance studies of a marketed

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therapeutic agent have been completed to address these safety concerns, the extent to which GLP-2 analogs can be developed for treatments beyond orphan indications such as SBS will be limited. The orphan drug status of teduglutide in SBS patients requiring TPN will provide an avenue to assess many of these potential liabilities over a long-term course of treatment. Furthermore, if teduglutide shows proven clinical efficacy in chronic inflammatory disorders, in the absence of enhanced risk of carcinogenesis, then it is an attractive option as a 'first-in-class' treatment for IBD employing a very different mechanisms of action to restore mucosal integrity in the absence of direct immune-suppression.

(C) AIM OF THE WORK

GLP-2 is a pleiotropic intestinotrophic hormone that has been shown therapeutically attractive in digestive diseases. But to date, several essential questions in this field remained unanswered. First, the mechanisms underlying the regulatory actions of GLP-2 and its signaling pathways have not yet been sufficiently elucidated. For instance, nothing so far has been reported about the signaling of GLP-2 in enteroendocrine cells. Second, the expression pattern and the regulatory mechanisms of GLP-2R are not thoroughly explored. For example, the sites expression of GLP-2R and their biological significance inside and outside the gastrointestinal tract are not well defined. Also, potential regulation of GLP-2R expression under pathophysiological conditions has never been estimated. Third, major discrepancies in GLP-2 action have been revealed between animal models and clinical studies. The findings suggested that both the low and the high dose of teduglutide were more effective than the intermediate dose whereas studies in normal mice have indicated that the intestinotropic effects of GLP-2 are dose dependent. Thus, extending GLP-2 findings in animal models to humans seems to be complicated. Fourth, potential GLP-2 treatment side effects, notably extra-intestinal effects and enhanced carcinogenesis warrants further investigation.

We believe that the field of GLP-2 research has disregarded fundamental information about GLP-2/GLP-2R action and expression, and was mostly concerned by the therapeutic management and the drug development of GLP-2 analogs. In this study, we underlined the importance of elucidating the cellular expression and the tissue distribution of GLP-2R, as safety endpoint for further functional and clinical studies. To this aim, the expression of GLP-2R transcript was examined in different human, mouse and rat cell lines. We investigated as well the expression and distribution of GLP-2R in 19 different mouse organs and tissues. To reveal potential regulation of GLP-2R in inflammatory states, we studied the intestinal expression of GLP-2R in two mice models of chemically-induced colitis and also in colonic and ileal biopsies from IBD patients. Enhanced knowledge about the sites of expression of GLP-2R and its regulation would lead to a better comprehension of the biological functions of GLP-2 and therefore improved perception of drug management and development.

(D) PERSONAL WORK

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Glucagon like peptide 2 (GLP-2) receptor expression is not restricted to the gastrointestinal tract and is modulated by inflammation

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RUNNING HEAD: Extraintestinal GLP-2R expression and effect of inflammation

ABSTRACT

The dual function of glucagon like peptide 2 (GLP-2) as growth factor and anti-inflammatory agent, has led to its consideration as a therapeutic potential in various digestive diseases. However, the localization of GLP-2 receptor (GLP-2R) remaining controversial, we examined its expression in various cell lines, in murine tissues, and in human and murine intestinal samples in inflammatory conditions. The expression of GLP-2R was detected in human colonic myofibroblasts (CCD-18Co) but not in intestinal epithelial cell lines, lymphocytes, monocytes, and umbilical vein endothelial cells. GLP-2R was expressed in murine (GLUTag), but not human (NCI-H716) enteroendocrine cells. We also detected GLP-2R expression in primary culture of rat enteric nervous system but not in enteric glial cells. The screening of GLP-2R mRNA in mice organs revealed an increasing gradient of GLP-2R toward the distal gut. Unexpectedly, a significant expression was detected in the central nervous system, mesenteric adipose tissue, mesenteric lymph nodes, bladder, spleen, and in the liver, particularly in hepatocytes. Using two experimental mice models of colitis, we showed that expression of GLP-2R mRNA was decreased in the colon of mice with TNBS- and DSS-induced colitis. Also, GLP-2R mRNA was significantly downregulated in intestinal tissues of IBD patients, notably in inflamed areas. In conclusion, previous hypotheses limiting GLP-2R expression and function to proximal bowel need to be revisited, and further studies should address the extra-intestinal biological function of GLP-2.

ABBREVIATIONS

CD: Crohn's disease, DSS: dextran sulfate sodium, GLP: Glucagon-like peptide, IBD: inflammatory bowel disease, MLN: mesenteric lymph nodes, SBS: short bowel syndrome, TNBS: Trinitrobenzene sulfonic acid, UC: ulcerative colitis

KEYWORDS

Glucagon like peptide – Inflammatory bowel disease – Enteroendocrine cell

INTRODUCTION

Glucagon like peptide 2 (GLP-2) is a peptide hormone secreted by intestinal neuroendocrine cells and displaying intestinotrophic cytoprotective and neuroprotective properties. GLP-2 has mainly trophic effects on the intestine including enhancement of crypt cell proliferation, inhibition of apoptosis, and stimulation of nutrient digestion and absorption (25). GLP-2 regulates the size and integrity of the gut following insult, as well as in response to disease and altered nutrient status (6, 7). The apparent intestinal specificity of GLP-2 properties, unlike other mitogenic factors, renders this peptide attractive for clinical use in intestinal dysfunction conditions. Since GLP-2 half life is short (7 min), there has been a growing interest in the development of protease-resistant analogs such as Gly²-GLP-2 and teduglutide which would extend the half-life of GLP-2 and improve its efficacy. Notable intestinal actions of these long-acting GLP-2 analogs have been demonstrated in humans and they are currently considered in clinical trials for intestinal insufficiencies and diseases. Favorable effects were reported in recent clinical trials (phase 3) with Teduglutide (Gattex) in the treatment of short bowel syndrome (SBS) (13). It is currently under investigation for the treatment of Crohn's disease (CD) (phase 2), and also in preclinical development for gastrointestinal mucositis in patients under chemotherapy (2, 13, 14).

The integrative responses to GLP-2 are mediated via the GLP-2 receptor (GLP-2R), which is a member of the glucagon/secretin G protein-coupled receptor (GPCR) super-family (20). Precise tissue distribution of GLP-2R expression remains poorly known both in rodents and humans. It has been reported that GLP-2R expression is mainly confined to the intestinal tract and particularly to the stomach, duodenum and proximal small bowel (6). However, several studies have revealed weak expression of GLP-2R in the colon (23). Whether or not that may play a role in neoplastic development following exogenous GLP-2 administration remains unanswered (29). Also, extra-intestinal expression of GLP-2R has been reported notably in central nervous system, lung, and pancreas in rodents and humans (5, 18, 32). At the cellular level, GLP-2R is expressed in the enteroendocrine L cells, in intestinal subepithelial myofibroblasts, in enteric neurons of the submucosal and myenteric plexus, in pancreatic alpha cells and astrocytes in brain. (5, 11, 18, 31).

To enhance our knowledge concerning the potential functions of GLP-2 analogs, a better understanding for GLP-2R expression is considered necessary. We therefore realized a panel of GLP-2R expression in mice tissues and in several human, murine and rat cells lines. Given the therapeutic beneficial effects of GLP-2 analogs in intestinal disorders, we investigated the intestinal expression of GLP-2R in mice models of chemically-induced colitis and in inflammatory bowel disease patients.

MATERIALS AND METHODS

Cell culture

The human cell lines used in this study are intestinal epithelial cell lines (differentiated or undifferentiated) (Caco-2, HT-29, 5F7), colonic myofibroblast cell line (CCD-18Co), hepatocyte cell line (HepG2), T lymphocyte cell line (Jurkat), monocytic cell line (THP-1). The human enteroendocrine cell line (NCI-H716) was sympathetically offered by Pr. Bart Steals (U1011, Lille, France). Leukemia T cells were a generous gift from Pr. Bruno Quesnel (U837, Lille, France). Human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Eric Boulanger (EA 2693, Lille, France). Human primary hepatocytes and primary hepatic myofibroblasts were a precious gift from Dr. Filoména Conti (UPRES 1833, Paris, France) and Pr. sophie Lotersztajn (IMRB17, Paris, France). The embryonic intestinal epithelial cell line (FHC, CRL-1831) was an appreciated gift from Pr. Laurence Gamet-Payraastre (UMR 1089, Toulouse, France). The mouse enteroendocrine cell line (GLUTag) was kindly offered by Pr. Daniel Drucker (SLRI, Mount Sinai Hospital, Toronto). The rat cell lines primary culture of enteric nervous system (ENS), primary culture of enteric glial cells (JUG2) and immortalized glial cell line (CRL2690) were generously offered by Dr. Michel Neunlist (U 913, Nantes, France).

Isolation of hepatocytes

For hepatocytes isolation, mice were anesthetized by subcutaneous injection of ketamine (virbac, France)/xylazine (Ceva, France). A "U"-shaped incision was made through the skin of the lower abdomen to the lateral aspect of the rib cage and back to the skin over the chest. The cannula was inserted into the exposed vena cava, and an EDTA-containing wash medium was allowed to perfuse through the liver at 5ml/min for approximately 15 minutes. The wash medium was replaced by a collagenase type IV solution (Sigma-Aldrich, France) and a second perfusion was performed for 10 minutes. The entire liver was removed and dissociated on a petri dish-containing collagenase solution. Crude hepatocyte preparation was filtered through a gauze mesh filter (100 μ m) and centrifuged at 50g for 2 min. The supernatant was discarded and the wash procedure was repeated three to four times. Hepatocytes were then cultured in DMEM (Invitrogen, France) + gentamycine (Invitrogen, France) + 100 nM dexamethasone (Invitrogen, France) with 5% CO₂ at 37°C. After 6-8 h, hepatocytes were adhered to the plate and culture medium was renewed. The viability (80.10⁶/liver) and purity (50%) of hepatocytes were verified using trypan blue and RAL555 staining.

Mouse tissue collection

For the evaluation of GLP-2R expression in mice tissues, a total of 8 mice were sacrificed and carefully dissected. Samples from nineteen different tissues and organs: brain, hypothalamus, heart,

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lung, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, mesenteric adipose tissue, mesenteric lymph nodes, spleen, liver, muscle, kidney and bladder were collected. Samples were snap-frozen immediately for subsequent RNA extraction.

Human tissue collection

Human tissue collection was conducted according to the National Statement on Ethical Conduct in Human Research and consent forms were signed by patients for the present study. Intestinal specimens were collected during surgery for Crohn's disease (CD) patients (n=19; 9 males, 10 females, average age 41 years) and ulcerative colitis (UC) patients (n=15; 8 males, 7 females, average age 50 years). Negative controls were patients operated on for isolated colorectal cancer (n=5, 3 males, 2 females, average age 53 years). Patients did not receive any treatment at time of surgery. Intestinal specimens were whole transperietal colon and ileum samples from healthy and inflamed regions. Healthy specimens were checked for absence of inflammation following macroscopic and histological examination. Intestinal samples from controls, Crohn's disease and ulcerative colitis patients were always collected from distal ileum (20cm ahead of the cecum) and distal colon (25cm ahead of the rectum). Specimens from colorectal cancer patients were collected in the negative surgical margin.

Animal experimentation

Environmental conditions

Animal experiments were performed in accredited facilities at the Institut Pasteur (Lille, France) according to governmental guidelines. Animals were housed five per cage and had free access to standard mouse chow and tap water.

Trinitrobenzene sulfonic acid-induced colitis

C57bl6 mice, 9 weeks old, were anesthetized for 90–120 minutes and received an intrarectal administration of trinitrobenzene sulfonic acid (TNBS, 40 μ l, 150 mg/kg) dissolved in a 1:1 mixture of 0.9% NaCl with 100% ethanol. Control mice received a 1:1 mixture of 0.9% NaCl with 100% ethanol or a saline solution using the same technique. Animals harvest was performed 3 days after TNBS administration. Intestinal inflammation was evaluated macroscopically using the Wallace score. Colon samples were dehydrated and paraffin-embedded for histological analysis (Ameho score). For molecular analysis of GLP-2R expression, colon samples (2cm ahead of the rectum) were immediately frozen and stored at -80°C for RNA extraction.

Dextran sodium sulfate-induced colitis

Colitis was induced in 9 weeks BALB/c mice by one cycle of 5% dextran sulfate sodium (DSS) dissolved in sterile drinking water for 7 days. Animals were then harvested for clinical evaluation of colitis by assessing disease activity index including mice weight, stool consistency, bloody stool. Colon samples were dehydrated and paraffin-embedded for histological analysis. For molecular analysis of GLP-2R expression, colon samples (2cm ahead of the rectum) were immediately frozen and stored at -80°C for RNA extraction.

Primers design

GLP-2R primers (Table 1) for quantitative PCR were designed according to the nucleotide sequences published in Genebank (Human GLP-2R NM_004246, Mouse GLP-2R NM_175681, Rat GLP-2R NM_021848). Primers efficiency test was performed using serial dilutions of complementary DNA. The efficiency of the primers obtained was $\approx 98\%$ and the slope of the standard curve was equal to -3.2 ± 0.1 . The specificity of the primers was verified by sequencing the amplified product. Total RNA was isolated using NucleoSpin RNA kit (Macherey-Nagel) from human colonic myofibroblast cell line (CCD-18Co), mouse enteroendocrine cell line (GLUTag), and from primary culture of rat enteric nervous system (ENS) according to the manufacturer's instructions. First strand cDNA was synthesized by reverse transcription by means of random primers using the High capacity cDNA reverse transcription kit (Applied Biosystems). Complementary DNA was amplified using Taq DNA polymerase Native (Invitrogen) using specific oligonucleotides (Table 1). Amplified DNA was allowed to migrate on 3% agarose gel for 1 hour. Later, DNA bands were cut off from the gel and purified using Nucleospin PCR cleanup (Macherey-Nagel). DNA sequencing was performed by GenoScreen (Lille, France). Amplified sequences were 100% specific for the target gene.

Real-Time Polymerase chain reaction

Total RNA from cell lines, from human and mice tissues was isolated using NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions. RNA quantification was performed using spectrophotometry. First strand cDNA was synthesized by reverse transcription of 1 μg RNA using the High capacity cDNA reverse transcription kit (Applied Biosystems) as prescribed by the supplier. Complementary DNA was stored at -20°C . Gene expression was quantified using SYBR green Master Mix (Applied Biosystems, Courtaboeuf, France) with specific oligonucleotides (Table 1) in a GeneAmp ABIprism 7000 (Applied Biosystems, Courtaboeuf, France). In each assay, calibrated and no-template controls were included. SYBR green dye intensity was analyzed using the ABIprism 7000 SDS software (Applied Biosystems, Courtaboeuf, France). All results were normalized to the beta-actin.

Agarose gel electrophoresis

Total RNA was isolated using NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions. RNA quantification was performed using spectrophotometry. First strand cDNA was synthesized by reverse transcription of 1 µg RNA using the High capacity cDNA reverse transcription kit (Applied Biosystems) as prescribed by the supplier and complementary DNA was amplified using Taq DNA polymerase Native (Invitrogen). Amplified DNA was allowed to migrate on 3% agarose gel for later visualization using Gel documentation system. The size of the amplified products (Human transcript: 150bp, murine transcript: 185 bp) was verified by co-electrophoresis of a 100 base pair nucleotide marker (GibcoBRL).

Statistical Analysis

Statistical analyses were performed using the Wilcoxon Test for two independent samples. Statistics were calculated using SPSS software (SPSS Inc., Chicago, USA). Differences were considered statistically significant if the *p* value was <0.05. Values were represented as mean ± standard error of the mean (SEM).

RESULTS

GLP-2R mRNA expression in rodent and human cell lines

The expression of GLP-2R was examined in different primary and transformed cell lines of human, murine and rat origin (Figure 1). We first investigated the expression of GLP-2R mRNA in intestine-derived cells. Among cell lines tested, the presence of GLP-2R mRNA was detected in human intestinal subepithelial myofibroblasts (CCD-18Co), in murine enteroendocrine cell line (GLUTag) and in primary culture of rat enteric nervous system (ENS). Noticeably, GLP-2R was expressed in colonic myofibroblasts (CCD-18Co) but not in human intestinal epithelial cell lines Caco-2, HT-29, and 5F7 whether they were in a differentiated or undifferentiated state. Regarding the intestinal endocrine system, GLP-2R mRNA was detected in the murine enteroendocrine cell line GLUTag but not in the human analogous cell line NCI-H716 even if differentiated on a matrigel. The expression of GLP-2R was found in the primary culture of the enteric nervous system (ENS) but not in primary (JUG2) and transformed glial cells (CRL2690).

Beside intestine-derived cells, we have also examined GLP-2R expression in cells of the immune system. Neither human primary T lymphocytes nor Jurkat and THP-1 cell lines express GLP-2R transcript. GLP-2R mRNA was as well absent from human umbilical vein endothelial cell culture (Figure 1).

GLP-2R mRNA expression in mouse tissues

We investigated the expression of GLP-2R mRNA by RT-PCR and agarose gel electrophoresis in nineteen different mice organs and tissues in a total of eight mice. The highest GLP-2R expression was unexpectedly detected in the distal gut (colon and rectum) and bladder (Figure 2A). Along the intestinal tract, the profile of GLP-2R mRNA expression followed an increasing gradient from the duodenum to the distal colon (Figure 2A, 3A). There was no detectable expression in the esophagus.

Our data also revealed a widespread extraintestinal expression of GLP-2R transcript. Beside previously reported expression in the brain of mice, particularly in the hypothalamus, an unexpected expression was observed in the bladder, mesenteric adipose tissue, mesenteric lymph nodes, and to a lesser extent in the spleen and liver. There was no detectable expression in muscle, lung, kidney and heart samples (Figure 2B). Surprisingly, we found significant expression of GLP-2R in the liver. We therefore decided to investigate this observation by identifying GLP-2R expressing cells in the liver. Figure 3.B,C showed that GLP-2R transcript was present in human and mouse hepatocytes but not in hepatic myofibroblasts. Of note, GLP-2R was expressed in primary but not in the immortalized hepatocytes HepG2 and HuH7.

Differential expression of GLP-2R mRNA in mouse and human tissues according to inflammation

In order to assess the impact of the inflammatory environment on GLP-2R expression, we examined GLP-2R mRNA expression in the colon of mice with trinitrobenzene sulfonic acid (TNBS)-induced colitis. As shown in figure 4 (A, B, C) macroscopic and histological examination showed that a significant inflammation was established in the colon of mice three days after intra-rectal administration of TNBS (histological score 4.52 ± 0.22 vs 0.1 ($p < 0.001$); macroscopic score 5.94 ± 0.29 vs 0.2 ± 0.13 ($p < 0.001$)). The expression of GLP-2R transcript in the colon of TNBS-induced colitis was decreased by 58% ($p < 0.05$) compared to controls (Figure 4D). To confirm the decreased colonic expression of GLP-2R under inflammatory conditions, another model of chemically-induced colitis was employed. Dextran sulfate sodium was administered to mice in drinking water for 5 days followed by animal harvest and colitis evaluation. The severity of intestinal inflammation was assessed by histological examination and clinical evaluation of disease activity (mice weight, stool consistency, and presence of blood in the stool). As shown in figure 5 (E, F, G), DSS treated mice had significant colonic inflammation (histological score 11.8 ± 0.49 vs 0 ($p < 0.001$); macroscopic score 9 ± 1.45 vs 0 ($p < 0.001$)). Similarly to TNBS-treated mice, the expression of GLP-2R mRNA in the colon of mice with DSS-induced colitis was decreased by 53% ($p < 0.05$) compared to control mice without colitis (Figure 5H).

We extended the assessment to human samples and we examined the expression of GLP-2R transcript in ileal and colonic tissues of IBD patients. First, we compared the colonic versus ileal expression of GLP-2R in healthy controls, CD and UC patients. Colonic and ileal samples were collected 25cm ahead of the rectum and 20 cm ahead the cecum in regions displaying no signs of inflammation following macroscopic and histological examinations. The expression of GLP-2R in the ileum of healthy controls was reduced by 60% compared to colonic expression ($p < 0.01$). Interestingly, non-inflamed intestinal regions of IBD patients displayed similar expression pattern of GLP-2R with 60% ($p < 0.05$) and 90% ($p < 0.001$) decrease in the ileum of CD and UC patients compared to the colonic expression, respectively (Figure 5A). Thus, in line with the data observed in mouse intestine, the colon displayed higher GLP-2R expression than the ileum.

Regarding the expression of GLP-2R in intestinal samples of IBD patients compared to controls. The expression of GLP-2R in healthy colon of CD patients was decreased by 50% ($p < 0.01$) compared to controls. This expression was further decreased by 80% in inflamed colon of CD patients ($p < 0.005$). Similarly, both healthy and inflamed ileal samples from CD patients displayed 65% ($p < 0.01$) and 85% ($p < 0.005$) decrease in GLP-2R expression compared to controls (Figure 5B).

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Therefore, in CD patients, inflamed colonic and ileal samples displayed further reduced expression of GLP-2R compared to non-inflamed samples (61% and 67%; $p < 0.05$ for colon and ileum respectively).

In UC patients, the expression of GLP-2R was decreased by 70% in inflamed colonic samples compared to controls. However, healthy colonic samples from UC patients displayed similar GLP-2R expression to samples from controls. Conversely, a decrease of 90% in GLP-2R expression was detected in healthy ileal samples from UC patients as compared to healthy patients (Figure 5C).

Overall, these data demonstrate first an enhanced expression of GLP-2R in the colon as compared to ileum in both healthy subjects and IBD patients, and secondly, a reduced expression of GLP-2R in normal tissues as well as in inflamed zones of colon and ileum of IBD patients.

DISCUSSION

Although there is a wealth of information about the ultimate effects of GLP-2 on intestinal physiology, conclusive data about the expression level and the localization of GLP-2R are still lacking. In this study we demonstrated that GLP-2R seems to be more widely expressed than it was estimated. Beside GLP-2R presence in the central nervous system as previously reported by *Lovshin, J. et al.* (18, 19), we have detected an unexpected expression of GLP-2R mRNA in the bladder, mesenteric adipose tissue, as well as in the mesenteric lymph nodes, spleen and liver (Figure 2). The expression pattern of GLP-2R has been the subject of ongoing debate and disagreement in the literature. GLP-2R was shown by *Lovshin et al.* to be expressed in hypothalamic and extrahypothalamic regions in mice but was completely absent from the central nervous system in the work of *Drucker D.J.* (18, 32). In addition, *de Heer et al.* have localized GLP-2R on alpha cells of both human and rat pancreas but *Yusta et al.* failed to detect GLP-2R transcript in pancreatic mouse tissue and rat insulinoma cell lines (5, 32). The latter group has also reported GLP-2R expression in rat but not in mouse lungs. Furthermore, *Thulesen, J. et al.* have reported positive accumulation of GLP-2 in rat liver but were not able to define whether this detection represented a specific receptor binding or a non-specific metabolic fate (28). On the other hand, the group of *Drucker D.J.* has shown by Northern blot that GLP-2R transcript was absent from mouse and rat liver tissues (32). Thus, an agreement on GLP-2R expression has not yet been established. The reasons behind these discrepancies would be from one side the characteristic cell-specific location of GLP-2R and from the other side the lack of specific tools to assess this particular expression. Aware of these difficulties and to reinforce our data, we opted for a particular strategy for GLP-2R primers design. The oligonucleotides were thoroughly designed and the amplicon was sequenced to confirm the specificity of the detection (see materials and methods). In our work, we demonstrated that GLP-2R mRNA was expressed in various organs displaying important physiological functions. Therefore, although pharmacological studies have characterized GLP-2 analogs devoid of noticeable extraintestinal actions, the concern that GLP-2 agonists could have potential effects on various physiological systems warrants further investigation as safety endpoints in clinical trials.

It was widely believed that GLP-2R is predominantly expressed in the stomach and proximal small bowel (5, 11, 32). Nevertheless, exhaustive quantification of GLP-2R distribution along the intestinal tract is still lacking. Our results showed that the expression of GLP-2R transcript in mice increased gradually while proceeding distally and appeared to be the highest in the colon and rectum (Figure 2.A and 3.A). More importantly, in man, the colonic expression of GLP-2R in human samples was as well significantly higher than ileal expression (Figure 5A). The predominant expression of GLP-2R in the distal bowel was in accordance with the known distribution in the colon of GLP-2R

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expressing enteroendocrine L cells (32). Our observations were also in line with data by *Taylor-Edwards C.C. et al.* who have evaluated the expression of GLP-2R transcript in various intestinal compartments of ruminants. They have shown that there was a gradient of expression of GLP2R mRNA from forestomach tissues to the distal colon (27). Therefore, the expression of GLP-2R and the biological activities of GLP-2 in the colon should not be underestimated anymore. Also, whether there is a link between the high GLP-2R colonic expression and enhanced risk of colorectal cancer following exogenous GLP-2 administration warrants further investigation (29).

Preclinical studies in animal models of intestinal disorders and clinical trials in humans have suggested an important role for GLP-2 in intestinal adaptation and repair during inflammatory events (10, 13, 15). However, in these studies nothing has been reported about the potential regulation of GLP-2R expression under inflammatory conditions. Here, we demonstrated that the intestinal expression of GLP-2R was significantly decreased in experimental mice models of colitis and in IBD patients (Figure 4 and 5). We showed that, non-inflamed regions in IBD patients displayed reduced colonic and ileal GLP-2R expression compared to healthy controls. Whether or not this diminished expression is due to pre-existent background or is secondary to the active disease remains to be determined. Moreover, in inflamed zones of CD and UC patients, the expression of GLP-2R mRNA was further decreased compared to non-inflamed regions. Whether the downregulation of GLP-2R is a cause or a consequence of the inflammatory milieu warrants further exploration. In fact, the profile of intestinal inflammation in the two models of TNBS- and DSS-induced colitis employed in this study is quite different. Whereas TNBS induced a T-cell mediated response in the colon, DSS exhibited a direct toxicity on epithelial cells. Thus, the fact that GLP-2R expression was decreased in two different experimental models of colitis (and also in IBD patients), implies that this downregulation could be secondary to inflammatory signals. Indeed, severe intestinal inflammation is characterized by mucosa destruction which could result in potential loss of GLP-2R expressing cells (enteroendocrine cells and subepithelial myofibroblasts). Thus, until profoundly investigated, the decrease in intestinal expression of GLP-2R mRNA in IBD patients could explain the limited efficacy of GLP-2 analogs in the treatment of CD as compared to its recognized efficiency in short bowel treatment which is usually not in majority an inflammatory condition (2, 13).

At the cellular level, we showed as others that GLP-2R was not expressed in intestinal epithelial cell lines but prominently present in mouse intestinal myofibroblastic cell line, CCD-18Co and in rat primary culture of enteric neurons (ENS) (Figure 1). Within this context, It was proposed that GLP-2 exerts its intestinotrophic actions indirectly, via downstream mediators derived from GLP-2R-expressing cells (1, 11, 23, 32). GLP-2 signaling through subepithelial myofibroblasts and enteric

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neurons induced the release of several growth and anti-inflammatory factors mediating the proliferative and cytoprotective effects of GLP-2 (8, 9, 11, 12, 16, 17, 21, 23, 26). Concerning the enteroendocrine system, immunostaining studies have well documented the presence of GLP-2R in neuroendocrine cells in human and porcine intestine (11). In our study, we showed that GLP-2R was expressed in the murine enteroendocrine cell line GLUTag cells but completely absent from the human analogous cells NCI-H716 (Figure 1). This suspects the initially documented endocrine functions of NCI-H716 cells (4). In fact, the classification of NCI-H716 as a human enteroendocrine cell line has been recently revisited. Several mediators (Insulin, phorbol myristate acetate) known as regulators of proglucagon gene expression in primary rodent islet cell lines, had no effect on the human proglucagon gene expression. Also, human proglucagon promoter was shown to be transcriptionally inactive in NCI-H716 cells (3, 22, 24). Altogether, these data do not support the adequacy of NCI-H716 cells as a valid model of human enteroendocrine system.

CONCLUSION

Our work has shown that GLP-2R expression was expressed in the whole digestive tract, but predominantly in the distal bowel. We have also shown that GLP-2R was expressed outside the digestive tract including the bladder, liver, spleen, mesenteric lymph nodes. Therapeutic trials have shown that GLP-2 analogs will likely require chronic administration given that its growth-promoting effects were reversed upon withdrawal of treatment (14, 30). Thus, long-term surveillance studies are needed to weigh the benefits of GLP-2 treatment versus potential adverse outcomes.

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REFERENCES

1. **Bjerknes M, and Cheng H.** Modulation of specific intestinal epithelial progenitors by enteric neurons. *Proceedings of the National Academy of Sciences of the United States of America* 98: 12497-12502, 2001.
2. **Buchman AL, Katz S, Fang JC, Bernstein CN, and Abou-Assi SG.** Teduglutide, a novel mucosally active analog of glucagon-like peptide-2 (GLP-2) for the treatment of moderate to severe Crohn's disease. *Inflammatory bowel diseases* 16: 962-973, 2010.
3. **Cao X, Flock G, Choi C, Irwin DM, and Drucker DJ.** Aberrant regulation of human intestinal proglucagon gene expression in the NCI-H716 cell line. *Endocrinology* 144: 2025-2033, 2003.
4. **de Bruine AP, Dinjens WN, Pijls MM, vd Linden EP, Rousch MJ, Moerkerk PT, de Goeij AF, and Bosman FT.** NCI-H716 cells as a model for endocrine differentiation in colorectal cancer. *Virchows Archiv B, Cell pathology including molecular pathology* 62: 311-320, 1992.
5. **de Heer J, Pedersen J, Orskov C, and Holst JJ.** The alpha cell expresses glucagon-like peptide-2 receptors and glucagon-like peptide-2 stimulates glucagon secretion from the rat pancreas. *Diabetologia* 50: 2135-2142, 2007.
6. **Drucker DJ, Erlich P, Asa SL, and Brubaker PL.** Induction of intestinal epithelial proliferation by glucagon-like peptide 2. *Proceedings of the National Academy of Sciences of the United States of America* 93: 7911-7916, 1996.
7. **Dube PE, and Brubaker PL.** Frontiers in glucagon-like peptide-2: multiple actions, multiple mediators. *Am J Physiol Endocrinol Metab* 293: E460-465, 2007.
8. **Dube PE, Forse CL, Bahrami J, and Brubaker PL.** The essential role of insulin-like growth factor-1 in the intestinal tropic effects of glucagon-like peptide-2 in mice. *Gastroenterology* 131: 589-605, 2006.
9. **Dube PE, Rowland KJ, and Brubaker PL.** Glucagon-like peptide-2 activates beta-catenin signaling in the mouse intestinal crypt: role of insulin-like growth factor-I. *Endocrinology* 149: 291-301, 2008.
10. **Garrison AP, Dekaney CM, von Allmen DC, Lund PK, Henning SJ, and Helmrath MA.** Early but not late administration of glucagon-like peptide-2 following ileo-cecal resection augments putative intestinal stem cell expansion. *American journal of physiology Gastrointestinal and liver physiology* 296: G643-650, 2009.
11. **Guan X, Karpen HE, Stephens J, Bukowski JT, Niu S, Zhang G, Stoll B, Finegold MJ, Holst JJ, Hadsell D, Nichols BL, and Burrin DG.** GLP-2 receptor localizes to enteric neurons and endocrine cells expressing vasoactive peptides and mediates increased blood flow. *Gastroenterology* 130: 150-164, 2006.
12. **Guan X, Stoll B, Lu X, Tappenden KA, Holst JJ, Hartmann B, and Burrin DG.** GLP-2-mediated up-regulation of intestinal blood flow and glucose uptake is nitric oxide-dependent in TPN-fed piglets. *Gastroenterology* 125: 136-147, 2003.
13. **Jeppesen PB, Gilroy R, Pertkiewicz M, Allard JP, Messing B, and O'Keefe SJ.** Randomised placebo-controlled trial of teduglutide in reducing parenteral nutrition and/or intravenous fluid requirements in patients with short bowel syndrome. *Gut* 60: 902-914, 2011.
14. **Jeppesen PB, Sanguinetti EL, Buchman A, Howard L, Scolapio JS, Ziegler TR, Gregory J, Tappenden KA, Holst J, and Mortensen PB.** Teduglutide (ALX-0600), a dipeptidyl peptidase IV resistant glucagon-like peptide 2 analogue, improves intestinal function in short bowel syndrome patients. *Gut* 54: 1224-1231, 2005.
15. **Kato Y, Yu D, and Schwartz MZ.** Glucagonlike peptide-2 enhances small intestinal absorptive function and mucosal mass in vivo. *Journal of pediatric surgery* 34: 18-20; discussion 20-11, 1999.
16. **Leen JL, Izzo A, Upadhyay C, Rowland KJ, Dube PE, Gu S, Heximer SP, Rhodes CJ, Storm DR, Lund PK, and Brubaker PL.** Mechanism of action of glucagon-like peptide-2 to increase IGF-I mRNA in intestinal subepithelial fibroblasts. *Endocrinology* 152: 436-446, 2011.

17. **Liu X, Murali SG, Holst JJ, and Ney DM.** Enteral nutrients potentiate the intestinotrophic action of glucagon-like peptide-2 in association with increased insulin-like growth factor-I responses in rats. *American journal of physiology Regulatory, integrative and comparative physiology* 295: R1794-1802, 2008.
18. **Lovshin J, Estall J, Yusta B, Brown TJ, and Drucker DJ.** Glucagon-like peptide (GLP)-2 action in the murine central nervous system is enhanced by elimination of GLP-1 receptor signaling. *J Biol Chem* 276: 21489-21499, 2001.
19. **Lovshin JA, Huang Q, Seaberg R, Brubaker PL, and Drucker DJ.** Extrahypothalamic expression of the glucagon-like peptide-2 receptor is coupled to reduction of glutamate-induced cell death in cultured hippocampal cells. *Endocrinology* 145: 3495-3506, 2004.
20. **Munroe DG, Gupta AK, Kooshesh F, Vyas TB, Rizkalla G, Wang H, Demchyshyn L, Yang ZJ, Kamboj RK, Chen H, McCallum K, Sumner-Smith M, Drucker DJ, and Crivici A.** Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2. *Proceedings of the National Academy of Sciences of the United States of America* 96: 1569-1573, 1999.
21. **Nelson DW, Murali SG, Liu X, Koopmann MC, Holst JJ, and Ney DM.** Insulin-like growth factor I and glucagon-like peptide-2 responses to fasting followed by controlled or ad libitum refeeding in rats. *American journal of physiology Regulatory, integrative and comparative physiology* 294: R1175-1184, 2008.
22. **Nian M, Drucker DJ, and Irwin D.** Divergent regulation of human and rat proglucagon gene promoters in vivo. *The American journal of physiology* 277: G829-837, 1999.
23. **Orskov C, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, and Holst JJ.** GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors. *Regul Pept* 124: 105-112, 2005.
24. **Powers AC, Philippe J, Hermann H, and Habener JF.** Sodium butyrate increases glucagon and insulin gene expression by recruiting immunocytochemically negative cells to produce hormone. *Diabetes* 37: 1405-1410, 1988.
25. **Rowland KJ, and Brubaker PL.** The "cryptic" mechanism of action of glucagon-like peptide-2. *American journal of physiology Gastrointestinal and liver physiology* 301: G1-8, 2011.
26. **Sigalet DL, Wallace LE, Holst JJ, Martin GR, Kaji T, Tanaka H, and Sharkey KA.** Enteric neural pathways mediate the anti-inflammatory actions of glucagon-like peptide 2. *Am J Physiol Gastrointest Liver Physiol* 293: G211-221, 2007.
27. **Taylor-Edwards CC, Burrin DG, Matthews JC, McLeod KR, Holst JJ, and Harmon DL.** Expression of mRNA for proglucagon and glucagon-like peptide-2 (GLP-2) receptor in the ruminant gastrointestinal tract and the influence of energy intake. *Domestic animal endocrinology* 39: 181-193, 2010.
28. **Thulesen J, Hartmann B, Orskov C, Jeppesen PB, Holst JJ, and Poulsen SS.** Potential targets for glucagon-like peptide 2 (GLP-2) in the rat: distribution and binding of i.v. injected (125)I-GLP-2. *Peptides* 21: 1511-1517, 2000.
29. **Trivedi S, Wiber SC, El-Zimaity HM, and Brubaker PL.** Glucagon-like peptide-2 increases dysplasia in rodent models of colon cancer. *American journal of physiology Gastrointestinal and liver physiology* 302: G840-849, 2012.
30. **Tsai CH, Hill M, and Drucker DJ.** Biological determinants of intestinotrophic properties of GLP-2 in vivo. *The American journal of physiology* 272: G662-668, 1997.
31. **Velazquez E, Ruiz-Albusac JM, and Blazquez E.** Glucagon-like peptide-2 stimulates the proliferation of cultured rat astrocytes. *Eur J Biochem* 270: 3001-3009, 2003.
32. **Yusta B, Huang L, Munroe D, Wolff G, Fantáske R, Sharma S, Demchyshyn L, Asa SL, and Drucker DJ.** Enteroendocrine localization of GLP-2 receptor expression in humans and rodents. *Gastroenterology* 119: 744-755, 2000.

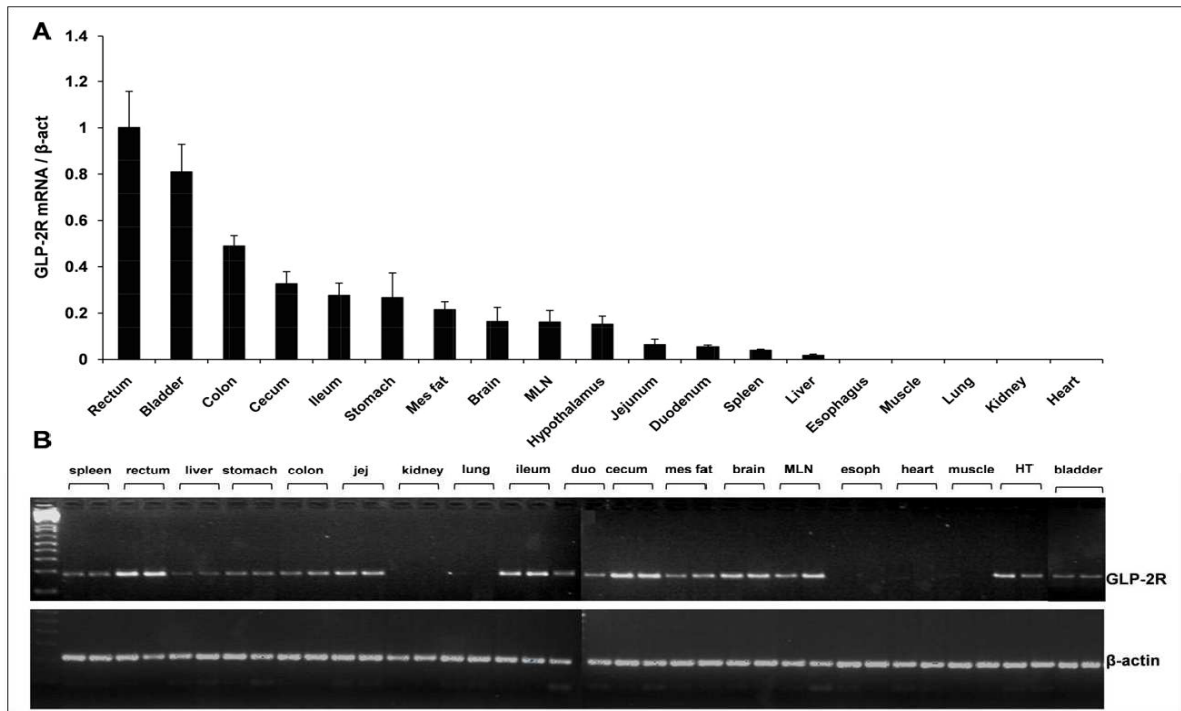


FIGURE 2: Glucagon-like peptide 2 receptor mRNA expression in mouse tissues.

A. Expression of GLP-2R mRNA/ β -actin in mouse tissues. GLP-2R mRNA expression was expressed as relative expression with respect to the rectum (highest expression) normalized to 1. GLP-2R is predominantly expressed in the distal gastrointestinal tract. An unexpected expression was detected in the mesenteric adipose tissue, mesenteric lymph nodes, bladder, spleen and liver.

B. Agarose gel electrophoresis for GLP-2R transcript in mouse tissues. GLP-2R mRNA is not detectable in the kidney, lung, esophagus, muscle, and heart tissues (Abbreviations; MLN: mesenteric lymph nodes; jej: jejunum; duo: duodenum; mes fat: mesenteric fat; esoph: esophagus; HT: hypothalamus). Values are expressed as mean \pm SEM in 8 samples.

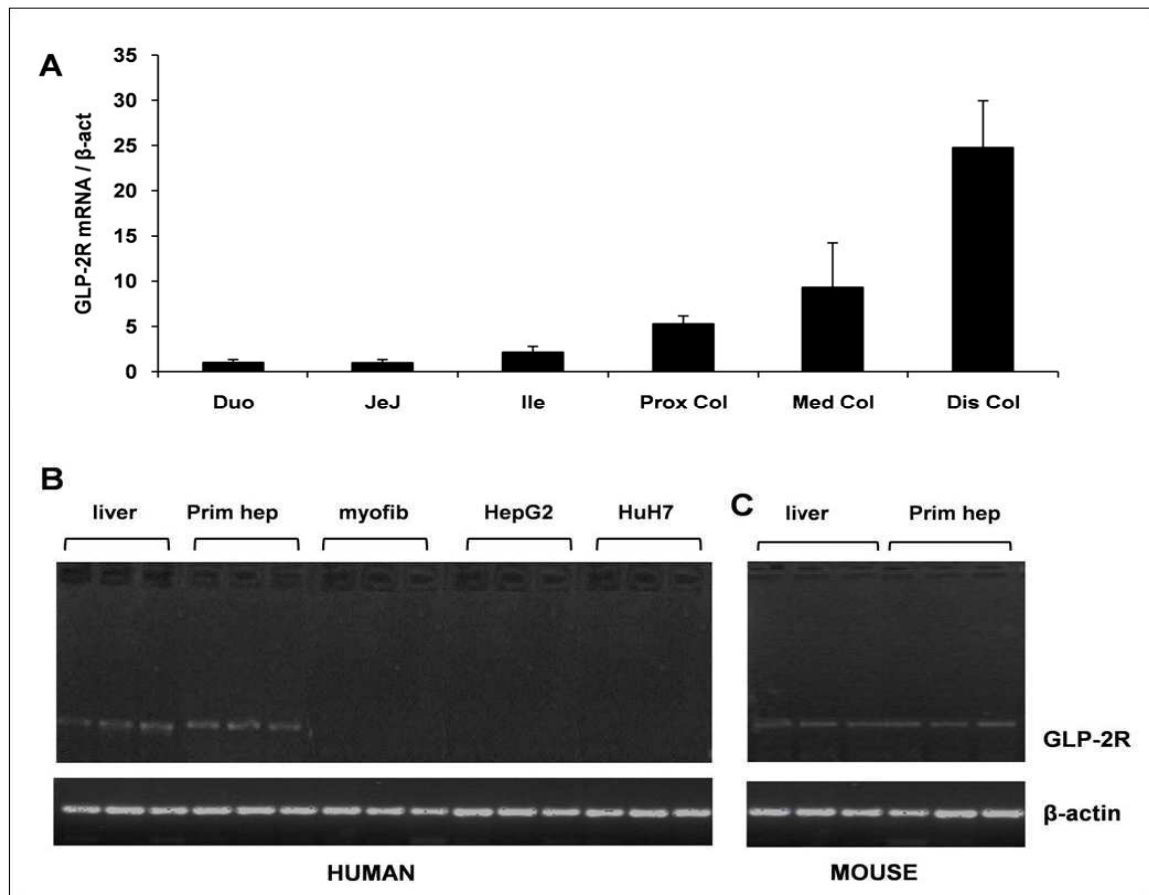


FIGURE 3: Glucagon-like peptide 2 receptor mRNA expression in intestinal and hepatic samples.

A. Expression of GLP-2R mRNA/ β -actin in mouse intestinal samples from the duodenum (Duo), jejunum (JeJ), ileum (Ile), proximal colon (Prox Col), medial colon (Med Col), and distal colon (Dis Col). The expression of GLP-2R mRNA increases gradually from duodenum to distal colon. GLP-2R mRNA expression was expressed as relative expression with respect to the duodenum normalized to 1. **B.** Agarose gel electrophoresis for GLP-2R transcript in human total liver, primary culture of hepatocytes, primary culture of hepatic myofibroblasts and in HepG2 and HuH7 cell lines. GLP-2R is expressed in human liver and only detectable in primary hepatocytes. **C.** Agarose gel electrophoresis for GLP-2R transcript in mouse total liver and primary culture of hepatocytes. GLP-2R is also expressed in murine primary hepatocytes.

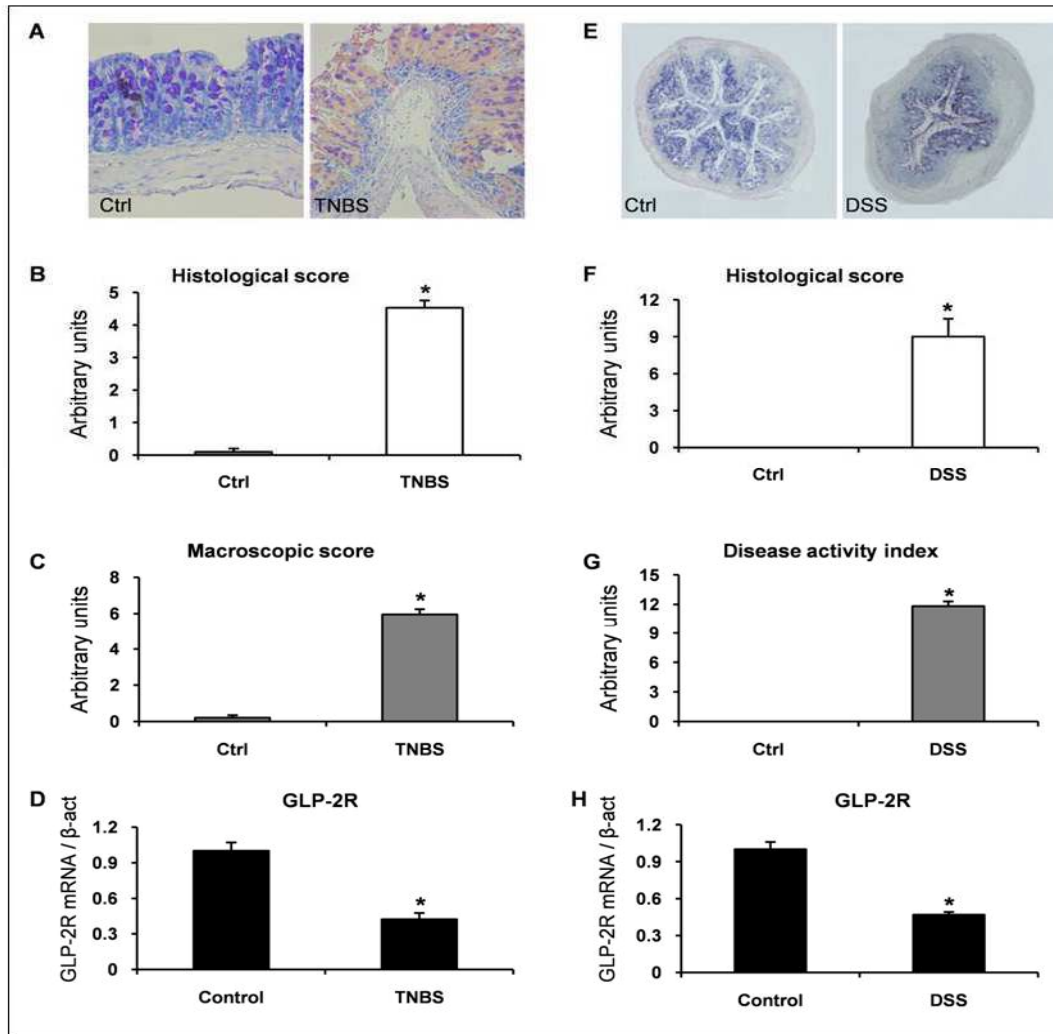


FIGURE 4: Glucagon-like peptide 2 receptor mRNA expression in experimental colitis. A-D Model of TNBS-induced colitis (n=60 mice). Intestinal inflammation was induced by intra-rectal administration of TNBS. The severity and extent of intestinal inflammation was assessed at day 3 post TNBS by **(A,B)** histological and **(C)** macroscopic score. **D**, Relative expression of GLP-2R mRNA/ β -actin in colonic tissues from TNBS-induced colitis. GLP-2R mRNA was expressed as relative expression with respect to control mice (no colitis) normalized to 1. Values are expressed as mean \pm SEM. $p < 0.05$. **E-H** Model of DSS-induced colitis (n=60 mice). Intestinal inflammation was induced by administration of DSS in drinking water for 7 days. The severity and extent of intestinal inflammation was assessed at day 7 by **(E,F)** histological and **(G)** disease activity index. **H**, Relative expression of GLP-2R mRNA/ β -actin in colonic tissues from DSS-induced colitis. GLP-2R mRNA was expressed as relative expression with respect to control mice (no colitis) normalized to 1. Values are expressed as mean \pm SEM. $p < 0.05$.

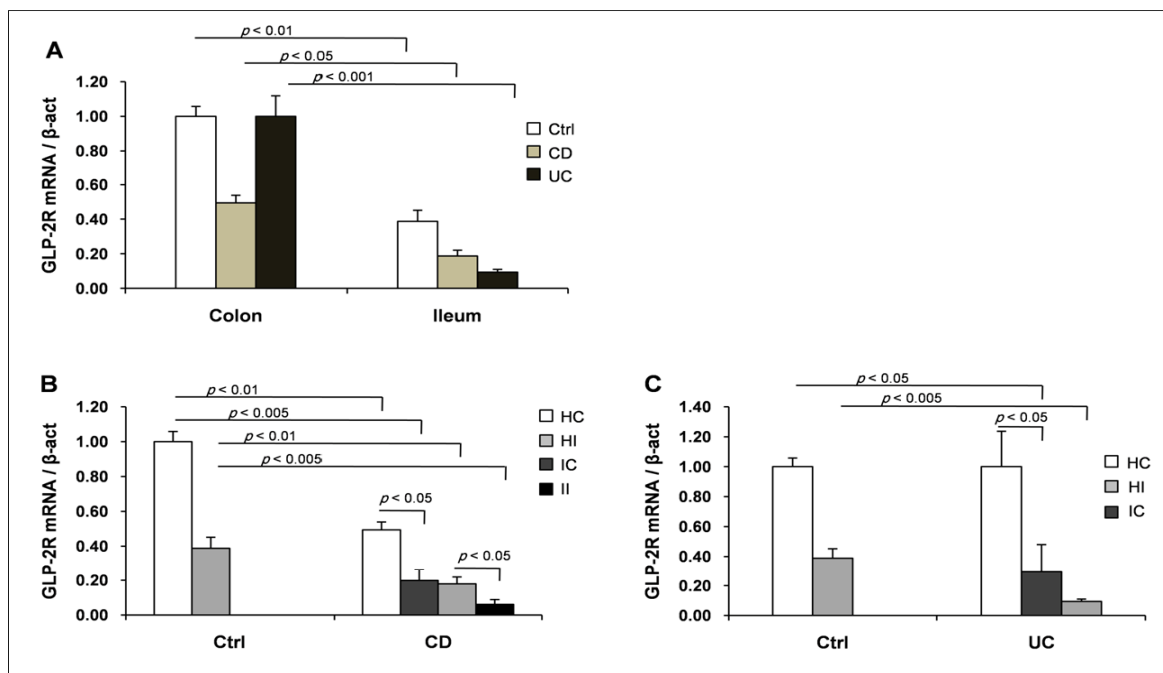


FIGURE 5: Glucagon-like peptide 2 receptor expression in intestinal tissues of inflammatory bowel disease patients. **A.** Expression of GLP-2R mRNA/ β -actin in healthy colon and ileum samples (n=10 for each condition) from controls, Crohn’s disease and Ulcerative colitis patients. GLP-2R mRNA was expressed as relative expression with respect to colonic samples from healthy controls normalized to 1. **B and C.** Expression of GLP-2R mRNA/ β -actin in healthy (healthy colon: HC; healthy ileum: HI) and inflamed (inflamed colon: IC; inflamed ileum: II) intestinal samples from CD (**B**) and UC (**C**) patients compared to samples from healthy controls. GLP-2R mRNA was expressed as relative expression with respect to colonic samples from healthy controls normalized to 1. Values are expressed as mean \pm SEM.

TABLES

Table 1: List of primers

Human primers	(5' → 3')
β-actin	F - TCACCCACACTGTGCCCATCTACG R - CAGCGGAACCGCTCATTGCCAATG
GLP-2R	F - CTGGTCCTCATTCCCTTTATTGGG R - GGCAAAACCATACTGCAAGGC
Mouse primers	(5' → 3')
β-actin	F - CCATGTCGTCCCAGTTGGTAA R - GAATGGGTCAGAAGGACTCCTATGT
GLP-2R	F - TGCTCGAGAAACCTTCTGGT R - CTCTTGCTTCTGCCAAGTCC
Rat primers	(5' → 3')
β-actin	F - AGCCATGTACGTAGCCATCC R - CTCTCAGCTGTGGTGGTGAA
GLP-2R	F - CAGTGCCCAGTAGATGCAGA R - TGTTGGGAGTGGAGAGGAAC

(E) CONCLUSION AND PERSPECTIVES

Since the original report of the intestinotrophic actions of GLP-2 by Drucker and colleagues [5], a number of studies have demonstrated the therapeutic efficacy of GLP-2 and the protease-resistant GLP-2 analogs in several animal models as well as in patients with intestinal dysfunction [150]. Currently, there is now mounting clinical evidence that GLP-2 can provide a new therapeutic strategy in SBS patients in whom it may pave the path towards reducing parenteral nutrition dependence [69].

However, there are still several gaps in our knowledge regarding the intestinal response to GLP-2. This is mainly due to the lack of information about the expression pattern and the regulation of GLP-2R. To enhance our knowledge concerning the potential functions of GLP-2 analogs, a better understanding for GLP-2R expression is considered necessary. In this work, we showed that the expression of GLP-2R is not by any means restricted to the proximal bowel as it was believed. We showed that at least six organs out of the gastrointestinal tract exhibit detectable expression of GLP-2R including, the bladder, mesenteric adipose tissue, mesenteric lymph nodes, central nervous system, spleen and liver. These observations suggest that the intestinal functions of GLP-2 may be functionally linked to currently unidentified whole body actions. Indeed, recent studies have identified bone as a target of GLP-2 action, although how these effects are mediated is completely unknown [151]. Thus, even though clinical studies have so far identified GLP-2 analogs as therapeutically attractive in gastrointestinal diseases, hypotheses pretending that GLP-2 or GLP-2 analogs are devoid of extra-intestinal effects need to be revisited. Clinical trials have demonstrated that GLP-2 appears safe and well tolerated in the short and medium term. Nevertheless, this would not exclude the appearance of adverse outcomes following long term treatment in the intestine or others organs carrying the receptor. This observation is particularly important since it has been shown that GLP-2 analogs will likely require chronic administration given that the growth-promoting effects of teduglutide in patients with SBS, and those of GLP-2 in mice also, are reversed upon withdrawal of treatment [36, 46]. Thus, future studies will have to consider the full range of GLP-2 effects, as well as all the sites of GLP-2R expression, to elucidate the multiple mechanisms of GLP-2 action.

On the other hand, this study showed that the maximal GLP-2R expression in the gastrointestinal tract was observed in the distal bowel (colon and rectum). There was a gradient of GLP-2R expression increasing from the duodenum toward the distal colon of mice. Similarly, healthy and IBD patients displayed significantly higher GLP-2R expression in the colon than in the ileum. Logically, GLP-2 actions would be therefore more pronounced in the colon than in the proximal bowel. The high colonic expression of GLP-2R might reveal undefined yet interesting therapeutic potentials for GLP-2 in the colon. But this therapeutic benefit is limited by concern that it predisposes patients to gastrointestinal cancers, or their re-occurrence in cancer patients. This may be especially important in IBD patients, who already possess an increased risk of gastrointestinal cancer due to chronic inflammation as well as in patients with chemotherapy-induced mucositis. Thus, long-term colonic surveillance studies must be considered to weigh the benefits of GLP-2 treatment versus the enhanced risk of carcinogenesis.

Most importantly, the absence of studies addressing the potential regulation of GLP-2R under states of intestinal dysfunction and/or inflammation is intriguing. Effectively, the presence of unrevealed GLP-2R regulation might redirect the therapeutic use of GLP-2 analogs in several digestive diseases. In the study, we showed that the intestinal expression of GLP-2R was significantly decreased in experimental mice models of colitis and in IBD patients. Since this is the first work showing a dysregulation GLP-2R expression in IBD, it would be interesting to determine whether this decrease is a consequence of pre-existing genetic background or secondary to disease activity. However, since this observation was consistent in two mice models of colitis, differentially mediated, as well as in IBD intestinal tissues; it's more likely that the inflammatory milieu in the intestine influence the expression of GLP-2R. What support this hypothesis is that inflamed colonic and ileal samples from IBD displayed a further reduced GLP-2R expression compared to non-inflamed zones. Thus, beside the potential of GLP-2-induced increased carcinogenesis in IBD patients (who are already at high risk) the biological effect of GLP-2 in these patients might be overestimated. Overall, this observation warrants further exploration and could explain the limited efficacy of GLP-2 analogs in the treatment of CD as compared to its recognized efficiency in short bowel treatment which is usually not in majority an inflammatory condition [69, 71].

Extraintestinal GLP-2R expression and effect of inflammation

It has become clear that GLP-2 functions through multiple interrelated pathways that defy simple definition. Each new discovery in this field raises important new questions, both for the actions of GLP-2 and for the integrated physiology of the intestine. Therefore, with the aim of accelerating mechanistic discoveries of the proglucagon and its derived peptides (GLP-1, GLP-2), several intestinal endocrine cell lines have been developed. Of note, NCI-H716 is the most commonly used human enteroendocrine cell line. In fact, these cells were classified as enteroendocrine based on their ability to secrete the glucagon in a regulated manner. However, we have shown that these cells do not express the GLP-2R mRNA, an observation that contradicts the classification of NCI-H716 as a human enteroendocrine cell line. Besides, many studies have already questioned the relevance of NCI-H716 as an human enteroendocrine system since these cells exhibit a transcriptionally inactive promoter and altered responses to well-recognized control factors of the rodent proglucagon [152-154]. Thus, further studies need to address the function of the GLP-2R in intestinal endocrine cells, although it is tempting to hypothesize a role in the regulation of intestinal peptide hormone and/or serotonin secretion.

Finally, the optimal duration and concentration requirements for GLP-2 to induce beneficial effects on intestinal secretion, motility, morphology, and, most importantly, absorption, are yet not known. What add a further level of complexity, are the major discrepancies in GLP-2 action between animal models and clinical studies. The findings suggested that both the low (0.05 mg/kg/day) and the high (0.2 mg/kg/day) dose of teduglutide were more effective than the intermediate dose (0.1 mg/kg/day) whereas previous studies in normal mice have indicated that the intestinotropic effects of GLP-2 are dose dependent [36, 38]. Therefore, extending GLP-2 findings in animal models to human seems to be complicated. Thus, future studies have to show if a more frequent administration, higher dose, longer duration of treatment, or possibly co-administration with other growth factors will further improve intestinal function. However, because the effects of GLP-2 and teduglutide are reversed following withdrawal, lifelong administration may be required.

Overall, a major goal of future research should therefore be to discover not only novel therapeutic usages of GLP-2 but also to exactly determine the sites and the levels of

Extraintestinal GLP-2R expression and effect of inflammation

GLP-2R expression as well as the unrevealed GLP-2 actions yet. This will enhance our understanding about the complex paracrine and neurocrine signaling induced by GLP-2 and help avoiding potential adverse outcome.

INTESTINAL HOMEOSTASIS – SECTION 2

The 5-Aminosalicylic Acid (5-ASA) Anti-neoplastic Effect in the Intestine is Mediated by Peroxisome Proliferator-Activated Receptor Gamma

PREFACE

Our interest in the intestinal homeostasis was not restricted to the field Glucagon-like peptide 2 and its appropriate receptor. Another research focus that currently represents a hot topic with intense clinical relevance is the promising preventive role of 5-aminosalicylic acid (5-ASA) in colorectal cancer associated with inflammatory bowel disease (IBD).

Less than two decades ago, inflammatory bowel diseases including – Crohn’s disease (CD) and ulcerative colitis (UC) – were described as ‘important and disabling diseases, still under-researched. Since then, whilst the etiology remained unknown, the understanding of the molecular mediators and mechanisms of tissue injury have greatly advanced, and certain features of these diseases have suggested several areas of possible importance, such as genetic, infectious, and immunological factors. Histological observations of affected tissue from IBD patients have revealed lymphocytic and neutrophilic infiltration, the hallmarks of inflammation. Medical therapy for IBD has advanced dramatically in the last decade with the introduction of targeted biologic therapies, the optimization of older therapies, including drugs such as immunomodulators and 5-aminosalicylic acid (5-ASA), as a result of a better understanding of the mucosal immune system and the genetics involved in the pathogenesis of IBD. Since then and for years, the treatment of IBD has been mainly limited to 5-aminosalicylic acid, corticosteroids and tumor necrosis factor blockers, empirically employed to control the inflammatory activity of IBD.

Because of chronic inflammation, IBD patients especially those with UC, are threatened by a higher risk of colorectal cancer (CRC) compared to normal population. Indeed, relationship between inflammation and cancer has been suggested for a long time. Since B. J. Marshall and J. R. Warren, who discovered *Helicobacter pylori* and reported its

infection closely associated with gastric cancer development, won the Nobel Prize in Physiology or Medicine in 2005, there have been an increasing number of reports as to the relationship between inflammation and carcinogenesis in a variety of tissues. In terms of the large bowel, it has been found that the risk of colorectal cancer increases in relation to the degrees of inflammation and the disease duration. Therefore, chemopreventive measures are highly recommended in IBD patients. Regular colonoscopic surveillance aiming at early detection of dysplasia is relatively a limited technique since only 20-50% of neoplasms are detected by endoscopic surveillance. On the other hand, epidemiological studies have suggested that IBD patients, who were also regular users of the anti-inflammatory drug 5-ASA, displayed reduced risk of CRC. Since then, an emerging chemopreventive role for 5-ASA has been addressed and later subjected to further investigations. Molecular and pharmacological studies have thoroughly examined the anti-neoplastic properties of 5-ASA and several mechanisms have been proposed, notably regulation of cell proliferation and induction of apoptosis. However, to date, the receptor the 5-ASA anti-tumorigenic effects remains undetermined. In the present work, we examined whether the peroxisome proliferator activated receptor gamma (PPAR γ), which mediated the anti-inflammatory effects of 5-ASA, would also mediate its anti-neoplastic properties. To this aim, we investigated the anti-proliferative and pro-apoptotic effects of 5-ASA *in vitro* as well as in two animal models of intestinal carcinogenesis. The involvement of PPAR γ was assessed by the addition of a specific antagonist for this receptor.

(A) ABSTRACT

Background: Epidemiologic evidences suggest that long-term 5-ASA therapy may prevent the development of colorectal cancer in inflammatory bowel disease (IBD). The mechanism by which 5-ASA may exert this protective effect is unknown. **Aim:** To evaluate if PPAR γ mediates *in vivo* and *in vitro* the anti-neoplastic effects of 5-ASA. **Methods:** *In vitro*, HT-29 and Caco-2 cells were treated either by 5-ASA, rosiglitazone or etoposide in the presence or not of the specific PPAR γ antagonist GW9662. Epithelial cell growth, proliferation and apoptosis were assessed by cell count, Ki-67 staining and TUNEL assay respectively. *In vivo*, the anti-neoplastic effect of 5-ASA was evaluated in a xenograft tumor model in SCID mice and in azoxymethane (AOM)-induced colon carcinogenesis in A/J01aHsd mice. In both models, the role of PPAR γ was examined by administration of GW9662. **Results:** *In vitro*, compared to untreated cells, incubation of HT-29 or Caco-2 cells for 24, 48 and 72h with 5-ASA resulted in a $60\pm 8\%$ inhibition of cell growth ($p < 0.001$). Similar results were obtained with the two positive controls rosiglitazone and etoposide. Growth inhibitory activities of 5-ASA and rosiglitazone were abolished by administration of GW9662. In comparison to untreated cells, the Ki67 immunostaining of HT-29 cells treated by 5-ASA for 48h showed an inhibition of 63% of cell proliferation. This effect was blocked following GW9662 treatment. Similarly, the pro-apoptotic capacity of 5-ASA induced in $75\pm 5\%$ of cells was also abolished by GW9662. In the xenograft model of HT-29 cells, daily treatment with 5-ASA induced a decrease of about 80% of tumor weight and volume in SCID mice after 3 weeks compared to untreated mice. Moreover, 5-ASA suppressed colon carcinogenesis by significantly decreasing the number of aberrant crypt foci (75%) and aberrant crypts (22%) induced by AOM treatment. The anti-tumorigenic effect of 5-ASA was completely abolished by simultaneous administration of GW9662 to mice. **Conclusion:** 5-ASA exerts *in vitro* and *in vivo* potent anti-neoplastic effects which are mediated by PPAR γ . These data reinforce the clinical interest of 5-ASA as a chemopreventive agent in patients with IBD.

(B) BACKGROUND

I- Inflammatory Bowel Disease

I-A. Overview

Inflammatory Bowel Disease (IBD) refers to a group of idiopathic inflammatory disorders affecting the gastrointestinal tract [155]. It is typified by Crohn's disease (CD) and ulcerative colitis (UC), and can affect both adults and children [156]. IBD currently has a worldwide prevalence rate of approximately 40–100/100,000 people [157]. Furthermore, patients with longstanding IBD may be predisposed to the development of colorectal cancer (CRC) [158]. The significant community impact indicates an acute need for the development of novel treatment strategies for IBD. CD, in contrast to UC, can affect any region of the gastrointestinal tract, extending from the mouth through to the anus [159]. Gastrointestinal symptoms depend on the location, extent and severity of involvement of the damaged region (Figure 1).

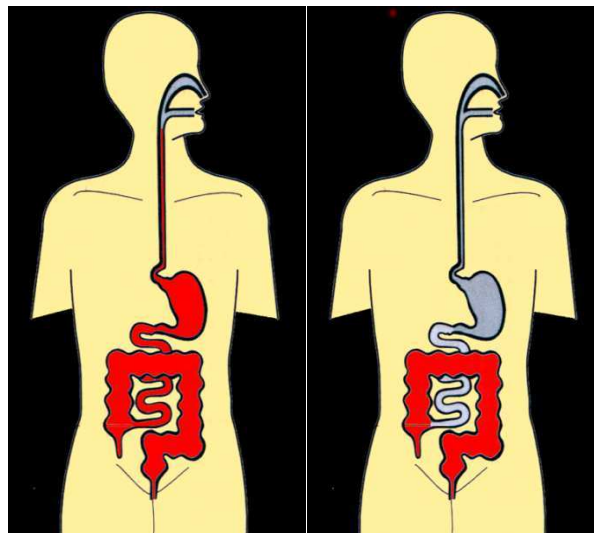


Figure 1. Crohn's disease can affect the entire gastrointestinal tract, extending from the mouth through to the anus while ulcerative colitis engages the colon only.

Generally, symptoms can present as abdominal pain, early satiety, nausea, epigastric pain and dysphagia [155, 159]. In its most severe forms, IBD may result in anorexia, diarrhoea and weight loss. While CD displays transmural pattern of inflammation, UC is

typified by superficial mucosal inflammation which consists of continuous ulceration, edema, and haemorrhage of varying severity (Figure 2). The most consistent physical presentation of UC is the presence of blood and mucus mixed with the stool, accompanied by lower abdominal cramping [159].

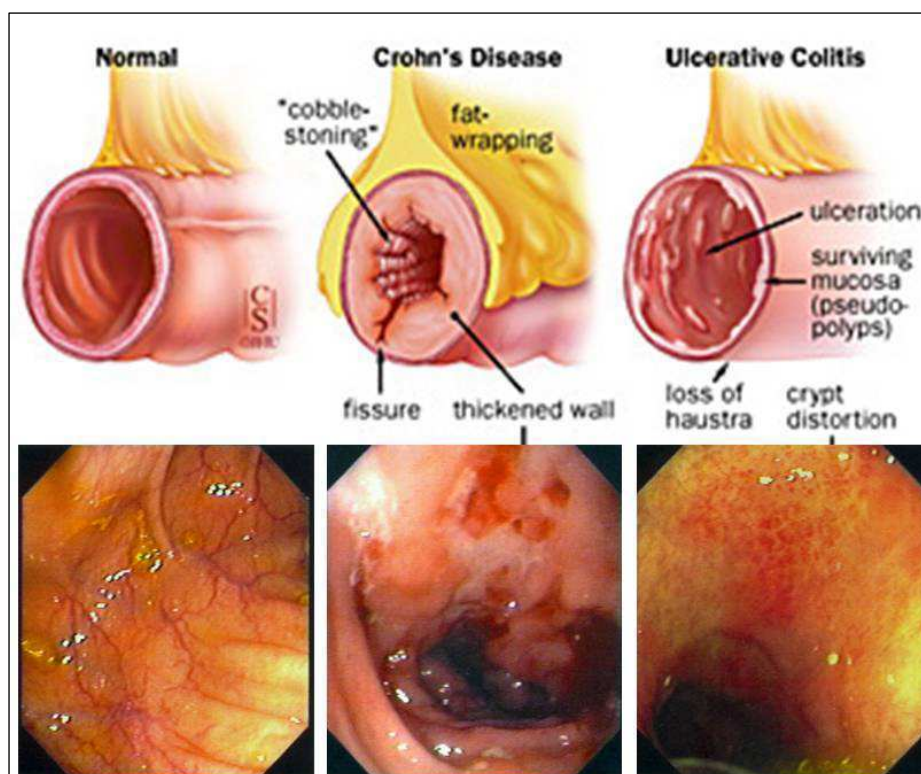


Figure 2. Comparison of the appearance of normal, Crohn's, and ulcerative colitis mucosa; gross (top); endoscopic (bottom). Updated from (<http://www.hopkins-gi.org>)

Although the cause of IBD is yet to be determined, it is believed to be a complex interplay of genetic and environmental factors, leading to inflammation of the gut mucosa [160]. This inflammation results from a persistent immune response, driven by gut bacterial flora. Current treatments for IBD include aminosalicylates, corticosteroids, antibiotics, immunosuppressants and biological therapies [161]; however, relapse rates of up to 50% for both UC and CD patients were reported. These treatment strategies are also often associated with a number of adverse and undesirable side effects [162]. Thus, there is an acute need for the development of new treatment strategies effective not only at reducing inflammatory episodes, but also at repairing the often extensive damage to the intestinal mucosa, ideally with minimal effects on quality of life.

I-B. History

Crohn's disease was first seen by German surgeon Wilhelm Fabry in 1623 [163], and was later described by and named after the US physician Burril B Crohn [164]. Ulcerative colitis was first described by the British physician Sir Samuel Wilks in 1859 [12].

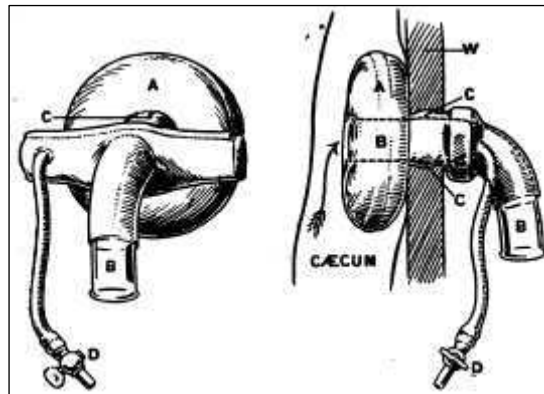


Figure 3. Old artificial anus [12]

I-C. Epidemiology

The highest incidence rates and prevalence of ulcerative colitis and Crohn's disease have been reported from northern Europe, the UK, and North America, where the rates are beginning to stabilize. Rates continue to rise in low-incidence areas such as southern Europe, Asia, and most developing countries. The incidence of IBD reported in North America ranges from 2.2 to 14.3 cases per 100,000 person/year for UC and from 3.1 to 14.6 cases per 100 000 person/year for CD, and, in Europe, respectively, from 8.7 to 11.8 (UC) and from 3.9 to 7.0 (CD) cases per 100,000 person/years. UC is more prevalent than CD, irrespective of the geographic area considered, with an estimated number of 780,000 individuals having UC and as many as 630,000 suffering from CD in the United States [165].

In North America, prevalence rates of Crohn's disease for Hispanic (4.1 per 100,000) and Asian people (5.6 per 100,000) is much lower than those for white individuals (43.6 per 100,000) and African-American people (29.8 per 100,000) [166]. The importance of race and ethnic origin in risk for inflammatory bowel disease are lent further support by a study showing racial differences in disease location and extraintestinal disease complications

[167]. IBD are more prevalent in Jewish people than in any other ethnic group, and important epidemiological differences exist between Jewish people living in Israel and those living elsewhere [168, 169]. This change in prevalence after migration has also been shown for other ethnic groups (eg, Chinese people living in Hong Kong compared with mainland China) [170]. The observation that geographic variability in incidence parallels the gradient in economic growth and the rise in the *pro capite* income, together with the change observed in prevalence in some ethnic groups after migration to other geographic areas, further support the theory that environmental factors and lifestyle play a role in disease occurrence [165].

Long before genome-wide analysis studies [171], familial clustering of cases and twin studies had already established the role of genetic factors in the occurrence of these disorders, and a positive family history is still the most important independent risk factor [172]. Cigarette smoking affects these two diseases in a different way: smokers are at an increased risk of CD and tend to present more severe clinical course whereas former smokers and non-smokers are at greater risk of UC [173]. Finally, the incidence of both CD and UC appears to follow a bimodal age distribution with signs and symptoms frequently manifesting in early adulthood as well as from 50 to 70 years of age.

I-D. Etiopathogenesis

Despite all the advances in our understanding of the pathophysiology of inflammatory bowel disease, we do not know the cause. It is now becoming increasingly accepted that dysregulated immunity, genetic predisposition, and environmental factors play an important role in the predisposition, modulation, and perpetuation of IBD.

I-D.1 Genetic predisposition

In IBD, several studies have shown the presence of a disorder of immune tolerance to luminal antigens [174-176]. The conditions leading to dysregulation of the immune response toward the microbiota remain unknown. However, following the initial experimental studies showing the presence of a loss of immune tolerance, genetic studies

revealed an increased risk of CD or UC associated with polymorphisms in genes encoding components of innate and adaptive immune response. In CD, a meta-analysis of three CD genome wide association studies (GWAS) has identified more than 30 loci associated with the disease, with odds ratio ranging from 1.08 to 3.99 [177]. Prominent among these genetic studies, for the strength of the association and the insights into the mechanism of the disease, are those related to polymorphisms in genes related to adaptive immunity (IL-23R, Signal Transducer and Activator of Transcription 3 (STAT-3)) [178] or to genetic mutations related to a disturbed surveillance of bacteria of the microflora by the intestinal mucosa (Nucleotide Oligomerization Domain (NOD2)) [179-182], or to deficient autophagy (Autophagy-related protein 16-1 (ATG16L1), Immunity-related GTPase family M protein (IRGM)) [183-185]. It is important to stress here that these variants do not appear to be either unique or necessary for the disease to express. Attempts have been made to link the associated genetic variants with the classic clinical CD sub-phenotypes. So far, the association of NOD2 variants with ileal disease location has been described [186, 187] but data from a recent study indicate a poor relationship between the genetic based subgroups and the clinical sub-phenotypes used [188].

I-D.2 Environmental factors

a) Geographic, temporal, and seasonal variability

The highest incidence rates and prevalence for ulcerative colitis and Crohn's disease are reported from North America and northern Europe. The lowest incidence rates are reported from South America, Southeast Asia, Africa (with the exception of South Africa), and Australia [165]. Although these data suggest that a gradient exists from north to south, they could also indicate variation in access to, and quality of, health care as well as different extents of industrialization, sanitation, and hygiene. Different incidence rates could also result from different genetic backgrounds of the residents of these parts of the world. More important factors, however, seem to be other environmental ones. This hypothesis is supported by increasing incidence rates among immigrants from low-incidence regions moving to developed countries.

Several large epidemiological studies in North America and Europe have shown an accumulation of cases of inflammatory bowel disease in urban compared with rural communities. As with the north to south gradient, this pattern probably does not implicate geographical as much as other environmental factors, such as industrialization, sanitation and hygiene, or differences in access to specialized health care. Early epidemiological studies from the USA and Scandinavia suggested that ulcerative colitis is more common during the autumn and winter seasons [189, 190]. These data are contradicted by more recent studies from the USA and Italy [191, 192]. Since all studies examined hospital admission rates, their results are difficult to interpret and might indicate patients' seasonality of access to health care.

b) Lifestyle and nutrition

Breastfeeding confers immunity while the child's intestinal immune system is still developing [193]. A recent meta-analysis of 17 studies of breastfeeding and the risk of developing inflammatory bowel disease showed heterogeneous results, probably because of poor design and recall bias of mothers. However, a subgroup analysis of high quality studies for Crohn's disease and ulcerative colitis showed the pooled odds ratio was 0.45 (95% CI 0.26–0.79) for Crohn's disease and 0.56 (0.38–0.81) for ulcerative colitis, lending support to the notion that breastfeeding provides protection against inflammatory bowel disease in offspring [194]. Breastfeeding itself does not contribute to disease exacerbation whereas drug cessation post partum during breastfeeding does [195].

The association between excessive consumption of carbohydrates and development of inflammatory bowel disease, especially Crohn's disease, probably indicates the differences in sugar consumption in Asia compared with Western Europe and North America rather than a true contributing factor [196]. Other studies have found an association between an increased intake of polyunsaturated fats or margarine with an increased risk of inflammatory bowel disease [197]. Whereas in the past, lipid mediators derived from arachidonic acid were commonly perceived as proinflammatory, new data suggest otherwise [198]. Most dietary studies have weak methods and poor patient compliance or recall, which makes interpretation of findings difficult. No final conclusions

about the role of nutrition or dietary intervention in inflammatory bowel disease can yet be made.

c) Hygiene hypothesis

The traditional low incidence of inflammatory bowel disease and other chronic inflammatory disorders in developing countries, which is now on the rise, might be related to socioeconomic changes affecting hygiene [199]. For example, a lower risk of developing inflammatory bowel disease, particularly Crohn's disease, has been reported for lower birth rank, absence of tap water, absence of hot water, large or poor families with several children, crowded living conditions, or consumption of contaminated foods [200-202]. Excessive sanitation might limit exposure to environmental antigens and impair the functional maturation of the mucosal immune system and induction of immune tolerance, which ultimately result in inappropriate immune responses when re-exposed to these antigens later in life.

d) Smoking

Smoking cigarettes is associated with less frequent exacerbations of ulcerative colitis. By contrast, in Crohn's disease, smoking aggravates the course of the disease, promotes formation of fistulas and strictures, increases the rates of exacerbations and the need for corticosteroids, and accelerates the need for surgery after surgically induced remission [173]. Smoking cessation seems to be an effective therapeutic intervention in Crohn's disease, but nicotine patches or enemas failed to show efficacy in the management of ulcerative colitis [203, 204]. Experimental studies suggest that the beneficial effects of nicotine in ulcerative colitis is due to increased mucus production, decreased production of proinflammatory cytokines and nitric oxide, and improvement of the intestinal barrier function, whereas nicotine's detrimental effects in Crohn's disease seem to be related to an increased influx of neutrophils into the intestinal mucosa [173]. The data for passive smoking are contradictory, with some reports showing a reduced risk of ulcerative colitis when exposed to smoke in childhood, and others showing that such exposure results in an increased risk of both ulcerative colitis and Crohn's disease.

e) Appendectomy

Epidemiological studies suggest that appendectomy might be protective against ulcerative colitis, with one study finding significantly reduced risks of colectomy or the need for immunosuppressive therapy in patients who had undergone appendectomy before diagnosis [205, 206]. The opposite is true for Crohn's disease, with appendectomy being associated with an increased risk of developing strictures [207, 208]. Potential explanations for these contrasting findings might relate to the handling of microbes by the mucosal immune system, the hygiene hypothesis, and a failure to develop immune tolerance after appendectomy.

f) Infection

Inflammatory bowel disease is more common after gastrointestinal infections and people with the disease generally have higher concentrations of mucosal bacteria than do healthy people. Mucosal bacteria concentrations increase progressively with the severity of disease in both the inflamed and non-inflamed colon. Adhesive bacteria might predominate, although no single species is exclusively causative [209-211]. Indeed, the evidence does not provide unequivocal support for any microbe being a causative agent in inflammatory bowel disease. However, these data underscore the impaired handling of microbial antigens by the intestinal immune system in inflammatory bowel disease. On the other hand, the absence of exposure to intestinal parasites (helminthes) appears to be an important environmental factor contributing to development of IBD. Helminths interact with both host innate and adoptive immunity to stimulate immune regulatory circuitry and to dampen effector pathways that drive aberrant inflammation.

g) Others

Several case-control studies have reported a weak association of the disease with contraceptive use (insignificant differences) [212]. Moreover, non-steroidal anti-inflammatory drugs were shown to exacerbate inflammatory bowel disease [213]. Psychological stress has anecdotally been reported to increase activity of inflammatory

bowel disease. Adverse life events, chronic stress, and depression seem to increase the likelihood of relapse in patients with quiescent disease [214]. New experimental evidence suggests involvement of direct interactions of the nervous and immune systems.

I-D.3 Immunobiology

Increasing evidence suggests that IBD results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host [28, 215]. Evidence for the validity of this hypothesis comes from the observation that almost all the experimental murine models of mucosal inflammation depend upon the presence of the microflora. In fact, inflammation does not occur if the mice are reared in a germfree environment. This is mainly due to defects in T-cell effector function and defects in regulatory T-cell function [216]. The importance of microflora is also confirmed by findings in humans with IBD. In these patients, the disease can be ameliorated by the administration of antibiotics and is abrogated by avoiding the enteric stream coming into contact with the area of inflammation [174]. This inappropriate inflammatory response can occur as a result of excessive activation and differentiation of immune effector T-cell subsets against harmless antigens (so called type 1 helper T cells (Th1) and Th17 for CD; Th2 and Th17 for UC) [175, 176, 217-219] and/or defective counter-regulation by regulatory T cells (Tregs) [220-222].

a) Intestinal Microbiota

An enormous number of micro-organisms are known to colonize the intestine and form complex communities or microbiota. The host-microbiota associations usually evolve into beneficial relationships. Bacteria in the gastrointestinal tract supply key nutrients and prevent colonization by opportunistic pathogens. Furthermore, they contribute to the anatomical development and function of the mucosal immune system [223]. However, the microbiota has the potential to exert both pro- and anti-inflammatory responses, and the composition of the bacterial communities, in the gut, is related to the correct functioning of the immune system [26]. Thus, the alterations in the development or composition of the microbiota (known as dysbiosis) disturb the partnership between the microbiota and the

human immune system, ultimately leading to altered immune responses that may underlie various human inflammatory disorders (Figure 4).

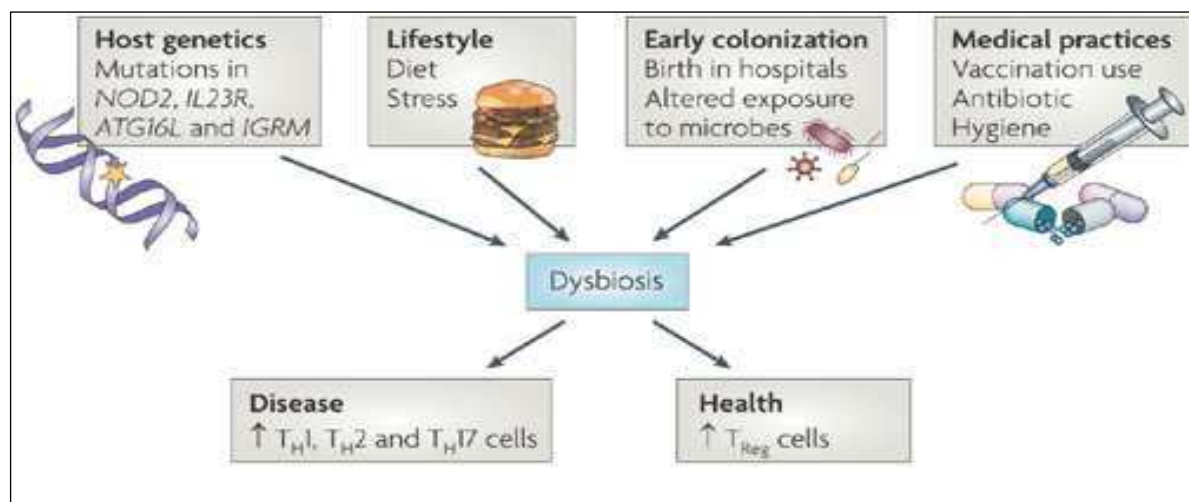


Figure 4. The composition of the microbiota can shape a healthy immune response or predispose to disease. Many factors can contribute to dysbiosis, including host genetics, lifestyle, exposure to microorganisms and medical practices. Host genetics can potentially influence dysbiosis in many ways. An individual with mutations in genes involved in immune regulatory mechanisms or pro-inflammatory pathways could lead to unrestrained inflammation in the intestine. Diet and stress also have the potential to influence the microbiota. Birth in the sterile environment of hospitals can protect from exposure to dangerous pathogens, but can also prevent early exposure to health-promoting bacteria. Overuse of vaccination and antibiotics, which do not distinguish between pathogenic or symbiotic microorganisms, could adversely alter the microbiota. ATG16L, autophagy-related gene 16-like; IGRM, immunity-related GTPase family, M; IL23R, interleukin-23 receptor; NOD2, nucleotide-binding oligomerization domain 2; T_H, T helper; T_{Reg}, regulatory T [26].

Several important effects of the microbiota on the host immune system have been determined by studies of gnotobiology, which is the selective colonization of germ-free (sterile) animals. Germ-free animals show extensive defects in the development of gut-associated lymphoid tissues, and in antibody production, and have fewer and smaller Peyer's patches and mesenteric lymph nodes (MLNs) compared with animals housed under specific pathogen free (SPF) conditions [26]. Together with various morphological tissue defects that are observed in the intestines of germ-free animals, it seems that the entire ultrastructural development of the gut is intimately connected to intestinal bacteria. For

example, intestinal epithelial cells (IECs), which line the gut and form a physical barrier between luminal contents and the underlying cells of the immune system, have altered patterns of microvilli formation and decreased rates of cell turnover in germ-free animals compared with wild-type animals [224]. Furthermore, gut bacteria have been shown to direct the glycosylation of lumenally exposed surface proteins of IECs [225]. Evidence for the involvement of gut bacteria in intestinal inflammation is provided by studies of animal models. Pre-treatment of mice with antibiotics has been shown to alleviate subsequent intestinal inflammation in several animal models [226]. HLA-B27-transgenic rats, IL-10- and IL-2-deficient mice raised in conventional conditions spontaneously develop chronic colitis, whereas they do not develop intestinal inflammation if raised in germ-free conditions [227, 228]. In a model of colitis induced by the adoptive transfer of pathogenic T cells into immunodeficient recipient mice, colonization of animals with intestinal pathogens such as *Helicobacter hepaticus* was found to exacerbate inflammation [229]. Moreover, colitis can be induced in healthy animals through the adoptive transfer of T cells that are reactive against specific commensal organisms [230].

Dysbiosis has been as well implicated in IBD in humans, with several studies showing a significant alteration in the microbiota of patients with IBD. Specifically, the microbiota of IBD patients showed abnormal microbial composition that was characterized by depletion of two phyla of bacteria, the Firmicutes and Bacteroidetes, which are both prominently represented in non-IBD controls. These observations were associated with early postoperative recurrence in patients with CD [231, 232]. Thus, if some bacteria are actively shaping a healthy immune system, does the absence of these organisms lead to disease? It has recently been proposed that the total information encoded by the mammalian genome is not sufficient to carry out all functions that are required to maintain health and that products of our microbiome are crucial for protection from various diseases [233]. On the other hand, other studies have shown that a subset of patients with CD harbors a potentially proinflammatory “adherent-invasive” strain of *Escherichia coli* in the small intestine [234]. However, whether these modifications contribute to the disorder or merely reflect secondary changes caused by the inflammation remains to be established.

Regardless of the primary or aggravating role of the alteration of microbiota in the pathogenesis of IBD, the interactions observed between the microbiota and the mucosal immune system stress the importance of the mucosal response to commensal microorganisms and how genetic factors may affect this response. In “non-inflammatory” conditions, the mucosal immune response to the microbiota is controlled by the cooperation between the innate and adaptive immune response. This cooperation results in a state of “immunological equilibrium” defined as tolerogenic response [235] (Figure 5).

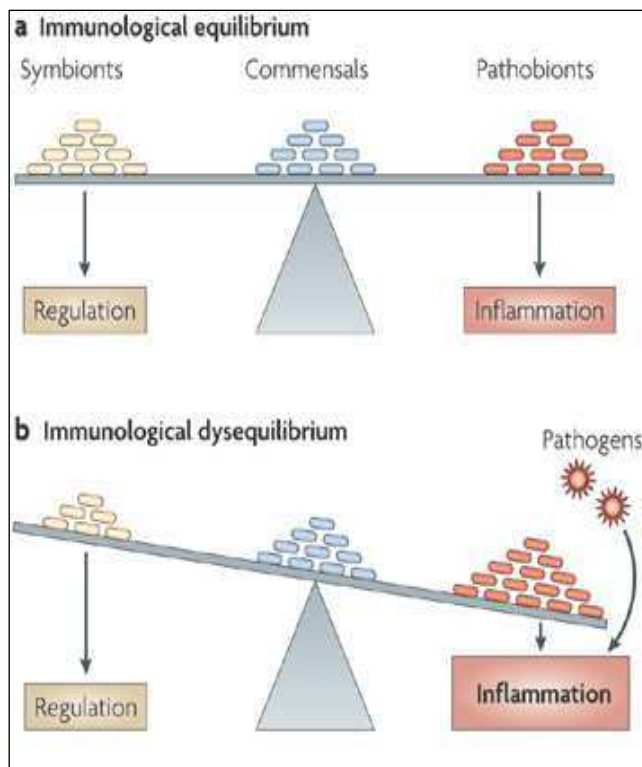


Figure 5. a .A healthy microbiota contains a balanced composition of many classes of bacteria. Symbionts are organisms with known health-promoting functions. Commensals are permanent residents of this complex ecosystem and provide no benefit or detriment to the host. Pathobionts are also permanent residents of the microbiota and have the potential to induce pathology. **b**. In conditions of dysbiosis there is an unnatural shift in the composition of the microbiota, which results in either a reduction in the numbers of symbionts and/or an increase in the numbers of pathobionts [26].

b) Immunohomeostasis in the healthy gut

Cells and their soluble products belonging to the innate response and the epithelial barrier represent the first line of host-luminal bacteria interactions. Specialized cells (goblet cells and Paneth cells) within the epithelial layer, which is sealed by the tight junctions, produce mucus and antimicrobial peptides (i.e. defensins) that limit the entry of bacteria into the intestinal mucosa [211, 236]. At the same time, the epithelial cells are able to “sense” microbial components through the pattern recognition receptors (PRRs), which can be located either on both the cell surface and the intracellular compartment (Toll like

receptors (TLRs) or exclusively in the intracellular compartment (nucleotide-binding oligomerization domain (NOD) proteins)). These receptors recognize evolutionary conserved microbial components. The same receptors are present on dendritic cells (DCs) in the lamina propria and, restricted to the expression of some TLRs, on T cells [237, 238]. These microbial sensors are functionally intersected, as NOD may affect TLR signaling [239, 240], and they cooperate in the autophagy pathway [185, 241] (Figure 6).

In the steady state, epithelial cells produce mainly molecules and cytokines (IL-10, IL-25, transforming growth factor- β (TGF- β) and probably retinoic acid (RA)) [242-247] that promote in the human lamina propria macrophages and DCs (i.e. professional antigen-presenting cells (APCs)), a default state of hypo-responsiveness to several inflammatory stimuli, including commensal components [248, 249]. Such APCs conditioning is likely to be related to commensal signaling in intestinal epithelial cells (IECs) through PRRs. This type of conditioning is further reinforced by the phagocytosis of apoptotic cells (epithelial cells, lymphocytes) that represent an important potential source of bioactive TGF- β capable of enhancing TGF- β production from DCs and macrophages [250, 251]. All these factors are combined in generating the tolerating milieu that normally characterize the intestinal mucosa, and consistently, lamina propria T cells show a constitutive reduced proliferation upon polyclonal stimulation and a high activation-induced apoptosis rate compared to the circulating T cells [252, 253]. DCs loaded with antigens migrate in secondary lymphoid organs (Peyer's patches and mesenteric lymph nodes (MLNs)) where they trigger an adaptive response by presenting antigens to naïve T cells. At these inductive sites of immune response, APCs have been increasingly thought to possess flexibility in their function because of their ability to modulate differentiation of CD4⁺ T-cell subsets with opposite function and characteristic cytokine profiles either inducible T regulatory cells (iTregs) or effector helper T cells (Th1, Th2, and Th17) depending on the local signals received (either regulatory or inflammatory) [254] (Figure 6).

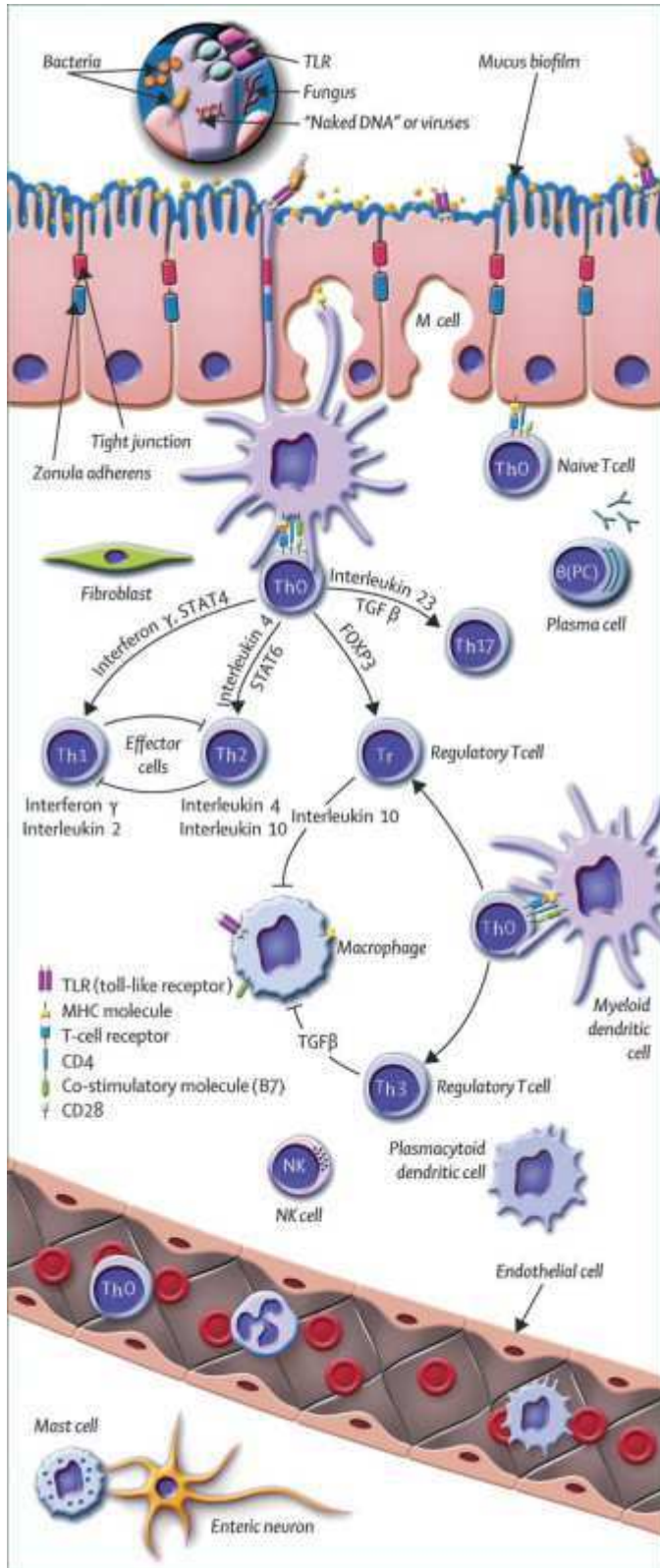


Figure 6. Intestinal immune system in healthy state. The intestinal immune system represents a complex network of different lymphoid and non-lymphoid cell populations and humoral factors. Luminal antigens, such as commensals, pathogens, and nutrients, are sampled by professional and nonprofessional antigen-presenting cells. Although non-professional antigen-presenting cells, such as intestinal epithelial cells, might interact with naive T cells (Th0) through major histocompatibility complex II receptors, they do produce co-stimulatory signals in the absence of inflammation, thereby suppressing or inducing anergy in mucosal T cells. Dendritic cells control both the adaptive immune response, such as the balanced differentiation of naive T cells into effector T cells (Th1, Th2, Th17) required to fight off pathogens and regulatory T cells. In the presence of commensals and absence of inflammation, a balance between effector and regulatory immune subpopulations is maintained through a tightly controlled cytokine network. Th=T helper cells. Th0=naive T cell. Th, Th1, Th2, Th17=effector T cells. Tr, Th3=regulatory T cells. B=B cell. B(PC)=plasma cell. NK=natural killer cell. NKT=natural killer T cells [28]

For a long time, T helper responses have been defined according to the relevant cytokine production as Th1 (IL-12 driven-interferon (IFN)- γ production) or Th-2 (IL-4 production in the absence of IL-12) [255]. Few years ago, an additional subset of T helper cells producing IL-17 (Th17) has been characterized [256, 257]. This subset is believed to differentiate from naïve T cells in the presence of TGF- β and the inflammatory cytokines IL-1 β and IL-6 and be dependent upon IL-23 for expansion [258-261]. Human Th17 response is generally recognized (and currently defined) as characterized by the production of several proinflammatory cytokines namely IL-17A, IL-17F, IL-6, IL-21, IL-22, and TNF- α involved in acute inflammatory response as well as in chronic autoimmune response and, in some circumstances, in regulatory responses. Indeed, the Th17 response is characterized by a high degree of plasticity, because IL-17 producing cells can co-express, under different circumstances, the regulatory cytokine IL-10 or the inflammatory cytokine IFN- γ [255, 262].

Increasing evidence suggests that different subsets of DCs are present in the gut-associated lymphoid tissue; in particular a subset expressing the surface marker CD103 has been demonstrated to preferentially induce the generation of regulatory Foxp3⁺ cells via a TGF- β and RA-dependent mechanism [263]. Tregs actively control autoimmunity and tissue homeostasis. TGF- β and IL-10 are the molecules associated with their development and function [244, 264]. Treg populations include thymic-derived natural Tregs (nTreg), responsible for maintaining self-tolerance, and inducible Tregs (iTreg) that are generated in the periphery to prevent inappropriate and exuberant immune responses to microbial or tissue antigens [265]. The hallmark of nTreg cells is the expression of the Foxp3 transcription factor, which is required for maintaining Treg cell function [266]. In the presence of TGF- β , Foxp3 can also be induced in naïve T cells in the periphery, and the resulting iTregs exhibit a suppressive phenotype similar to that in nTreg cells [267]. The most intriguing observation on Foxp3⁺ Treg cells is the increased evidence of their ability, in inflammatory conditions; to switch from a regulatory to an effector phenotype, mainly to IL-17- producing cells [268]. In particular, it has been reported, in *in vitro* studies and in animal models, that under lymphopenic and / or inflammatory conditions, Tregs may lose Foxp3 and / or acquire different effector functions, especially in the intestine, which may contribute to uncontrolled inflammation [269]. IL-23 appears to play a central role in orchestrating the mucosal immune response through modulation of both the inflammatory

and regulatory arms of Th cell response. Indeed, IL-23 is able to promote the expansion of Th17 cells to induce the co-expression of IFN- γ in Th17 lymphocytes and to directly inhibit Tregs expansion [270, 271]. Thus, the IL-23-Th17 axis and its relation with regulatory cells represent currently the focus point of research aimed at elucidating the genesis of inflammation. After priming in gut-associated lymphoid tissues, T cells enter the systemic circulation. During priming by DCs, T cells acquire the surface expression of enterotropic molecules (a4b7) that guarantee the gut homing of primed lymphocytes once they enter the systemic circulation [272, 273]. These activated CD4⁺ T cells can, thereby, leave the circulation to enter the intestinal lamina propria, where they carry out their own specific functions. In the steady state, the reactivity to mucosal antigens is finely tuned by regulatory mechanisms so that effector cells producing IFN- γ (Th1 response) or IL-4, IL-5, IL-13 (Th2 response) or IL-17 (Th17 response) are controlled by regulatory cells (Figure 6). In IBD, this balance is broken, resulting in chronic inflammation.

c) Dysregulated immunity in IBD

The inflammatory response in IBD is characterized by an increased production of inflammatory cytokines (TNF- α , IL-6, IL-1 β) sustained by T cell-derived cytokines (Figure 7). In CD, the IL-12 / 23-induced IFN- γ production is recognized as having the major role in sustaining the inflammation (Th1-mediated inflammation). In UC, increased IL-5 and IL-13 production have been reported to play a significant role in the generation and perpetuation of the inflammation (Th2-like-mediated inflammation) [217, 274, 275]. Irrespective of the pathway of inflammation (Th1 or similar Th2), both in CD and UC the local activation of anti-apoptotic pathways in pathogenic lamina propria T lymphocytes as a secondary consequence of inflammation leads to further expansion of these cells in the lamina propria and, consequently, further increase in inflammatory mediators, inhibition of the regulatory function and perpetuation of IBD [276, 277]. In CD and UC, an increased expression of IL-17 and IL-23 has been shown [218, 278, 279], although IL-23 appears to differently regulate the effector Th subsets balance in CD and UC, enhancing the production of the Th1 signature cytokine IFN- γ by lamina propria CD4⁺ T cells in patients with CD, and the production of IL-17 in UC [218]. Thus, in IBD, the increased production of IL-17 has been reported both in CD and in UC, but its role in the two diseases has not been completely clarified. Right after

experimental data have linked the Th17 response to human chronic inflammatory and autoimmune diseases, and the effect of IL-23 mainly to the Th17 response, the concept of IBD as a Th17 co-mediated disease and of “IL-23 / IL-17 axis” as central in this pathological condition has immediately emerged. This paradigm, although well fitting with the reported genetic association of both forms of IBD with variants in the IL-23R and STAT-3 (the predominant downstream mediator of IL-23 signaling), appears to require further confirmation. In animal models, IL-17-deficient T cells are not impaired in their ability to induce colitis, suggesting that Th17 cell responses are not essential for colitis [271, 280]. Moreover, IL-23 seems to directly inhibit the Foxp3+regulatory cells suggesting that, inhibition of regulatory cells, more than the increase in effector mechanisms, may account for the development of chronic inflammation [271]. Thus, the paradigm of the pathogenetic IL-23 / IL-17 mucosal axis might change to that of IL-23 / IFN- γ or IL-23 / Tregs axis. With regard to regulatory cells, studies performed in IBD did not show a reduction in the percentage of regulatory cells infiltrating the mucosa. On the contrary, it has been reported that regulatory Foxp3+ T cells are increased during the active phases of the diseases and are not different from controls during remission. Furthermore, in contrast to many mouse models of IBD, regulatory T cells in human IBD appear to be completely capable of suppressing effector T-cell proliferation [221]. It should be emphasized here that the ability of intestinal regulatory cells to suppress effector T-cell proliferation has been tested using, as effector cells, T cells from peripheral blood. T effector cells in an inflamed site might show a different response to suppression. Indeed, it has been shown that effector cells in active IBD and in experimental colitis present increased Smad7 expression (an intracellular inhibitor of TGF- β signaling) that might prevent the TGF- β -mediated suppressive effect of regulatory T cells [281, 282]. Thus, the possibility exists that regulatory function might be ineffective as a consequence of the presence, during the active phases of the disease, of effector cells resistant to inhibition by regulatory cells. In summary, the experimental findings obtained so far indicate an increased local production of pro-inflammatory cytokines from infiltrating lamina propria mononuclear cells in patients with IBD when compared to controls, but evidence of primary phenomena is still lacking. Recently, new data confirmed a previous observation that revealed the presence, in patients with CD, of a weak acute inflammatory response, resulting in the development of chronic inflammation because of partial immunodeficiency [283-285]. However, as the observations emerging

from those studies were obtained in patients with anatomical lesions, albeit in clinical remission, it is not possible to rule out the possibility that they were secondary to the presence of chronic inflammation.

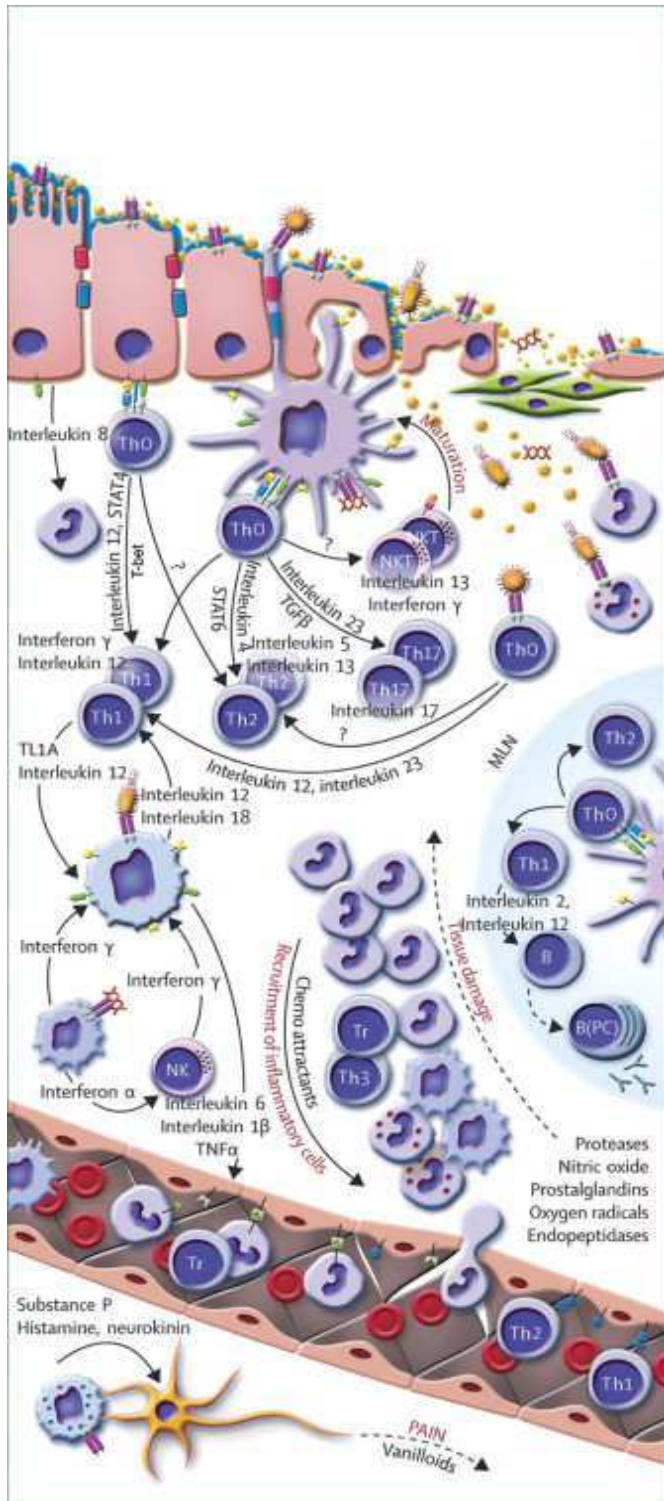


Figure 7. Intestinal immune system in IBD. In inflammatory bowel disease, the well controlled balance of the intestinal immune system is disturbed at all levels. Luminal antigens gain access to the underlying mucosal tissue via a leaky barrier. Innate and adaptive immune cells express a different profile and number of molecular pattern-recognition receptors. Microbial antigens from commensals trigger and maintain an inflammatory response through several different pathways involving dendritic cells, epithelial cells and effector T cells. Overall, in active inflammatory bowel disease effector T cells (Th1, Th2) predominate over regulatory T cells. In Crohn's disease, naive T cells preferably differentiate into Th1 cells. In ulcerative colitis, these cells differentiate into Th2 cells. The proinflammatory cytokines secreted by activated effector T cells stimulate macrophages to secrete large amounts of TNF- α , IL-1, and IL-6. In addition to their failure to balance the adaptive immune response in inflammatory bowel disease, dendritic cells might also be responsible for a dysregulated innate immune response [28].

As a summary, several, not mutually exclusive, pathways, as shown in figure 7, might result in the inflammatory cascades observed in IBD and could underlie, at least partially, the pathogenesis described above:

- 1- The epithelial barrier is leaky in people with inflammatory bowel disease. Several studies have shown a lowered epithelial resistance and increased permeability of the inflamed and non-inflamed mucosa in Crohn's disease and ulcerative colitis [286].
- 2- Second, people with inflammatory bowel disease have disturbed innate immune mechanisms of the epithelial layer. In these people, mucosal epithelial cells have a different pattern of TLR expression. TLR3 is significantly down regulated in active Crohn's disease, but not in ulcerative colitis. By contrast, TLR4 is strongly upregulated in both diseases [287]. TLR5 signaling is as well suppressed in IBD; however, in the injured inflammatory bowel disease mucosa, flagellin—TLR5 ligand—can engage the receptor and thereby aggravate inflammation [288]. An upregulation of NOD2 in epithelial cells, which can augment itself further in a feedback loop, when the NF κ B cascade is activated, has also been reported [289].
- 3- Antigen recognition and processing by professional antigen-presenting cells is disturbed in people with inflammatory bowel disease. Increased expression of TLR4 by myeloid dendritic cells in inflammatory bowel disease has been reported [290]. Studies animal models and in inflammatory bowel disease patients have shown mature activated dendritic cells, which prolong their survival thereby maintaining inflammation [290, 291].
- 4- Atypical antigen-presenting cells become potent effector-T-cell activators in people with inflammatory bowel disease. Non-professional antigen-presenting cells, such as epithelial cells, which normally induce anergy in CD4⁺ T cells, acquire an activated phenotype with increased histocompatibility molecule expression in the presence of inflammatory cytokines such as IFN- γ and TNF- α [292]. Intestinal epithelial cells from patients with inflammatory bowel disease also express alternative costimulatory

molecules, which might transform them into functional antigen-presenting cells [293].

- 5- Patients with inflammatory bowel disease have disturbed clearance of overreactive or autoreactive T-cell populations. Due to a failure of central (thymic) and peripheral tolerance, activated T cells persist and do not undergo apoptosis [277, 294].

- 6- Psychosocial stress might trigger or augment the inflammatory cascade through neuroimmunological interaction. Stress—ie, overactivation of the sympathetic nerve—has been shown in patients with ulcerative colitis and in turn, causes increased colonic paracellular permeability involving mast-cell degranulation, overproduction of IFN- γ , and altered the expression of tight junction proteins [295, 296]

I-E. Treatment of inflammatory bowel disease: "Bottom-Up" Approach

Current drug treatments aim to induce and maintain the patient in remission and ameliorate the disease's secondary effects, rather than modifying or reversing the underlying pathogenic mechanism. Corticosteroids, aminosalicylates, and immunosuppressive agents, such as azathioprine, are routinely used. Other drugs, such as metronidazole and broad-spectrum antibiotics, are helpful in some cases, while colestyramine, sodium cromoglycate, bismuth and arsenical salts methotrexate, and fish oils provide alternative therapies [297]. A new approach for the treatment of IBD using humanized monoclonal antibody preparations has produced encouraging results and may eventually provide a welcome alternative to traditional treatments because these antibody treatments modify the affected biochemical inflammatory pathways. Some specific goals of pharmacotherapy in IBD include controlling acute exacerbation of the disease, maintaining remission, and treating specific complications such as fistulas. Specific drugs may be better suited to one or more of these aims. For example, steroids are the treatment of choice for moderate to severe flare-ups but are inappropriate for long-term use because of their side effects and inability to maintain remission. Other immunosuppressants, such as azathioprine, that require several weeks to achieve their therapeutic effect have a limited

role in the acute setting but are preferred for long-term management [298] (Figure 8). A more thorough appreciation of the intricacies of the inflammatory response and improved biotechnology has led to the development of biological agents that can target single steps in the immune cascade. Drug delivery to the appropriate site(s) along the gastrointestinal tract also has been a major challenge, and second-generation agents have been developed with improved drug delivery, increased efficacy, and decreased side effects.

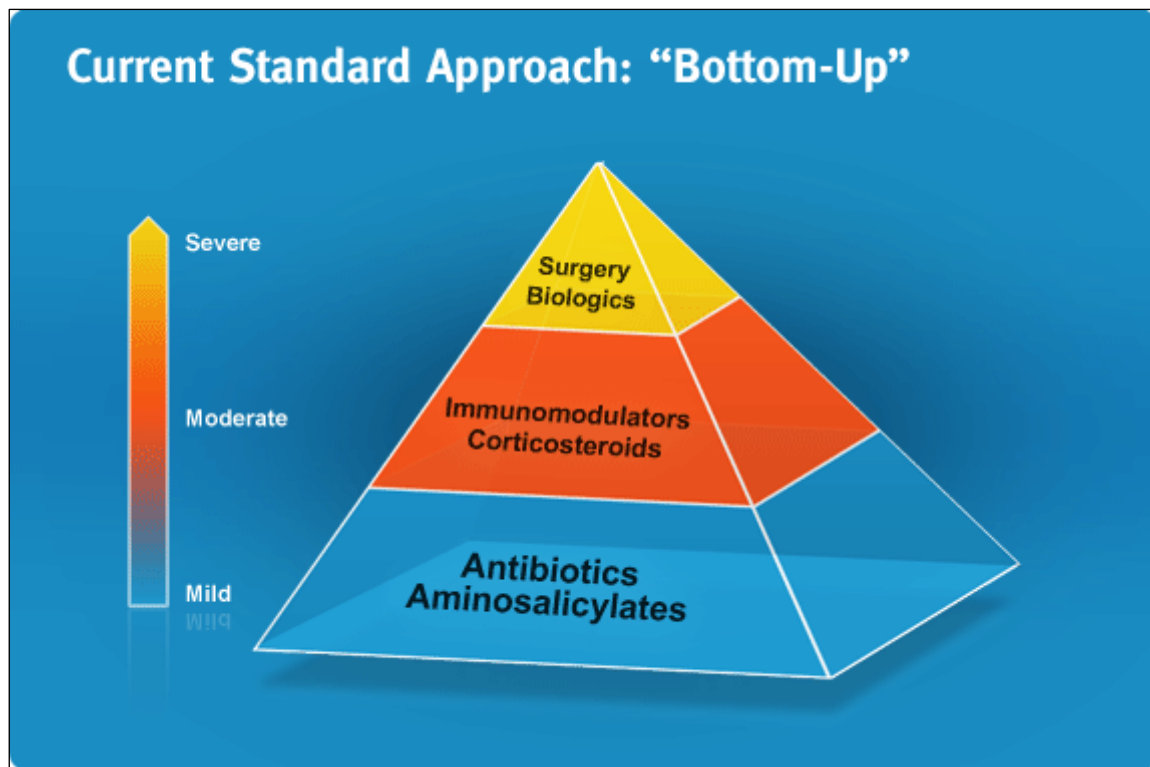


Figure 8. Bottom-Up approach. The current standard approach for treating IBD is the "Bottom-Up" approach. This involves assessing how severe a patient's symptoms are, and choosing treatment based on that severity (<http://www.ibdetermined.org>).

I-E.1. Immunomodulators

Several drugs initially developed for cancer chemotherapy or as immunosuppressive agents in organ transplants have been adapted for the treatment of IBD. Immunosuppressant drugs can be an invaluable adjunct therapy for the treatment of patients with intractable inflammatory bowel disease or complex, inoperable perianal

disease. Although immunosuppressant agents have significant side effects, they are safer and better tolerated than long-term corticosteroid therapy. However, these agents should not be used in young patients who are candidates for surgery or in patients who are noncompliant and refuse to return for periodic monitoring.

a) Azathioprine and mercaptopurine

Since the early 1970s, azathioprine and mercaptopurine have been used to treat IBD. These drugs are superior to the placebo, but their full effects may not become apparent for as long as three months. Azathioprine and mercaptopurine impair purine biosynthesis and inhibit cell proliferation. Both are prodrugs; azathioprine is converted to mercaptopurine, which is subsequently metabolized to 6-thioguanine nucleotides, which are the presumed active moiety [299]. Azathioprine and mercaptopurine are used to treat patients with severe IBD or those who are steroid-resistant or steroid-dependent and are beneficial in 50 to 70% of patients with intractable perianal CD [298]. These drugs effectively maintain remission in CD and UC; they may also prevent or delay recurrence of CD after surgical resection. Because of concerns of side effects, the decision to initiate immunosuppressive therapy depends on an accurate assessment of the risk to benefit ratio. Mild leukopenia suggests that the drug is effective and therefore more likely to benefit the patient. It is prudent to obtain a complete blood count every two weeks during the initial treatment phase in patients with active disease and every three months in patients on maintenance therapy [300]. Drug-induced pancreatitis occurs in 3 to 5% of the patients, invariably during the first six weeks of azathioprine or mercaptopurine therapy [301]. Pancreatitis is a contraindication for continued use of these agents.

b) Methotrexate

Methotrexate was engineered to inhibit dihydrofolate reductase, thereby blocking DNA synthesis and causing cell death. First used in cancer treatment, methotrexate was subsequently recognized to have beneficial effects in 70% of patients with severe IBD [302]. The response to methotrexate appeared to be more rapid than the response to mercaptopurine. As with azathioprine-mercaptopurine, methotrexate is generally reserved

for patients whose IBD is either steroid-resistant or steroid-dependent [298]. In CD, it both induces and maintains remission, generally with a more rapid response than observed with mercaptopurine or azathioprine. Only limited studies have examined the role of methotrexate in UC. Side effects are rare and include leukopenia and hypersensitive interstitial pneumonitis. Hepatic fibrosis is the most severe potential side effect of long-term therapy. Patients with concomitant alcohol abuse and/or morbid obesity are more likely to develop hepatic fibrosis and therefore should not be treated with methotrexate [303].

c) Cyclosporine

Cyclosporine is effective in treating fistulous complications of CD and in severe UC that has failed to respond adequately to glucocorticoid therapy. In such patients, intravenously administered cyclosporine is highly effective for rapid disease control, and it may allow patients to avoid surgery. However, after one year, 70 to 80% of these patients may still require surgery. Thus, in many patients, the role of cyclosporine is to change a risky emergency operation into a less urgent procedure [304]. Between 50 and 80% of these severely ill patients improve significantly (generally within 7 days) in response to intravenous cyclosporine (2 to 4 mg/kg daily), which sometimes allows them to avoid an emergent colectomy. Oral cyclosporine is less effective as a maintenance therapy in IBD, perhaps because of its limited intestinal absorption. In this setting, long-term therapy with a microemulsion formulation of cyclosporine with increased oral bioavailability may be more effective, but this has not been fully studied. Cyclosporine can be used to treat fistulous complications of CD. A significant, rapid response to intravenous cyclosporine has been observed; however, frequent relapses accompany oral cyclosporine therapy, and other medical strategies are required to maintain fistula closure. Thus, it is generally used to treat specific problems over a short term while providing a bridge to long-term therapy [305]. Cyclosporine is lipid-bound and thus is associated with an increased risk of seizures when it is administered to acutely ill, severely malnourished patients who have low serum cholesterol/lipid levels. Oral maintenance with cyclosporine has, at best, limited benefit, and the relapse rate is high. The drug has a significant side effect profile that includes renal insufficiency and hypertension [305].

I-E.2. Corticosteroids

The glucocorticoid properties of hydrocortisone and prednisolone are the mainstay of IBD treatment. The preferred steroid is prednisolone, administered orally, rectally or parenterally in emergency situations. Corticosteroids can be used either alone or in combination with a suitable aminosalicylates formulation to induce and maintain remission in inflammatory bowel disease [303, 306]. By binding to intracytoplasmic glucocorticoid receptors found in most cell types, glucocorticosteroids activate glucocorticoid-responsive elements (GREs), resulting in a broad spectrum of effects on the immune system including inhibition of the recruitment and proliferation of lymphocytes, monocytes and macrophages, migration of neutrophils to sites of inflammation, and decreased production of soluble inflammatory mediators including cytokines, leukotrienes, and prostaglandins [307].

The response to steroids in individual patients with IBD divides them into three general classes: steroid-responsive, steroid-dependent, and steroid-unresponsive. Steroid-responsive patients improve clinically, generally within one to two weeks, and remain in remission as the dose of steroids is tapered and discontinued. Steroid-dependent patients also respond to glucocorticoids but experience a relapse of symptoms as the steroid dose is tapered [308]. Steroid-unresponsive patients do not improve even with prolonged high doses of steroids. Approximately 40% of patients are steroid-responsive, 30 to 40% have only a partial response or become steroid-dependent, and 15 to 20% of patients do not respond to steroid therapy [309]. Steroids are sometimes used for prolonged periods to control symptoms in steroid-dependent patients. However, failure to respond to steroids with prolonged remission (i.e., a disease relapse) should prompt consideration of alternative therapies, including immunosuppressants and infliximab. Steroids are not effective in maintaining remission in either UC or CD; thus, their significant side effects have led to an increased emphasis on limiting the duration and cumulative dose of steroids in IBD [310]. Systemic corticosteroids have an extensive side effect profile. Acute side effects include acne and severe mood changes, which are particularly common in young patients. Adrenal insufficiency can be triggered by an intercurrent infection in patients who are receiving low doses of systemic corticosteroids or in patients who have been recently tapered off of

corticosteroids. Visual changes can occur because of steroid-induced hyperglycemia. Early cataract formation is another possible side effect. Aseptic joint necrosis, which is the most dreaded side effect, usually occurs in patients receiving long-term, high-dose corticosteroid therapy. The incidence of this complication is 4.3% [311].

Budesonide is an enteric-release form of a synthetic steroid that is used for ileocecal CD [312]. It is thought to deliver adequate steroid therapy to a specific portion of the inflamed gut while minimizing systemic side effects caused by extensive first-pass hepatic metabolism to inactive derivatives. Topical therapies (e.g., enemas and suppositories) are also effective in treating colitis that is limited to the left side of the colon. While the topical potency of budesonide is 200 times higher than that of hydrocortisone, its oral systemic bioavailability is only 10%. In some studies, budesonide was associated with a lower incidence of systemic side effects than prednisone, although the data also indicate that systemic steroids are more effective in patients with higher CD activity index scores [313].

I-E.3. Biological agents - Tumor necrosis factor- α blockers

TNF- α is a proinflammatory cytokine with a central role in the pathogenesis of CD [314]. Infliximab is a chimeric monoclonal antibody directed against TNF- α which was introduced into clinical practice in the USA almost 12 years ago. Infliximab was the first TNF-blocker used for the treatment of moderately to severely active CD and UC in patients who do not respond despite complete and adequate therapy with a corticosteroid or an immunosuppressive agent. In the case of non-response to the standard three infusions at time 0, 2 and 6 weeks, further treatment with infliximab is not recommended. If remission is achieved, an attempt to withdraw or taper any concomitant corticosteroid therapy is appropriate. Treatment with corticosteroids, Azathioprine or mercaptopurine prior to and concomitantly with infliximab can reduce the formation of antibodies against infliximab. After successful remission, maintenance therapy is recommended, comprising an infusion every 8 weeks. In the case of a loss of effect, these intervals should be shortened up to 4 weeks, and if the patient is still therapy refractory, an increase in dose daily can be considered [315]. The use of infliximab is also recommended for the treatment of CD with fistulas. Before the administration of infliximab, delineation of the fistula anatomy is useful

to exclude the presence of an abscess [316]. Abscesses should be drained adequately before treatment with infliximab. Due to an increased risk of infection under immunomodulatory therapy with infliximab, it is contraindicated in patients with active tuberculosis and other serious infections or opportunistic infections.

Two additional anti-TNF molecules are currently used to treat IBD: adalimumab and certolizumab pegol [317]. To date, evidence for efficacy presented in clinical trials has been more robust for infliximab than for the other anti-TNFs. Sequential use of different anti-TNF agents in patients who have responded well to a first agent is applicable. Further clinical data is needed to decide whether early with TNF antibodies is generally efficient.

I-E.4. Antibiotics

There are experimental and clinical data demonstrating that colonic bacteria may either initiate or perpetuate the inflammation of IBD [318]. The standard antibiotics used for inducing remission in CD are ciprofloxacin and metronidazole. Ciprofloxacin is the antibiotic of choice and Metronidazole may be particularly effective for the treatment of perianal disease [319, 320]. Other antibiotics used for CD include trimethoprim/sulfamethoxazole and tetracycline. Studies have reported that either ciprofloxacin or metronidazole has produced yearlong remission rates of approximately 70%. Patients with severe perianal inflammation have responded to metronidazole treatment, experiencing less pain and tenderness, eventually decreased erythema and swelling, and wound healing. Specific CD-related complications that may benefit from antibiotic therapy include intra-abdominal abscesses and inflammatory masses, perianal diseases, bacterial overgrowth in the small bowel secondary to partial small bowel obstruction, secondary infections with organisms such as *Clostridium difficile*, and postoperative complications. Antibiotics can be used as (1) an adjunctive treatment along with other medications for treatment of active IBD, (2) a treatment for a specific complication of CD, or (3) prophylaxis for disease recurrence in postoperative CD.

I-E.5. Aminosalicylates

Aminosalicylates can be used in combination with steroids to induce and maintain remission in patients with inflammatory bowel disease. The first-line therapy for mild to moderate UC generally involves 5-aminosalicylic acid (5-ASA) [3]. The archetype for this class of medications is sulfasalazine, which consists of 5-ASA linked to sulfapyridine by an azo bond. Given individually, either 5-ASA or sulfapyridine is absorbed in the upper gastrointestinal tract; the azo linkage in sulfasalazine prevents its absorption in the stomach and small intestine, and the individual components are not liberated for absorption until colonic bacteria cleave the bond (Figure 9). 5-ASA is the active therapeutic moiety while Sulfapyridine is absorbed and metabolized by hepatic acetylation or hydroxylation followed by glucuronidation [3]. Although 5-ASA is a salicylate, its therapeutic effect does not appear to be related to cyclooxygenase inhibition; indeed, traditional nonsteroidal anti-inflammatory drugs may actually exacerbate IBD [321]. For patients with severe colitis, 5-ASA is of less certain value even though it is often used as an adjunct therapy to systemic glucocorticoids. Regardless of disease severity, this drug plays a useful role in preventing relapses once remission has been achieved [322].

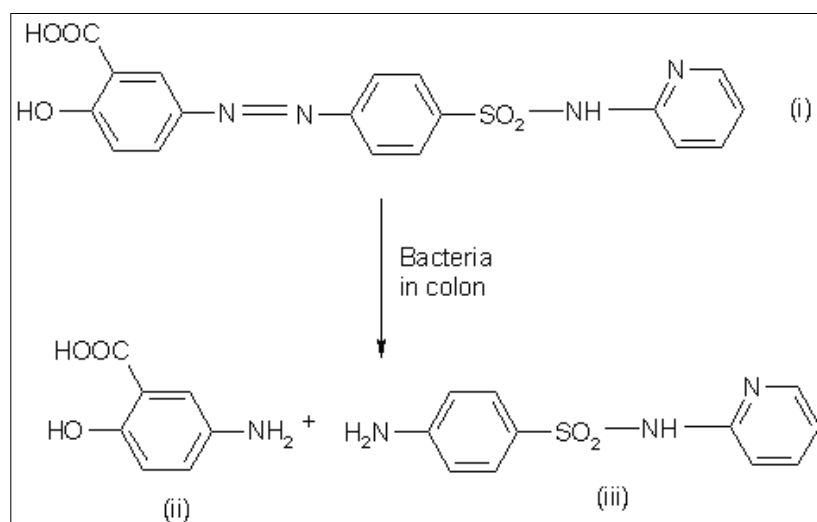


Figure 9. Hydrolysis of sulfasalazine (i) into 5-aminosalicylic acid (ii) and sulfapyridine (iii) [4].

5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

Although it is not active therapeutically, sulfapyridine causes many of the same side effects observed in patients taking sulfasalazine. To preserve the therapeutic effect of 5-ASA without the side effects of sulfapyridine, several second-generation 5-ASA compounds have been developed. As a result, formulations based on the active moiety (ie, 5-ASA) have been evaluated and two main methods of 5-ASA delivery have been used (Table 1).

Table1. Different dose and delivery system of 5-aminosalicylic acid (ASA) formulations [3]

Generic 5-ASA name	Commercial name	Dose per tablet, capsule or sachet, mg	Formulation	Site(s) of delivery
Mesalazine	Pentasa	500, 1000, 2000	Ethylcellulose-coated microgranules (slow, continuous release)	Duodenum to rectum
Mesalamine	Asacol	400, 800	Eudragit S-coated tablets (release at pH >7)	Terminal ileum, colon
Mesalamine	Mezavant/Lialda	1200	Eudragit S-coated multimatrix system	Terminal ileum, colon
Mesalazine	Salofalk	250	Eudragit L-coated tablets	Mid to distal ileum, colon
Mesalazine	Ipocol	400	Enteric coated with Eudragit S	Terminal ileum, colon
Sulfasalazine	Salazopyrin	500	5-ASA linked to sulfapyridine by azo bond	Colon
Balsalazide	Colazal	750	5-ASA linked to 4-aminobenzoyl-b-alanine by azo bond	Colon
Olsalazine	Dipentum	250	5-ASA dimer linked by azo bond	Colon

The first class is azobonded compounds that are controlled-release and pH dependent (pH 6 to 7). These molecules are non-absorbable prodrugs that are cleaved in the colon by the bacterial enzyme azoreductase and released. Drugs that use this delivery strategy include balsalazide (Colazal, Salix Pharmaceuticals, USA) and olsalazine (Dipentum, Celltech Pharmaceuticals Inc, USA). The second class is a composite (pH-dependent combined with controlled release), in which the formulation contains a gastroresistant coating and a pH/transit-dependent controlled-release that prevents 5-ASA release until the drug reaches the distal ileum. Agents that use this strategy are delayed-release mesalazine tablets (Asacol, Procter & Gamble Pharmaceuticals, USA), Salofalk tablets and Salofalk Granustix (Axcan Pharma Inc). Pentasa (Shire Inc, USA), in contrast to the formulations described above, releases 5-ASA from the duodenum to the rectum, and is often used 'off-label' to treat CD in addition to its indicated use for UC [323, 324].

Topical preparations of 5-ASA suspended in a wax matrix suppository or in a suspension enema are effective in active distal UC (Figure 10) [161]. They appear to be superior to topical hydrocortisone in this setting, with response rates of 75 to 90%. 5-ASA enemas should be used at bedtime and retained for at least eight hours; the suppository should be used two to three times a day with the objective of retaining it for at least three hours. Responses to local therapy with 5-ASA may occur within three to 21 days; however, the usual course of therapy is from three to six weeks. Once remission has occurred, lower doses are used for maintenance [3].

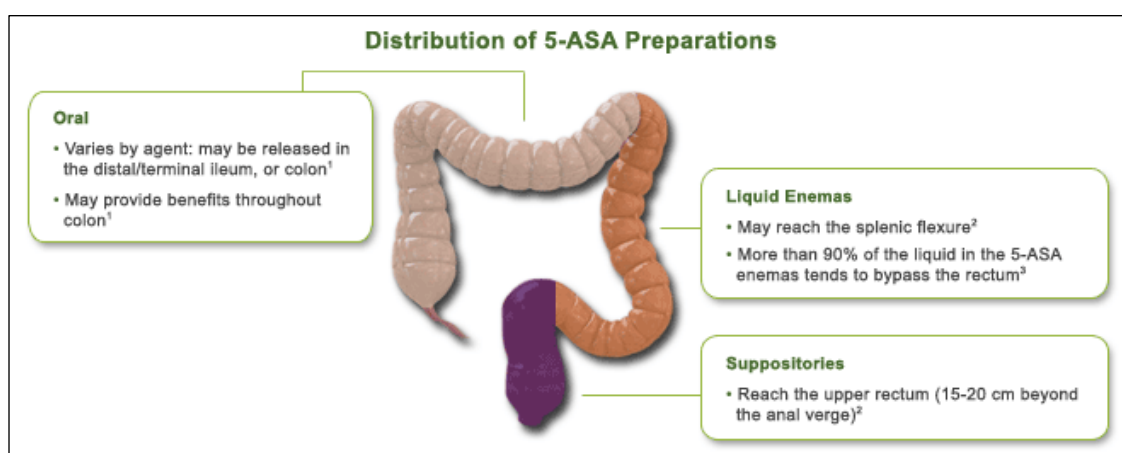


Figure 10. Choice of a 5-ASA formulation is partially based on the extent and location of disease (<http://www.canasa.com>).

Side effects of sulfasalazine occur in 10 to 45% of patients with UC and are primarily related to the sulfa moiety. Some side effects are dose-related, including headache, nausea, and fatigue. These reactions can be minimized by administering the medication with meals or by decreasing the dose. Other side effects include rash, fever, hepatitis, pneumonitis, hemolytic anemia, and bone marrow suppression. Sulfasalazine reversibly decreases the number and motility of sperm but does not impair female fertility. It also inhibits intestinal folate absorption; therefore, folate usually is given with sulfasalazine [321]. The newer 5-ASA formulations are generally well tolerated, and side effects are relatively infrequent and minor. Headache, dyspepsia, and skin rash are the most common side effects. Nephrotoxicity, although rare, is a more serious concern. 5-ASA has been associated with interstitial nephritis; while its pathogenic role is controversial, renal function should be monitored in all patients receiving these drugs. Both sulfasalazine and its metabolites cross the placenta but have not been shown to harm the fetus. Although they have not been studied thoroughly, the newer formulations appear to be safe also during pregnancy [161].

The mechanism of action of 5-ASA as an anti-inflammatory drug is diverse. It appears to act locally on colonic mucosa and reduces inflammation through a variety of anti-inflammatory processes. Several potential targets of 5-ASA action have been proposed. The current hypothesis is that 5-ASA activates a specific class of nuclear receptor, peroxisome proliferator-activated receptor (PPAR)-gamma [325]. PPAR γ , expressed in intestinal epithelial cells, was shown to mediate the effect of 5-ASA therapy in IBD by transrepressing several key target genes such as nuclear factor-kappa B (NF- κ B) and its downstream signaling pathway resulting in the inhibition of lipoxygenases and cyclooxygenases, and cytokine secretion. 5-ASA has also been recognized as a potent antioxidant and free-radical scavenger [326-330].

a) Potential role for Aminosalicylates in preventing colorectal cancer

After 5-ASA has been used for many years as an anti-inflammatory agent in the treatment of IBD, particularly UC, epidemiological studies have revealed that regular usage of 5-ASA by IBD patients was accompanied by decreased risk of colorectal cancer (CRC). In fact, colorectal cancer is considered the most serious complication in colonic IBD.

Furthermore, Patients with ulcerative colitis and Crohn's disease are at increased risk for developing colorectal cancer, and this is believed to be a result of chronic inflammation. Therefore, in recent years, several studies have been conducted to dissect the mechanisms by which 5-ASA interferes with CRC cell growth and survival. Some mechanisms have been revealed and others were remained undetermined.

II- Chemopreventive Role for 5-ASA in Inflammatory Bowel Disease

II.A- Colorectal Cancer: A Serious Complication of Inflammatory Bowel Disease

Patients with IBD, (CD and UC) are at increased risk of developing CRC (Figure 11). This increase in risk is more likely to result from chronic inflammation of the gastrointestinal mucosa than from any clear-cut genetic predisposition. Most CRCs, in general, develop from a dysplastic precursor lesion. In sporadic CRC, the dysplastic precursor is usually the adenomatous polyp (adenoma), a discrete focus of neoplasia that is typically removed by endoscopic polypectomy. In contrast, patients with IBD develop dysplastic lesions that can be polypoid, flat, localized, or multifocal; these are markers of colon inflammation and increased risk for neoplasia, and often indicate the need for surgical removal of the entire colon and rectum. These differences in morphology and biological behaviors of lesions not only make it a challenge to screen for CRC among patients with IBD, but they raise the important question of how chronic inflammation contributes to development of CRC. The progression from adenoma to carcinoma that occurs during development of sporadic colorectal tumors appears to be an inflammation–dysplasia– carcinoma sequence in IBD-associated CRC [331].

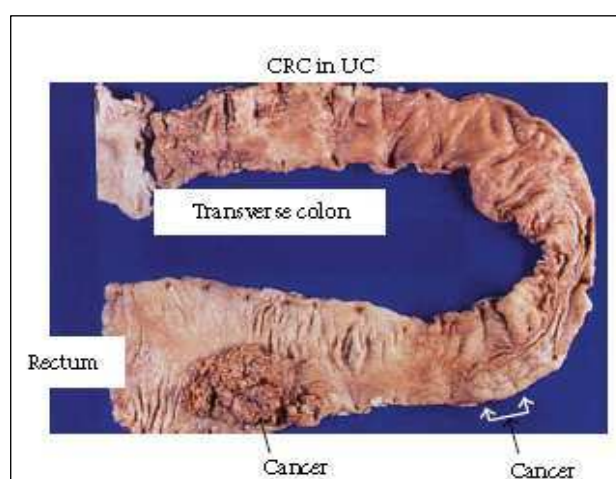


Figure 11. CRC in UC patients [13].

II-A.1. Incidence

Our understanding of the natural history of CRC in IBD has been gained largely from retrospective studies. The first CRC in patients with ulcerative colitis (UC) was described by Crohn and Rosenberg in 1925 [332]. In 1994, some UK data were published showing patients with extensive colitis showed an 18-fold increase in CRC risk [333]. CRC-complicating UC and CD accounts for 1–2% of all cases of CRC in the general population, but in patients with IBD approximately 10–15% of deaths are related to cancer [334]. A meta-analysis in 2001 including 116 studies from a wide array of centers and geographic sites estimated the cumulative risk of CRC in UC to be 1.6% at 10 years, 8.3% at 20 years and 18.4% at 30 years [335]. More recent studies estimate the risk of CRC in IBD patients to be approximately half that reported in the Eaden's meta-analysis. *Bernstein et al.* conducted a large population-based study in Manitoba, Canada, including 19,655 CD person-years and 21,340 UC person-years between 1984 and 1997. There was an increased incidence rate ratio of colon carcinoma for both CD patients (2.64) and UC patients (2.75) compared to the general population [336]. *Rutter et al.* from St. Mark's Hospital in the United Kingdom reported on their 30 year experience with the longest prospectively collected database on surveillance for dysplasia and cancer in UC. The cumulative incidence of CRC in this referral population was 2.5% at 20 years, 7.6% at 30 years and 10.8% after 40 years of disease [337]. In a population-based study in Hungary, in which 723 patients diagnosed with UC were followed over 30 years, an annual incidence of CRC of 0.15% was estimated and the cumulative incidence of CRC was found to be 0.6% after 10 years, 5.4% after 20 years and 7.5% after 30 years of chronic UC [338]. The reasons for this observed change in incidence may be the more widespread use of surveillance colonoscopy, a chemoprotective effect attributable to the more widespread use of maintenance therapies, more aggressive surgical intervention, and/or dietary or environmental factors [339]. **The statistics that made from CRC a threat to the lives of IBD patients is that more than 20% of IBD patients develop colitis-associated cancer (CAC) within 30 years of disease onset, and >50% of these will die from CAC [340].**

II-A.2. Risk Factors

Different studies have demonstrated that several inherent factors of the disease and/or possibly genetic factors may influence the risk of developing CRC in IBD. Identifying these factors will allow to better define a target population more likely to require colonoscopic surveillance and/or chemoprevention. Overall, factors that appear to increase the risk of CRC of patients with IBD include duration, severity, and extent of IBD; family history of CRC; primary sclerosing cholangitis; age at UC diagnosis; degree of histologic/endoscopic inflammatory activity; and presence of dysplasia of any grade (indefinite, low, or high) [337, 341-343]. However, it may be difficult to compare the exact relative risks for these various factors because of differences in the patient populations and how the analyses were performed.

a) Age of onset and duration of IBD

Some studies indicate that patients for whom the onset of disease occurs at an early age have a higher risk of developing CRC. This is the case for UC, for which the risk of CRC 35 years after disease onset was estimated by *Ekbom et al.* at 40% among patients diagnosed before the age of 15, whereas it was estimated at only 25% for those aged from 15 to 39 years at diagnosis, but also for CD, for which the relative risk of CRC among patients with isolated colitis increased from 5.6 to 20.9 for those aged under 30 years at diagnosis [344]. The role of age at onset, alone, remains however controversial. Most authors agree that the increased incidence among patients having developed IBD early in life results probably, or at least partly, from a longer duration of disease: before 8 to 10 years of disease progression, CRC risk is generally assumed to be not higher than in the general population [335, 337]. It is only later that it increases by approximately 0.5 to 1% each year, reaching cumulative incidence rates. However, a recent study shows that a substantial proportion of CRCs may occur before 8-10 years of disease [345].

b) Disease extension

Colonic extent of the disease has been identified as an independent risk factor. In UC, CRC occurs essentially in patients presenting with pancolitis or a history of colitis beyond the left angle of the colon (extensive colitis). When colitis does not affect beyond the left angle, the risk of CRC is low with a relative risk of 2.8 in the case of left-sided colitis *versus* 14.8 in the case of pancolitis, and very low when disease is limited to the rectosigmoid with a relative risk of 1.7, and virtually inexistent in the case of proctitis [344].

c) Chronic inflammation

The link between persistent inflammation and risk of CRC has long been controversial. Nevertheless, recent clinical and experimental data demonstrate that chronic inflammation is a key factor contributing to colon carcinogenesis in IBD. In addition, features indicative of previous severe inflammation, such as pseudopolyps, and features indicative of chronically active colitis, such as a shortened or tubular colon and stricture formation are all associated with a significant increase in the risk of CCR [346].

d) Family history of colorectal cancer

A family history of CRC increases the risk of CRC in UC and in CD [347, 348]. Conversely, the presence of a first-degree relative presenting with an IBD does not increase the risk of developing CRC among other healthy family members [347].

e) Presence of reflux ileitis

Heuschen et al. suggested that the presence of reflux ileitis may be an independent risk factor for CRC in UC [349]. These results were, however, not reproduced in a recent case-control study published by *Rutter et al.* [346].

f) Association with primary sclerosing cholangitis

Many studies have demonstrated that patients with primary sclerosing cholangitis (PSC) associated with UC have a higher risk of developing CRC [350-352]. For example, a case-control study conducted by *Broome et al.* observed a cumulated risk of CRC of 9% after 10 years of disease progression among patients presenting both diseases, of 21% after 20 years and 50% after 25 years, compared with 2%, 5% and 10% respectively among patients with UC, but not PSC [350]. Furthermore, studies have shown that this risk remains even after liver transplantation for PSC [352].

g) Dietary factors

Risk factors for CRC include obesity, lack of exercise, alcohol and tobacco consumption, and dietary factors, such the Western diet (large amounts of red meat and fat, low amounts of vegetables, fruit, and fiber) [353]. Several other compounds have been found to reduce CRC risk, including carbohydrates, unsaturated n-3 fatty acids, vitamins, minerals (calcium and selenium), and phytochemicals (resveratrol, curcumin) [354]. Although the molecular mechanisms of these compounds are unknown and probably numerous, there is evidence that they reduce inflammation and decrease the activity of oncogenic signaling pathways. For example, vitamin D reduces the amount of activated β -catenin and can therefore reduce expression of c-myc in certain cell lines [355, 356]. Butyrate, a derivate of inulin and oligofructose (carbohydrates) that is produced by certain colonic bacteria, seem to modulate TGF- β , cyclooxygenase-2 (COX2), and IFN- γ signaling by unknown mechanisms [357, 358]. Curcumin, resveratrol, and green tea polyphenols have a wide range of antitumor activities, affecting inflammation by reducing activation of NF- κ B [359]. However, their beneficial activities might arise from their antioxidant properties [360].

II-A.3. Pathophysiology

Colitis-associated cancers develop in chronically inflamed mucosa and are believed to develop in a sequence of no dysplasia–indefinite dysplasia–low-grade dysplasia–high-

grade dysplasia–carcinoma (Figure 12). Tumor progression in patients can skip one or more of these steps [361]. Like sporadic CRC, colon carcinogenesis in patients with IBD occurs through a sequence of events, such as mutations in somatic cells followed by their clonal expansion. However, unlike sporadic CRC, which develops from dysplasia in 1 or 2 foci of the colon, cancer arising in colitic mucosa often develops from multifocal dysplasia, indicating a field change effect. Studies of patients with chronic UC that used repeated colonoscopic examination to follow aneuploid cells, a marker of genomic instability, indicated that the cell populations remained in the same locations of the colon, but spread over time, occupying larger areas of the mucosa [362]. Moreover, within a specific area of aneuploidy, subclones of aneuploid cells appeared to emerge from their predecessors. The subclones became increasingly dysregulated, with greater amounts of genomic instability, as they expanded at the expense of the normal surrounding epithelium. For the most part, in patients with IBD, neoplastic lesions arise within inflamed areas of the colonic mucosa. The observed re-epithelialization of large patches of colonic mucosa by abnormal clones is a consequence of the healing response to ulceration from chronic inflammation. Because aneuploidy is often more widespread than dysplasia, substantial genomic alterations must occur in colonic mucosa without disturbing morphology.

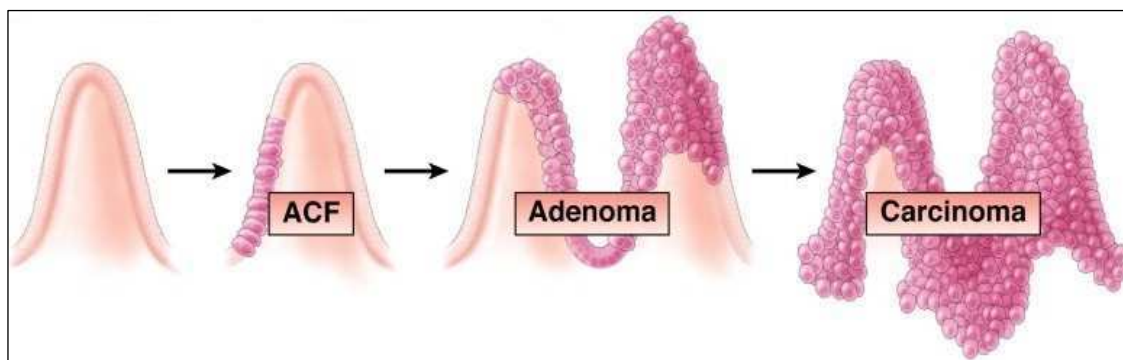


Figure 12. Common steps of colon development. Adapted from [1].

a) Genetic instability

Perhaps not surprisingly, many of the cellular defects associated with development of sporadic colorectal carcinomas are associated with colitis-associated colon carcinogenesis

[363]. In colitis-associated cancer, the frequencies of chromosome instability and microsatellite instability (MSI) are 85% and 15%, respectively—approximately the same as in sporadic colorectal carcinomas [364]. Progression of sporadic colorectal cancer differs from that of IBD-associated CRC, however, in the timing and frequency of these alterations (Figure 13). For example, loss of adenomatous polyposis coli (APC) function, an early event in progression of sporadic colorectal carcinomas, is less frequent and usually occurs later in the development of colitis-associated dysplasias and carcinomas [365-367]. Mutations in APC are rarely, if ever, detected in cells of the colitic mucosa that are negative or indefinite for dysplasia, and <14% of tissues with low-grade dysplasia or cancer have mutations in APC [365, 366, 368]. Likewise, allelic deletion of APC occurs in <33% of colitis-associated neoplasms [368].

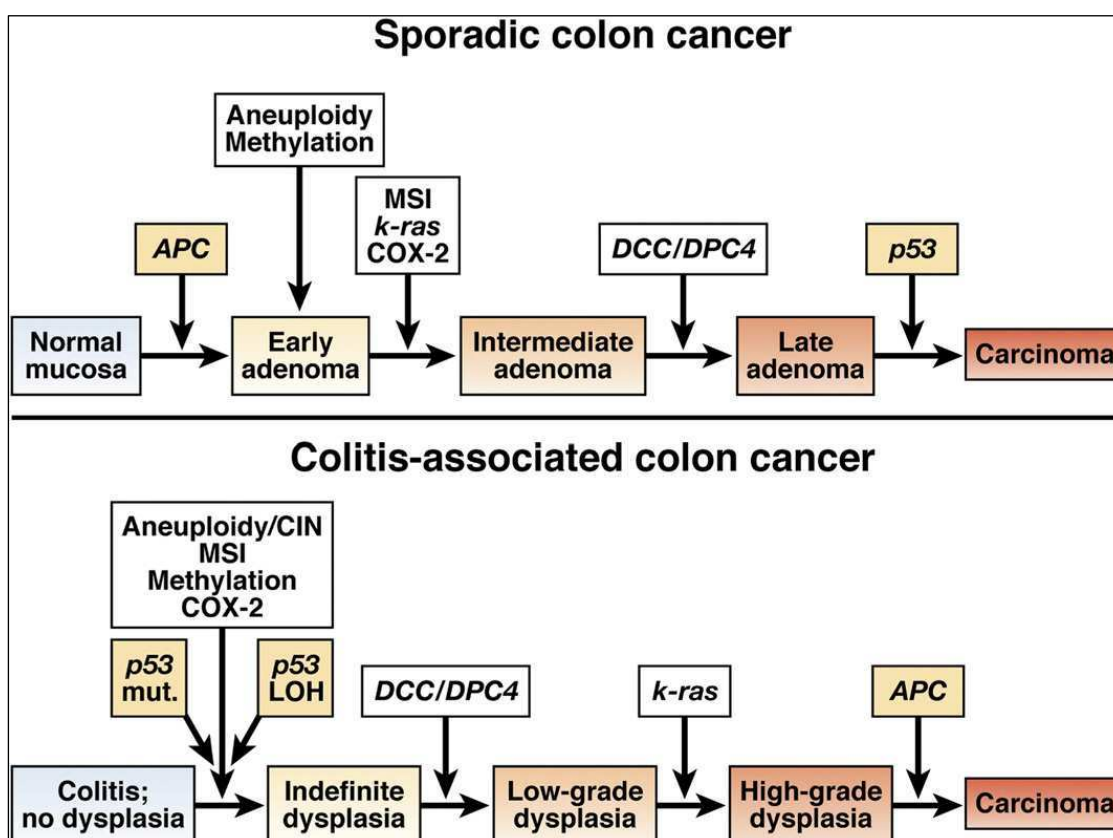


Figure 13. Molecular pathogenesis of sporadic colon cancer and colitis-associated colorectal cancer. There are similarities between the pathways, including the development of aneuploidy, microsatellite Instability, DNA methylation, activation of the oncogene *k-ras*, activation of *COX-2*, and mutation and eventual loss of heterozygosity of *p53*, and adenomatous polyposis coli (*APC*). However, the frequency and sequence of these events differs between the cancers [21].

Loss of p53 function is an important step in progression of colitis-associated cancer. Allelic deletion of *p53* was observed in approximately 50%–85% of colitis-associated tumors [369, 370]. Loss of heterozygosity at *p53* correlates with malignant progression—it was detected in 6% of biopsy samples without dysplasia, 9% with indefinite dysplasia, 33% with low-grade dysplasia, 63% with high-grade dysplasia, and 85% with cancer [369]. *p53* mutations are found in colitic colon tissue and often in mucosa that is non-dysplastic or only indefinite for dysplasia [369, 371]. In carefully mapped colectomy specimens, p53 mutation was determined to occur early in the process of tumorigenesis, before aneuploidy (Figure 13) [371]. In fact, p53 mutations were found in inflamed mucosa from >50% of UC patients who did not have cancer, indicating that chronic inflammation might cause these mutations [372].

DNA methylation also contributes to development and progression of colitis-associated cancer (Figure 13). Methylation of CpG islands in several genes precedes dysplasia and can be detected throughout the mucosa of patients with UC [373]. Among neoplastic samples from patients with colitis, *hMLH1* hypermethylation was observed in 6 of 13 (46%) with high levels of MSI, 1 of 6 (16%) with low levels of MSI, and 4 of 27 (15%) without MSI, so it might cause MSI or contribute to its development [374]. Loss of expression of the cell cycle inhibitor p16INK4a is associated with sporadic CRC; this gene is frequently hypermethylated in neoplastic samples from patients with UC [375]. Approximately 10% of biopsy samples that do not have dysplasia have hypermethylation of the *p16* promoter; the rate increases with higher grades of dysplasia, reaching 100% in tumor samples. p14ARF is an indirect regulator of p53; it is encoded by the same gene as p16INK4a. Loss of expression of p14ARF, via hypermethylation of the gene that encodes it, has been reported in 50% of adenocarcinoma, 33% of dysplastic, and 60% of mucosa samples without dysplasia, collected from patients with UC [376].

Little is known about the exact cellular origin of colon cancer. If mutations occur in a differentiated cell type that does not proliferate, the mutations will not be propagated. Transformed stem cells at the crypt base might therefore be responsible for the development and maintenance of early neoplastic lesions. Loss of APC specifically from

colonic stem cells results in progressively growing neoplasms, whereas its loss from transit-amplifying cells (located near the crypt base) does not [377].

b) Oxidative stress

In addition to promoting chromosomal and microsatellite instability and CpG island methylation, inflammation contributes to colon carcinogenesis by producing oxidative stress. IBD has been considered to be an “oxyradical overload” disease, in which chronic inflammation increases the risk for cancer [378]. Oxidative stress causes cellular damage that contributes to pathogenesis of the colitis itself and to colon carcinogenesis (Figure 14). The initiation of tumor formation in chronically inflamed tissue might be mediated by reactive oxygen and nitrogen species (RONS), which are released by cells of the innate immune system. Inflamed tissues from patients with active UC or CD-associated colitis demonstrate increased expression of nitric oxide synthase (NOS) and RONS [379-381]. Furthermore, using glyceraldehyde-3-phosphate dehydrogenase as a molecular marker, *McKenzie et al.* showed that oxidation of thiols in the active site of glyceraldehyde-3-phosphate dehydrogenase, with subsequent inhibition of enzyme activity, occurred in colonic epithelial cells from inflamed mucosa of patients with CD or UC, but not from paired samples of unaffected mucosa [382]. Measurements of 8-hydroxydeoxyguanosine, a mutagen formed by the action of hydroxyl radical at the C8 position of deoxyguanosine DNA base, in mucosal biopsies of patients with UC were increased compared to normal controls, with levels even higher in UC patients who had dysplasia [383].

How does oxidative stress contribute to colorectal carcinogenesis? Free radicals can affect many metabolic processes, including those that regulate DNA, RNA, proteins, and lipids [378, 384]. When they affect genes or proteins that maintain colonocyte homeostasis, such as p53, dysplasia, and subsequent carcinoma arise. *Hussain et al* compared mutations at codons 247 and 248 of p53 in biopsy samples collected from inflamed colonic mucosa of UC patients with those of normal mucosa from individuals without UC (controls) [372]. They reported that >50% of the UC samples had a higher frequency of G-to-A transitions at the CpG site of codon 248, and a higher frequency of C-to-T transitions at the third base of codon 247, compared to controls. Moreover, in paired biopsies from UC patients, these

mutations were only observed in inflamed (not in non-inflamed) mucosa. Increased NOS-2 activity was associated with these p53 mutations. In colon cancer cell lines, hydrogen peroxide inactivated the mismatch repair system, apparently by damaging the protein complexes that perform the DNA repair [385]. Oxidative stress might therefore disable the mismatch repair system to cause MSI. MSI has been detected in chronically inflamed mucosa from UC patients, even in those with short disease duration before the risk of neoplasia ostensibly increases [386]. However, MSI was not found in normal colon mucosa from healthy controls, from patients with other types of benign inflammatory colitis, or patients with CD [386, 387]. Therefore, only specific types of chronic inflammation appear to cause MSI.

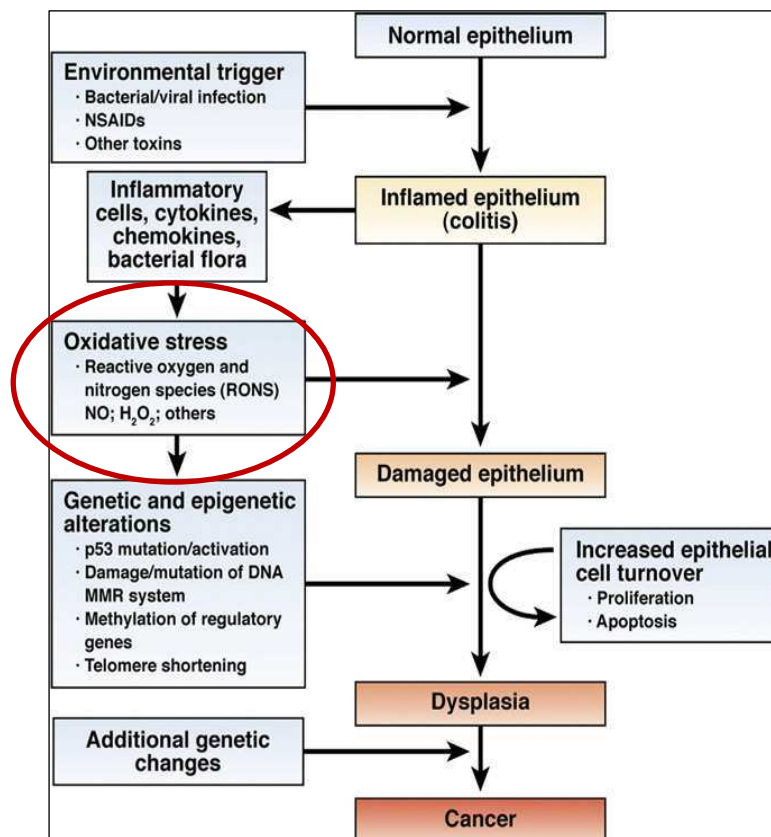


Figure 14. The sequence of events from inflammation to cancer in patients with inflammatory bowel disease [21].

c) The immune response

The interplay between cellular and acellular components that underlies and sustains the pathophysiology of colorectal cancer has been shown complex and multifaceted. It does not only involve almost all cell components but rather engages and exploits extracellular signals and nutrients to the benefit of the cancerous cell (Figure 15). The dissection of these pathways is beyond the scope of this section, it would rather discuss the major players involved in the pathogenesis of colitis-associated colorectal cancer.

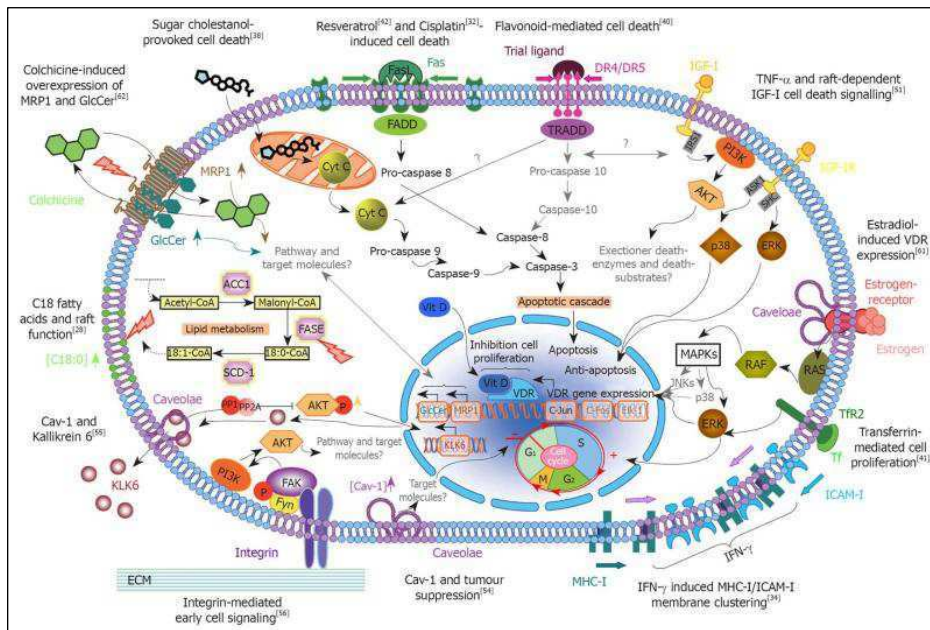


Figure 15. Scheme outlining the various intracellular signaling pathways in colorectal cancer epithelial cell [16].

Like other solid malignancies, colorectal and colitis-associated tumors are infiltrated by various types of immune cells. Cells of the innate immune system, such as neutrophils, mast cells, natural killer (NK) cells, dendritic cells (DC), and tumor-associated macrophages can be easily detected in these tumors (Table 2) [388]. In addition, advanced tumors recruit specific myeloid subsets that represent phenotypically heterogeneous but a functionally similar population of CD11b⁺Gr1⁺ cells, called myeloid-derived suppressor cells [389]. These cells share some characteristic with monocytes, macrophages, neutrophils, and DC and help suppress antitumor immune responses and tumor angiogenesis [389]. Cells of the adaptive immune system are also recruited into colorectal and colitis-associated tumors, where they

have either pro- or antitumorigenic roles. T cells, for instance, are required for inflammation, cancer development, and tumor progression, as well as for anticancer immunity [390-392].

Table2. Tumor-Promoting Immune Cells and Cytokines in Colon Cancer. DC, dendritic cells; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; IL, interleukin; MAPK, mitogen-activated protein kinase; MDSC, myeloid-derived suppressor cells; MMP, matrix metalloproteinase; NF- κ B, nuclear factor- κ B; NK, natural killer; STAT3, signal transducer and activator of transcription 3; TGF, transforming growth factor; TNF, tumor necrosis factor; Treg, regulatory T cells; VEGF, vascular endothelial growth factor [1].

Cell type	Function or mechanism
T cells (CD4 and CD8)	Cytokine production (IL-6, IL-10, TNF, IL-21, IL-17, IL-22, IFN- γ , lymphotoxin, RANKL)
Treg cells	Cytokine production (IL-10, TGF- β)
Macrophages, DC, MDSC, neutrophils	Cytokine production (IL-6, IL-1, VEGF, IL-23, TNF)
NK cells	Cytokine production (IFN- γ , IL-22, IL-17)
B cells	Cytokine production
Epithelial and tumor cells	IL-1, IL-6, TNF, EGF

Cytokine	Mechanisms/pathways in cancer and immune cells
TNF- α	Survival, activation, recruitment, growth. AP-1, MAPK and NF- κ B activation
IL-6	Survival, growth, T-cell survival and differentiation, myeloid cell recruitment. STAT3, ERK, and Akt
IL-11	Survival, growth. STAT3, STAT1, ERK
IL-23	T-cell differentiation (Th17) and interference with Tregs, production of IL-17 and IL-22 by immune cells.

(Continued Table 2)

Cytokine	Mechanisms/pathways in cancer and immune cells
IL-1 α , IL- β	Survival, growth, cytokines, chemokines, T-cell activation and differentiation. NF- κ B, MAPK
IL-22	Survival, mucosal integrity, chemokines. STAT3
IL-17A,F	Survival, chemokines, T-cell regulation, monocytes, and neutrophil recruitment. MAPK, NF- κ B
EGF	Survival, proliferation. MAPK, STAT3
IL-10	Anti-inflammatory, Treg stimulation. Unknown effects on cancer cells. STAT3, MAPK

What is the contribution of tumor immunosurveillance vs tumor-promoting inflammation in CRC and CAC? In CAC, the immune system seems to have a mostly protumorigenic role. In sporadic CRC, there seems to be a well-defined balance between immunosurveillance (executed by CD8⁺ T cells, NK cells, and CD4⁺ T cells) and tumor-promoting inflammation (executed by innate immune cells, B cells, and various subtypes of T cells) (Figure 16). In CRC and CAC, immunosurveillance could mediate the early detection and elimination of transformed cells and aberrant crypt foci, and also keep small tumors at dormant state. Immunosurveillance may be also important during metastasis, when small numbers or single metastatic cells travel and can be attacked by antitumor immune cells that are not inhibited by factors in the tumor microenvironment. In many other stages of colorectal and colitis-associated tumorigenesis, inflammation counteracts and outcompetes antitumor immunity by direct immunosuppressive effects, as well as by regulation of tumor-cell survival, proliferation, angiogenesis, and other hallmarks of tumorigenesis [1].

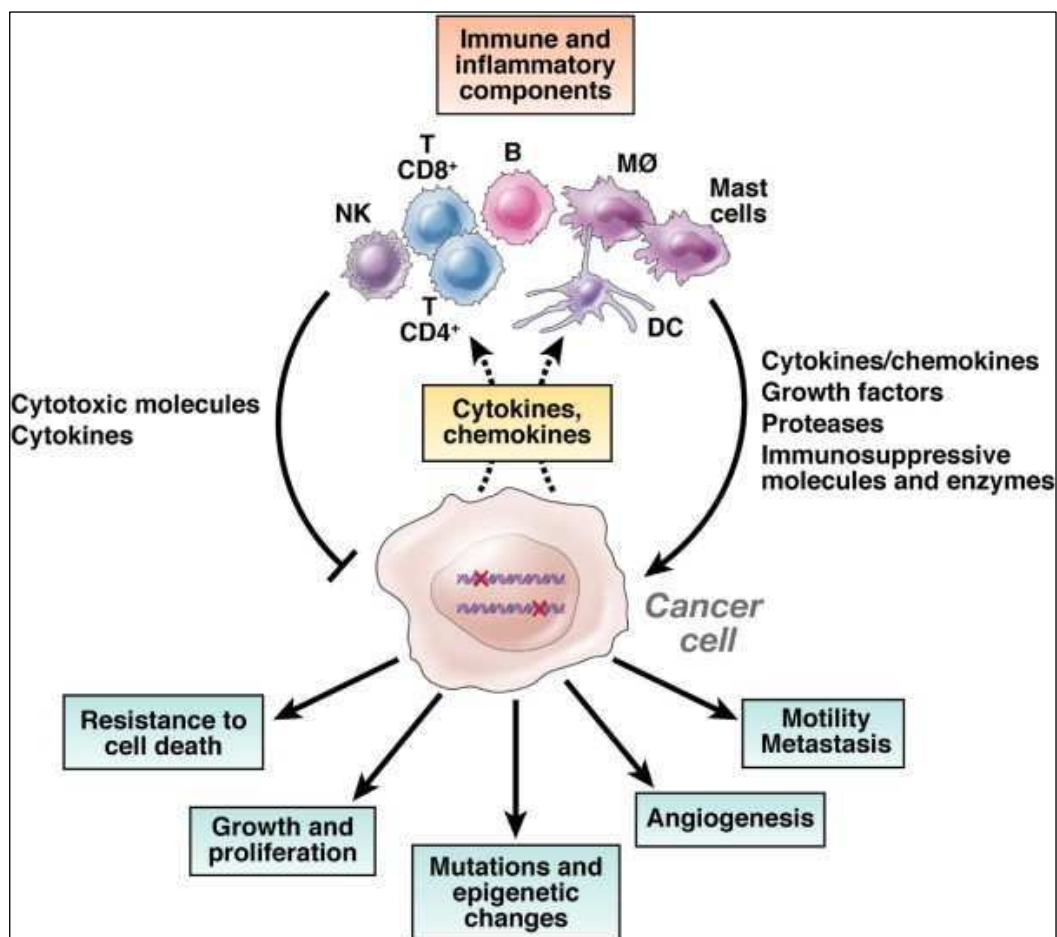


Figure 16. Immunosurveillance and inflammation in colorectal cancer (CRC) and colitis-associated cancer (CAC). Immunosurveillance is typically mediated by cytotoxic and helper T lymphocytes as well as natural killer (NK) cells. Innate and adaptive immune cells also promote tumor development. Inflammation can promote colitis-induced tumorigenesis [1].

In CAC, chronic inflammation precedes colitis-associated tumor development. Chronic inflammation causes oxidative damage to DNA, induces genetic mutations and microsatellite instability (as discussed in the section of genetic instability). At the cellular level, several inflammatory factors have been identified as key players in colitis-associated cancer. For instance, NF- κ B, TNF- α and IL-6 have been shown to promote and perpetuate inflammation and carcinogenesis in these settings. Most tumor-promoting cytokines are activated via NF- κ B transcription factors or (along with other inflammatory stimuli) activate NF- κ B signaling in premalignant cells and immune/inflammatory cells [393]. Aberrant NF- κ B activation was detected in >50% of colorectal and colitis-associated tumors and mouse studies have established a role for NF- κ B in CAC development [394, 395]. NF- κ B activation can support tumorigenesis by increasing cell proliferation and angiogenesis, inhibiting cell

death, and promoting cell invasion and metastasis [396]. The anti-apoptotic activity of NF- κ B is mediated via its activation of *Bcl2*, *Bcl-xL*, and *cFLIP*, along with other genes [396]. It has been shown that loss of function of NF- κ B function was characterized by decreased expression of different inflammatory cytokines and chemokines (IL-1, IL-6, TNF- α , COX-2) which are known to promote inflammation and carcinogenesis [397]. On the other hand, activation of NF- κ B in intestinal tissue with chronic colitis has been reported to up-regulate netrin-1, which provides antiapoptotic signals to the intestinal epithelium, to promote tumor progression [398, 399]. There are therefore many mechanisms by which NF- κ B promotes tumor development and progression (Figure 17). Interestingly, cancer cells with activated NF- κ B are resistant to chemotherapeutics and ionizing radiation; inhibition of NF- κ B activity greatly increases cell sensitivity to these agents [400]. IBD is associated with persistent NF- κ B activation in myeloid and epithelial cells of the colonic mucosa [401].

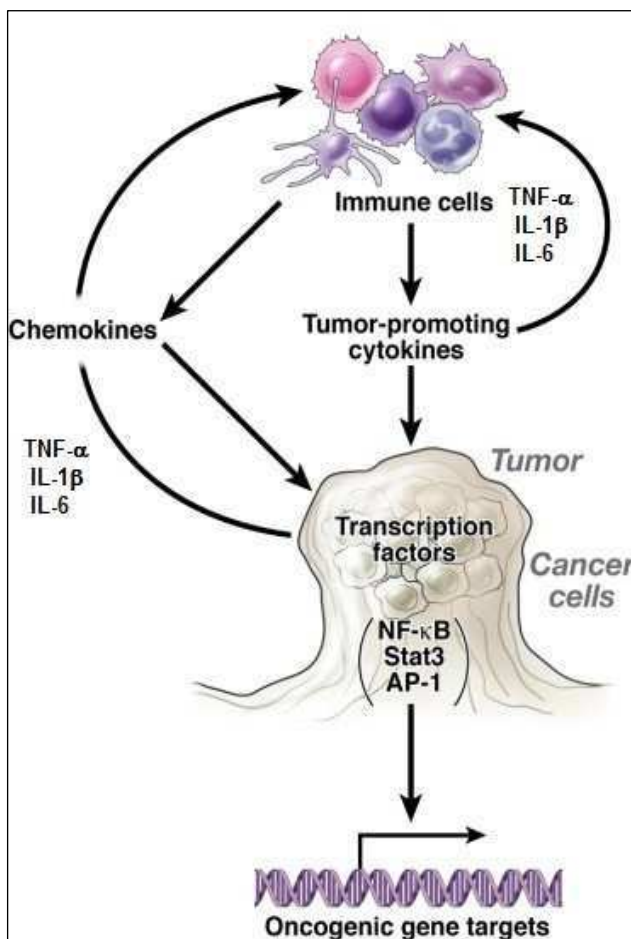


Figure 17. Role of NF- κ B in colon carcinogenesis. Tissue injury stimulates cytokine production by immune cells in the tumor microenvironment. Cytokines and growth factors in the tumor microenvironment activate NF- κ B and STAT3 which regulate genes that control cell proliferation, tumor growth, angiogenesis, and tumor progression and invasion. Activation of NF- κ B induces positive signaling loops that increase production of chemokines and cytokines that recruit inflammatory cells in the tumor microenvironment. Adapted from [1].

Most tumor-promoting cytokines are produced by lamina propria macrophages and DC during early states of CAC development or by T cells during late-stage tumor progression. TNF- α is released by activated macrophages and T cells; it binds to the TNF-receptor (TNF-R) and has been reported to promote inflammation and colitis-associated cancer. TNF- α can initiate carcinogenesis by promoting DNA damage, it can stimulate angiogenesis, and it can induce expression of COX-2, which also induces angiogenesis to promote tumor growth [402]. There are many downstream targets of TNF-R1 activation, but its activation of the transcription factor NF- κ B is likely to contribute to carcinogenesis [403]. Loss of function of NF- κ B function was characterized by reduced expression TNF- α , implying a dual role of TNF- α and NF- κ B in perpetuating inflammation and carcinogenesis [397].

Beside TNF- α , IL-6 is a potent stimulator of colon cancer cell proliferation and tumor growth [404]. For instance, high levels of circulating IL-6 have been reported in patients with CRC [405]. *Becker et al.* showed that IL-6 signaling to Stat3 promoted growth of colitis-associated cancer [406]. Subsequent studies showed that IL-6 produced by lamina propria myeloid cells protected normal and premalignant intestinal epithelial cells from apoptosis, an effect that was mediated by Stat3; the authors of this study proposed that IL-6 production by immune cells promotes epithelial tumor cell development and tumor growth in the setting of inflammation, whereas IL-6 production by epithelial cells contributes to epithelial carcinogenesis in the absence of inflammation [407]. A recent study reported increased epithelial cell expression of IL-6 and Stat3 in tissues from patients with active UC (compared to those with inactive UC), as well as in dysplasia and cancer samples [408]. Taken together, these findings implicate IL-6 signaling via Stat3 as an important pathway in colitis-associated neoplasia (Figure 17).

COX2 is an inducible mediator of prostaglandin synthesis and an important factor in colorectal tumorigenesis [409]. COX2 expression is upregulated in the colorectal tumors and in experimental models of CAC [410, 411]. Selective inhibitors (such as celecoxib) of COX were shown to reduce CRC incidence [411]. The pro-tumorigenic effects of COX2 are mediated by its major end product, PGE₂; and human colorectal tumors have increased levels of Prostaglandin E₂ (PGE₂) [412, 413]. In treating patients with IBD or CAC, the situation is more complex because COX2 inhibitors can exacerbate colitis and intestinal

injury. The pro-tumorigenic mechanisms of COX2 include inhibition of apoptosis, by increasing expression of Bcl2 via the mitogen-activated protein kinase or phosphoinositide 3-kinase-AKT signaling pathways [414-416]. PGE2 also activates β -catenin-dependent signaling, which promotes survival and proliferation. COX2 can also stimulate tumor angiogenesis by inducing production of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, and increase tumor dissemination by altering the adhesive properties of cells and increasing matrix metalloproteinase activity [417, 418]. Lastly, in myeloid cells, PGE2 signaling reduces expression of anti-tumorigenic IL-12 and increases expression of pro-tumorigenic IL-23 [419].

In addition to the innate immune response, the adaptive immune response has a role in pathogenesis of colitis-associated cancer. It has been demonstrated that Th17 and Th1 cells might be involved in inflammation-induced cancer [420, 421]. In samples from patients with CRC, increased T-bet expression, the transcription factor which controls development of the Th1 cell lineage, correlated with longer survival time and reduced lymphovascular invasion of the tumor [422]. Regulatory T (Treg) cells have anti-inflammatory effects and protect against colitis and associated cancer. Erdman and Poutahidis proposed that the ability of Treg cells to inhibit cancer depends upon gastrointestinal bacteria and IL-10, which maintain the Treg response. However, under proinflammatory conditions, Treg cells may fail to perform this inhibitory role and could contribute to Th17-cell-induced carcinogenesis [423].

d) Gastrointestinal microflora: lessons from animal models

If the microbiota is involved in cancer development, the colon must be its major site of action—the human intestine contains >500 different types of micro-organisms and the colon contains $>10^{13}$ bacterial cells [424]. Studies have shown that CAC development depends qualitatively and quantitatively on the intestinal microflora [425, 426]. Intestinal inflammation can result from an aberrant ratio of protective (tolerogenic) to aggressive (proinflammatory, damage-inducing, pro-tumorigenic) microflora (Table 3). Changes in the number, diversity, and stability of commensal bacteria (dysbiosis), particularly the *Clostridia* group, can alter normal physiological processes and lead to disease, including CRC [427]; the

quantity and quality of gastrointestinal microbiota also affects CAC development [425]. It is possible that only bacteria that induce a combination of tissue injury and inflammation appear to promote cancer, whereas inflammation without continuous cycles of injury and repair is insufficient for tumor induction. Other pathogens or conditional commensals also contribute to tumorigenesis. These can activate the immune system to produce either ROS, as in case of *Enterococcus faecalis*, or pro-survival and pro-angiogenic cytokines (IL-6, IL-8, IL-17), as in case of *Streptococcus bovis*, *B fragilis*, and other bacteria [428].

Table3. Microbes Linked to Colorectal Cancer [1].

Microbe	Mechanism of pathology
<i>Bacteroides fragilis</i> , enterotoxigenic	Activation of STAT3 in the colon, triggers injury and IL-17 production
<i>Bacteroides vulgatus</i>	MyD88 dependent signaling, possibly NF- κ B activation
<i>Bifidobacterium longum</i>	
<i>Clostridium butyricum</i>	Increased bacterial presence
<i>Mitsuokella multiacida</i>	
<i>Escherichia coli</i> , invasive	Intracellular colonization
<i>Enterococcus faecalis</i>	ROS production and DNA damage
Germ-free condition	Resistance to colon cancer
<i>Streptococcus bovis</i>	Production of IL-8, aberrant crypt formation, increased proliferation

The intestinal microbiota makes a significant contribution to the development of not only colitis, but also neoplasia. In several different rodent models of IBD, commensal bacteria or specific bacteria (such as *H hepaticus*) are required to initiate inflammation and for development of dysplasia or cancer [429]. Without these bacteria, neither colitis nor neoplasia develops. Under conventional conditions, IL-10-null mice develop spontaneous, generalized enterocolitis that requires IL-12 and the presence of enteric bacteria [430]. Under specific pathogen-free conditions, all the mice develop colitis after 3 months of age.

5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

After 3 months and 6 months, 25% and 60% of the mice, respectively, develop adenocarcinoma. Administration of IL-10 improved the colitis and reduced tumor development by 50%, even after the colitis had been established [431]. Probiotics reduced mucosal inflammation and the number of IL-10-null mice that developed colon tumors [432]; whereas pure cultures of *Enterococcus faecalis* induced colitis and, after 20 weeks, dysplasia and cancer of the rectum [433]. Mice that are deficient in T-cell receptor β chain have defective intestinal mucosal immune systems and develop colitis that resembles UC. When raised under conventional conditions, T-cell receptor β and p53 double-knockout mice develop chronic inflammation (90%, most limited to ileocecum and cecum), dysplasia (50% - 70%), and adenocarcinoma (70%) [434]. Germ-free conditions prevent chronic inflammation and tumor formation

Glutathione peroxidases (GPX) are intracellular antioxidant enzymes that reduce hydrogen peroxide and organic hydroperoxides by oxidizing glutathione. Mice with disruptions in *Gpx1* and *Gpx2* develop ileocolitis between 2 and 7 weeks of age; after 4 months of age, approximately 40% of animals develop tumors (28% adenocarcinomas) [435]. Higher rates of tumor development correlated with higher inflammation scores in the ileum but not the colon, indicating that inflammation is necessary but not sufficient to cause tumors. Tumor incidence was highest in colonies that were raised in non-specific pathogen-free conditions. Essentially no tumors developed in mice raised in germ-free or specific pathogen-free colonies, nor did tumors develop in animals that had at least one wild-type allele of *Gpx1* or *Gpx2*. Because these mice have intact immune systems and mucosal barrier function, these findings indicate the importance of antioxidants in defense against inflammation and neoplasia.

Adoptive transfer of CD4⁺CD45RB^{hi} T cells into *Rag2*^{-/-} mice induces colitis. Colitis is prevented by co-transfer (or adoptive transfer) of CD4⁺CD25⁺ or CD4⁺CD45RB^{lo} (Treg) cells, which inhibit intestinal inflammation. Colitis can also be induced in *Rag2*^{-/-} mice by infection with the enteric mouse pathogen *H hepaticus*; these mice develop colon tumors, including sessile tubular adenoma, dysplasia, and adenocarcinoma (100%) [436]. However, if they are maintained in a *H hepaticus*-free environment, the mice do not develop colon tumors or colitis. Adoptive transfer of CD4⁺CD45RB^{lo} (Treg) cells to *Rag2*^{-/-} mice prevents not only

colitis but also neoplasia, because these cells produce IL-10, which inhibits inflammation and carcinogenesis [437]. Long-term administration of antibiotics to TRUC (T-bet^{-/-}RAG2^{-/-} ulcerative colitis) mice prevented colitis and neoplasia, indicating the role for commensal flora [421]. Likewise, transfer of Treg cells from *H hepaticus*-infected donor mice to APC^{Min/+} mice protected them against cancer and reduced production of the proinflammatory cytokines TNF- α , IL-6, and IL-17 [423].

Infections are among the best-understood activators of inflammation [1, 438]. Microbes might contribute to colon carcinogenesis by disrupting the balance between protective (tolerogenic) and aggressive (proinflammatory, protumorigenic) flora or by changing the number, diversity, and stability of commensal bacteria (dysbiosis). Toxins produced by commensal organisms might also be involved; enterotoxigenic *B fragilis* colonizes Min^{APC716+/-} mice, causes colitis by activating Stat3 and Th17 cells, and increases colon tumor formation [420]. By contrast, nontoxigenic *B fragilis* colonized mice but did not cause inflammation or neoplasia. *Bifidobacterium lactis* prevents acute colitis and reduces the number of colonic neoplasms in a mouse model of colon carcinogenesis [439].

Finally, many researchers have studied activation of pattern recognition receptors (PRRs), particularly Toll-like receptors (TLRs), by microbial pathogens. TLR4 signaling might induce production of RONS. TLR4-deficient mice have smaller and fewer colorectal tumors following administration of carcinogenic agents [440]. TLR4 signals through myeloid differentiation primary response 88 (MyD88); in the absence of MyD88, colorectal tumors do not develop in IL-10 knockout mice following administration of carcinogenic molecules [425]. Because variants of NOD2, which mediates signaling by intracellular PRRs, have been associated with CD, along with variants in TLR4 (also with UC), studies are required to investigate the link between PRR signaling and human colon carcinogenesis.

e) Colitis-associated cancer: a complex interplay

Colitis-associated cancer is multifaceted process involving several factors and multiple steps. NF- κ B seems to play a central role in the pathophysiology of colitis-associated cancer. Important roles have been also attributed to inflammatory cytokines in

promoting and perpetuating inflammation and therefore carcinogenesis. Furthermore, the development of CAC is greatly influenced by the intestinal microflora and the role of inflammation-derived oxidative damage. Although the role of the factors cited above has been well-documented in the pathogenesis of IBD-associated cancer, establishing the exact timing and the “cause/consequence” requires further investigations (Figure 18).

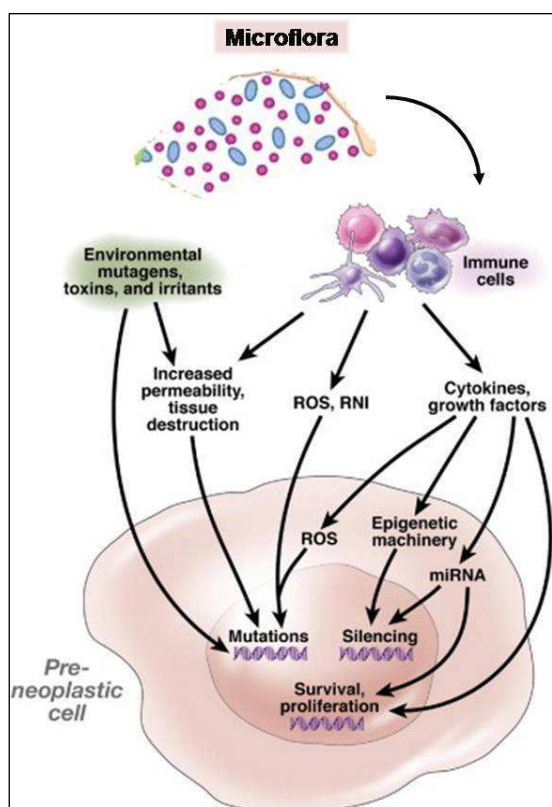


Figure 18. Initiation of colitis associated cancer. Reactive oxygen and species (ROS) and reactive nitrogen intermediates (RNI), produced by inflammatory cells, can cause DNA damage that in addition to other mutagens can initiate CAC. Cytokines from inflammatory cells can increase intracellular ROS and RNI in pre-malignant cells, resulting in epigenetic changes that silence tumor suppressors and promote tumor initiation. Evidences of signal perpetuation promoting carcinogenesis miRNA, microRNA; ROS, reactive oxygen species; RNI, reactive oxygen intermediates. Adapted from [1]

II-A.4. Colorectal Cancer Preventive Measures

The previously accepted increased risk of CRC in IBD has prompted medical society guidelines to endorse cancer prevention strategies. Today, prevention of CRC in IBD is principally based on endoscopic surveillance for the detection of dysplastic lesions. Nevertheless, preventive medical treatment, especially using 5-aminosalicylates (5-ASA), has also proved to be efficient in most studies.

a) Endoscopic Surveillance

According to the consensus guidelines, screening colonoscopies should begin 8–10 years after the onset of IBD symptoms in patients with UC pancolitis. The exception to this rule is that patients with concomitant PSC should begin yearly surveillance colonoscopies at the time PSC is diagnosed. The timing of follow-up surveillance colonoscopies depends on the presence of dysplasia. If the initial colonoscopy in UC and CD pancolitis is negative for dysplasia, repeat surveillance colonoscopy should be performed in 1–2 years. Once a patient has 2 negative surveillance colonoscopies, further surveillance colonoscopies should be performed every 1–3 years. After 20 years of disease duration, surveillance colonoscopies should be repeated every 1–2 years. The recommended surveillance biopsies are 4 quadrant biopsies every 10 cm with jumbo forceps on macroscopically normal mucosa, as well as in areas of mucosal irregularity, areas with altered mucosal colour, raised plaques or polypoid lesions. The probability of detecting dysplasia naturally increases with the number of biopsies performed. On the other hand, the cost of the examination also increases proportionately [441, 442].

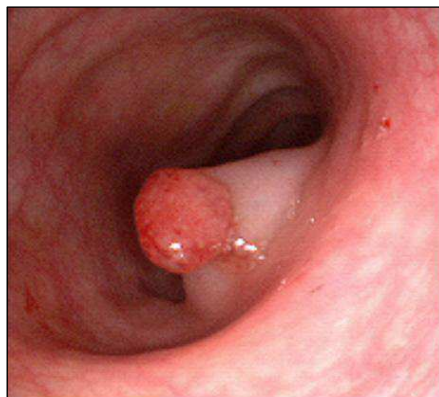


Figure 19. Endoscopic view of an invading colonic tumor.
(<http://www.colorectal-cancer.ca>)

The evidence that colonoscopy reduces CRC mortality in UC comes from case-control studies. *Choi et al.* demonstrated that, in their series, surveillance enabled the detection of early-stage CRC in 80% of patients against 41% in unmonitored UC patients. In this study, the mortality of the two groups, 5 years after CRC diagnosis, was also significantly improved

in the group of monitored patients (77% versus 36%, $P = 0.026$) [443]. Other studies have reported globally similar results [444, 445]. In addition, other data strongly suggest that patients who comply with surveillance colonoscopy have CRC detected at an earlier stage [446].

However, analysis of surveillance results is difficult, particularly in view of the methodological demands of such studies, requiring the inclusion of large numbers of patients and long-term follow-up (15 to 20 years), and of the difficulty in maintaining patient compliance to the study program, possible refusal to undergo colectomy by patients for whom low-grade dysplasia or high-grade dysplasia has been detected. Also, this strategy is often difficult to implement since it is not only inconvenient for the patient, but is also costly and involves a rate of morbidity which is by no means insignificant, all of which reduce its long-term observance. Furthermore, surveillance programs have not substantially prolonged survival in IBD patients, due to the technical limitations of recognizing dysplastic lesions in flat, normal-appearing mucosa [339]. Thus, the ideal solution would be to identify other, more accessible, risk markers for neoplastic degeneration, hence enabling the number of patients involved in endoscopic surveillance to be further reduced. An interesting prospect has been reported, based on the detection of molecular markers in faeces, potentially providing, in the future, a more accurate selection of patients with a high risk of presenting with CRC [447-449].

b) Chemoprevention

Despite the relative protection afforded by surveillance colonoscopies in IBD, there are still patients who develop CRC despite seemingly optimal surveillance. This raises the issue of whether chemoprevention in the form of either medications or dietary supplements might help reduce the risk of CRC in IBD [450]. Epidemiological studies have revealed that IBD patients, who were also regular users of 5-aminosalicylic acid, had decreased risk of colorectal cancer. In the last several years, reports have been emerging which suggest that 5-ASA therapy is associated with a protective effect against the development of CRC in longstanding colitis.

II.B- An Emerging Chemopreventive Role for 5-Aminosalicylic Acid

5-ASA therapy is the treatment of choice in active mild to moderate UC. In addition to its efficacy and safety as an anti-inflammatory therapy, 5-ASA is also believed to have unique chemopreventive properties.

II-B.1. Clinical Evidence of 5-Aminosalicylic Acid Chemoprevention

Multiple studies have assessed the utility of 5-ASA in preventing dysplasia and cancer in patients with UC, whereas fewer studies have assessed this endpoint in patients with CD. Looking across the UC studies, a meta-analysis of 9 observational studies involving a total of 1932 patients reported a protective association between 5-ASA use and CRC (OR 0.51; 95% CI: 0.37– 0.69) which equates to a 49% reduction in the risk of CRC or CRC/dysplasia with regular 5-ASA use [451]. The derivative studies generally reported similar findings, even though they were subject to their own unique limitations and strengths in how they were performed. In a UK community-based cohort study of 175 patients with UC (diagnosed between 1972 and 1981), compliance with sulfasalazine resulted in a significant reduction in the risk of CRC [452]. The crude proportion of patients who developed CRC was 3% (5 of 152) in the compliant group and 31% (5 of 16) in the noncompliant group (those not compliant with or advised to stop taking sulfasalazine), $P < 0.001$. In addition, *Rubin et al.* observed a 3.4-fold risk reduction of dysplasia and CCR in UC patients treated with 5-ASA at dosage $\geq 1.2\text{g/day}$ [453]. Also, *Eaden et al.* in a retrospective, matched case control study showed that mesalazine reduced the risk of CRC by 81% in individuals suffering from UC [335]. Another UK case-control study of 102 patients with UC and CRC plus matched controls showed that CRC could be substantially reduced by taking 5-ASA on a regular basis compared with no 5-ASA use (OR 0.47; 95% CI: 0.22–1.00) [454]. Likewise, a nested case-control study involving 18,969 patients with IBD in the UK General Practice Research Database (1987–2001) showed that regular 5-ASA users (defined as ≥ 6 prescriptions in the previous 12 months) had a significantly reduced risk of CRC compared with irregular 5-ASA users (adjusted OR 0.60; 95% CI: 0.38–0.96) [455]. In the latter 2 studies, regular mesalazine users achieved a reduction in CRC risk that was numerically greater than that achieved by regular sulfasalazine users.

On the other hand, some retrospective studies did not support 5-ASA as protective in preventing CRC [456-459]. According to the authors, the absence of protective effect against colitis-related CRC was mainly due to the heterogeneity of the populations analyzed and the short period of exposure to 5-ASA. Furthermore, because of the design of retrospective studies, some key areas have not been sufficiently addressed such as included concurrent use of other medications and accurate data on dosing.

II-B.2. Insights into the Mechanisms of 5-Aminosalicylic Acid Chemoprevention

In the recent years, a large amount of molecular data has accumulated supporting the notion that 5-ASA biological effects interfere with colorectal cancer development. These molecular pathways are of special interest in the search for 5-ASA's molecular target(s) and development of novel chemopreventive compounds.

a) Inhibition colon cancer cell growth and survival

The initial demonstration that 5-ASA can block the growth and promote apoptosis of CRC cells comes from *ex vivo* studies in patients with colonic adenoma. *Reinacher-Schick et al.* showed that 5-ASA administered orally to patients with sporadic polyps increased the apoptotic rate and decreased proliferation of cancer cells [460]. *Bus et al.* demonstrated that rectal administration of 5-ASA in patients with sporadic CRC enhanced apoptosis of tumor cells [461]. These observations were supported by studies in the mouse model of colitis-associated CRC, induced by administration of azoxymethane (AOM) followed by repeated oral administration of dextran sulfate sodium (DSS), which showed that 5-ASA reduced the number and size of neoplasms [462, 463].

5-ASA was demonstrated to directly regulate the activity of neoplastic cells. Indeed, it has been shown that 5-ASA inhibits the growth and enhances the apoptosis of several CRC cell lines in a time and dose-dependent manner [464]. 5-ASA-induced anti-proliferative effect is associated with modulation of replication checkpoints in CRC cells, which ultimately alter cell cycle progression. In this context, it has been shown that increasing concentrations of 5-ASA (5–30mM) cause a progressive accumulation of CRC cells in S phase, thereby

decreasing the percentage of cells in G2/M and G0/G1 [465]. 5-ASA-treated cells exhibit a reduced expression of the phosphatase Cell division cycle 25 A (CDC25A), but not CDC25B or CDC25C, and inactivation of Cyclin-dependent kinase 2 (CDK2), a target of CDC25A [465]. Since CDK2 binds to either cyclin E or cyclin A and regulates the G1/S transition and S phase progression, respectively, 5-ASA-induced CDK2 inactivation could be responsible for cell cycle block in S-phase [466]. The exact mechanism by which 5-ASA down-regulates CDC25A expression is not yet known. It is unlikely that Serine/threonine-protein kinase (CHK) 1 and 2, two upstream kinases that phosphorylate and promote degradation of CDC25A are involved in the negative regulation of CDC25A, because 5-ASA downregulates CDC25A, expression in CHK1- or CHK2-deficient cells [465]. By contrast, cells treated with 5-ASA displayed enhanced ubiquitination and proteasome- dependent degradation of CDC25A [465]. These findings together with the demonstration that 5-ASA does not affect CDC25A RNA expression strongly suggest that CDC25A is post-transcriptionally down-regulated in 5-ASA-treated cells. These results are consistent with those published by *Luciani and colleagues*, who showed that exposure of CRC cells to 5-ASA causes a CHK-1 dependent reversible accumulation of cells in S-phase [467]. Further work by other researchers has confirmed the ability of 5-ASA to interfere with cell cycle progression and shown that CRC cells can accumulate in various phases of the cell cycle in relation to the dose and time of exposure to the drug [468, 469].

b) Improvement of replication fidelity

IBD-related CRC is characterized by two major types of genomic instability, namely, chromosomal instability (CIN) also termed above aneuploidy, and microsatellite instability (MSI) [470]. CIN or aneuploidy is characterized by atypical segregation of chromosomes and abnormal DNA content with consequent loss of whole (or part of) chromosomes and function of critical tumor suppressor genes (e.g., adenomatous polyposis coli, p53). MSI is characterized by an increased rate of point mutations and is dependent on defects in the mismatch repair system, a mechanism involved in repairing DNA base-pair mismatches, which occur in the normal DNA replication. During this process, frameshift mutations, called microsatellites, tend to accumulate. Microsatellites occur mainly in intronic DNA sequences thus resulting in no gene function alteration. However, when microsatellites are located in

exonic gene regions, there can be a shift in the codon reading frame which results in a loss of protein function [471]. Studies performed by Gasche and Campregher demonstrated that 5-ASA improves replication fidelity in cultured CRC cells independently of the presence of mismatch repair system [472, 473]. The mechanism by which 5-ASA inhibits the generation of frameshift mutations remains unknown, even though it could in part rely on the ability of the drug to slow down DNA replication and cell division, because cell cycle regulation is one of the defense mechanisms that allow cells to either repair DNA damage or eventually undergo apoptosis thus safeguarding the integrity of the genome [467, 474].

c) Inhibition of Wnt/ β -Catenin pathway

An intriguing possibility that emerges from the available experimental data is that the anti-tumoral properties of 5-ASA reflect the ability of the drug to target several pathways that are both early and common in colorectal carcinogenesis. The Wnt/ β -catenin pathway, which is constitutively activated in the majority of CRC, is one of such targets. In this pathway, Wnt binds to the transmembrane Frizzled receptor, leading to the activation of the cytoplasmic disheveled (Dsh) protein. Dsh forms a complex with the β -catenin degradation complex, which consists of the APC gene product, glycogen synthase kinase-3 β (GSK-3 β), axin, and β -catenin. In the absence of Wnt, GSK-3 β phosphorylates β -catenin, thereby promoting its ubiquitination and degradation [475]. In response to Wnt signals, β -catenin is no longer targeted for degradation, and it accumulates in the cytoplasm and subsequently translocates to the nucleus, where it stimulates the expression of genes involved in tumor progression [475]. *Bos and colleagues* showed that 5-ASA inhibits the Wnt/ β -catenin pathway in CRC cells by increasing β -catenin phosphorylation and consequently reducing nuclear accumulation of β -catenin and expression of Wnt/ β -catenin target genes (e.g., cyclin D1, c-met, and c-Myc) [476]. The mechanism by which 5-ASA promotes β -catenin phosphorylation remains to be ascertained, but it is known that the drug inhibits the activity of the protein phosphatase (PP)2A, a known regulator of the β -catenin phosphorylation status in CRC cells [476, 477].

d) Inhibition of epidermal growth factor receptor activation

Another important target of 5-ASA in CRC cells is epidermal growth factor receptor (EGFR), the activation of which is followed by a range of intracellular events that eventually stimulate CRC growth and survival [478]. EGFR is overexpressed not only in sporadic CRC but also in the premalignant lesions of IBD-associated carcinogenesis [479, 480]. It has been demonstrated that 5-ASA suppresses EGFR phosphorylation/activation in CRC cells, as a result of its ability to enhance the activity of protein tyrosine phosphatases (PTPs) that control EGFR activation [481].

e) Inhibition of cyclooxygenases

Cyclooxygenase-2 (COX-2) is a major target of CRC chemopreventive programs, as it is over-expressed in IBD-related CRC tissue and its activation is known to trigger and/or amplify biological pathways that sustain CRC growth [409, 418, 482]. It has been shown that 5-ASA inhibits the growth of HT-115, a CRC cell line that expresses a functionally active COX-2, and that the anti-mitogenic effect of 5-ASA is associated with a marked down-regulation of COX-2 at the protein and mRNA level [483]. Consistently, treatment of HT-115 cells with 5-ASA causes a significant reduction in secretion of prostaglandin (PG) E₂, a product of COX-2 activity that positively regulates CRC cell growth. Moreover, mesalazine blocks the growth of DLD-1, a CRC cell line that does not express COX-2, which suggests that the effect of this drug on CRC cell growth is partially independent of inhibition of the COX-2/PGE₂ axis [483].

f) Scavenging reactive oxygen and nitrogen metabolites

5-aminosalicylic acid is a very efficient scavenger of reactive oxygen species (ROS) and its inhibitory effects against free radicals have been described in the literature [484]. As inflammation and inflammation-driven malignancies are accompanied by accumulation of ROS, treatment with 5-ASA may effectively prevent these events, especially when applied in IBD. Although inflammation is an important biological risk factor for cancer development, only recently has severity of inflammation been linked to carcinogenesis [343, 485]. 5-ASA showed significant $\cdot\text{O}_2^-$ scavenging effects [486]. Oxidized 5-ASA can regenerate by

endogenous compounds, e.g. ascorbic acid, cysteine, or glutathione, which may preserve the drug effects in tissues undergoing oxidative stress [487]. The antioxidant effect of 5-ASA was also shown on copper-mediated low-density lipoprotein oxidation [488]. Combined with another antioxidant N-acetylcysteine (NAC), 5-ASA reduces nitrate generation and suppresses nitric oxide synthase (NOS) activity [489]. The effects of 5-ASA on the expression and activity of haem oxygenase-1 (HO-1) have been evaluated in the colitis provoked by instillation of trinitrobenzene sulphonic acid (TNBS) [490]. HO-1 can modulate colonic inflammation by endogenous anti-oxidant and anti-inflammatory moieties. Intraluminal administration of 5-ASA induced colonic HO-1 protein expression and stimulated HO-1 enzyme activity suggesting that 5-ASA may exert its colonic anti-oxidant and anti-inflammatory effects *in vivo* in part through the upregulation of HO-1 enzyme expression and activity. Also, 5-ASA was shown to protect from radiation-induced ROS production and tissue damage [491]. The authors tested the radiation-protective effect of 5-ASA in mouse bone marrow. 5-ASA pre-treatment decreases death caused by radiation-induced gastrointestinal and haematopoietic syndromes. 5-ASA prevents radiation-induced depletion of antioxidant enzymes and lipid peroxidation, probably by quenching the hydroxyl radicals. Finally, beside its *in vitro* inhibition of NOS and NO production, treatment with 5-ASA induced a decrease in NOS expression and NO levels in a model of indomethacin-induced enteropathy [492, 493].

g) Inhibition of nuclear factor- κ B

As detailed previously, the NF- κ B signaling pathway has been shown to play a central role in promoting and perpetuating carcinogenesis in IBD-associated CRC. In human colon epithelial cells, 5-ASA inhibited the phosphorylation of Ser536 in the NF- κ B subunit p65, the critical residue for transcriptional activity of NF- κ B, and inhibited IL-1 β -dependent activation of NF- κ B [327, 494]. Furthermore, TNF- α -induced nuclear translocation of NF- κ B was prevented by 5-ASA through inhibition of I κ B α degradation [495].

h) Anti-microbial activity

5-aminosalicylic acid has also been implicated in antimicrobial activity [496]. There is increasing evidence that *E. coli* organisms are important in Crohn's disease pathogenesis [209, 234, 497]. Some adherent and adhesive mucosal *E. coli* isolates are able to invade colonic polyps and cancers resulting in activation of toll-like receptors, TNF, IL-8 and NF- κ B signaling [498]. 5-ASA inhibits MAPK-dependent IL-8 release triggered by mucosal *E. coli* isolates in a dose-dependent manner [499]. Moreover, bacterial invasiveness was decreased by 5-ASA. Although the exact mechanism of 5-ASA antibacterial activity remains unknown, recent finding suggested that 5-ASA has potent effects on bacterial gene expression which implies that intestinal bacteria could act as a pharmacological target of 5-ASA, a mechanism that might contribute to the therapeutic action of 5-ASA [500].

i) Activation of peroxisome proliferator activated receptor-gamma

The peroxisome-proliferator-activated receptor gamma (PPAR γ), a transcription factor belonging to the nuclear hormone receptor superfamily, is highly expressed in the colon where it regulates cellular proliferation, differentiation, and apoptosis [501]. PPAR γ activation inhibits formation of aberrant crypts foci and development of CRC in mice [502, 503]. *Rousseaux et al.* showed that PPAR γ is a target of 5-ASA in CRC cells [325]. Specifically, in competitive binding studies, 5-ASA displaces rosiglitazone and the selective PPAR γ ligand GW1929 from their binding sites on PPAR γ [325]. Activation of PPAR γ by 5-ASA is accompanied by induction of the tumor suppressor gene Phosphatase and tensin homolog (PTEN), activation of caspase-8 and caspase-3, and diminished expression of survivin and X-linked inhibitor of apoptosis protein [504].

III- The Role of PPAR- γ in Colorectal Cancer

III-A. Generalities

The peroxisome proliferator-activated receptor-gamma (PPAR γ) represents subtype (along with PPAR α and PPAR β/δ) from the PPARs family, nuclear receptors involved in the function and regulation of several physiological systems (Detailed description of PPARs

regulation and function is reported in section I of skin homeostasis) [505]. There are natural ligands for PPAR γ , including long chain polyunsaturated fatty acids, eicosanoids, components of oxidized low density lipoproteins, and oxidized alkyl phospholipids [506]. The prostaglandin J2 derivative, 15d-PGJ2 is the most potent endogenous ligand for the PPAR γ receptor. The antidiabetic thiazolidinedione (TZD) class of drugs including troglitazone, rosiglitazone, pioglitazone and ciglitazone are synthetic ligands for PPAR γ [506].

PPAR γ is expressed in adipose tissue, colon, immune system, hematopoietic cells, and retina. PPAR γ is also expressed in a variety of malignant tissues including prostate, breast, and colon [507-511]. This broad expression reflects multiple roles for PPAR γ . For instance, this receptor was shown to be involved in lipid anabolism, adipocyte differentiation, and control of inflammation, metabolic regulation, macrophage maturation, and embryo implantation [512]. Upon activation, PPAR γ forms heterodimer with retinoid X receptor (RXR) and mediate transcriptional activation by binding to the peroxisome proliferator-activated receptor response element (PPRE) [505, 513]. In the inactive state, association of various co-repressor molecules with PPAR γ (e.g., nuclear receptor co-repressor or silencing mediator for retinoid receptor) prevents this complex from binding to DNA. For transcriptional transactivation of PPAR γ , recruitment of co-activators (e.g., cyclic adenosine monophosphate response element-binding protein, PPAR γ coactivator-1, and PPAR γ binding protein) is required which replace co-repressors from the heterodimer complex. Transcriptional transrepression occurs through a genome independent mechanism and is mediated via physical association of the heterodimer with other activated transcription factors (STAT, NF- κ B, and AP-1) thereby blocking their functions [15].

III-B. Anti-neoplastic role of PPAR- γ in the Colon

Several studies have focused on the putative association between the various polymorphisms and mutations of the PPAR γ gene and the occurrence of cancer. It was described that 4 somatic PPAR γ gene mutations resulting in reducing its function occurred in 55 sporadic colon cancers [514]. However, *Ikezoe et al.* analyzed 397 clinical samples and cell lines including colon, breast, and lung cancers for mutations of PPAR γ gene and showed

the absence of PPAR γ gene mutations [515]. These data suggest that PPAR γ mutations may occur in cancers but they are rare. Furthermore, survival of patients with colorectal cancer is markedly better when PPAR γ expression is detectable in primary tumors than in patients with colorectal cancer who have no detectable PPAR γ expression in their primary tumors [516]. This is consistent with results showing that colon tumorigenesis is exacerbated in APC $^{\text{min}}$ / $+$ mice with genetic ablation of PPAR γ compared with control APC $^{\text{min}}$ / $+$ mice [517].

III-B.1. The Role of PPAR γ as a Tumor Suppressor in Colorectal Cancer

There has been substantial accumulation of experimental data supporting that PPAR γ ligands induce growth arrest and apoptosis in colon cancer cells [511, 512]. Most importantly, in the gastrointestinal tract, PPAR γ , expressed in intestinal epithelial cells, was shown to exhibit anti-tumorigenic properties via different mechanisms [505]. PPAR γ was shown to induce apoptosis, inhibit cell proliferation, suppress angiogenesis and promote cell differentiation (Figure 20).

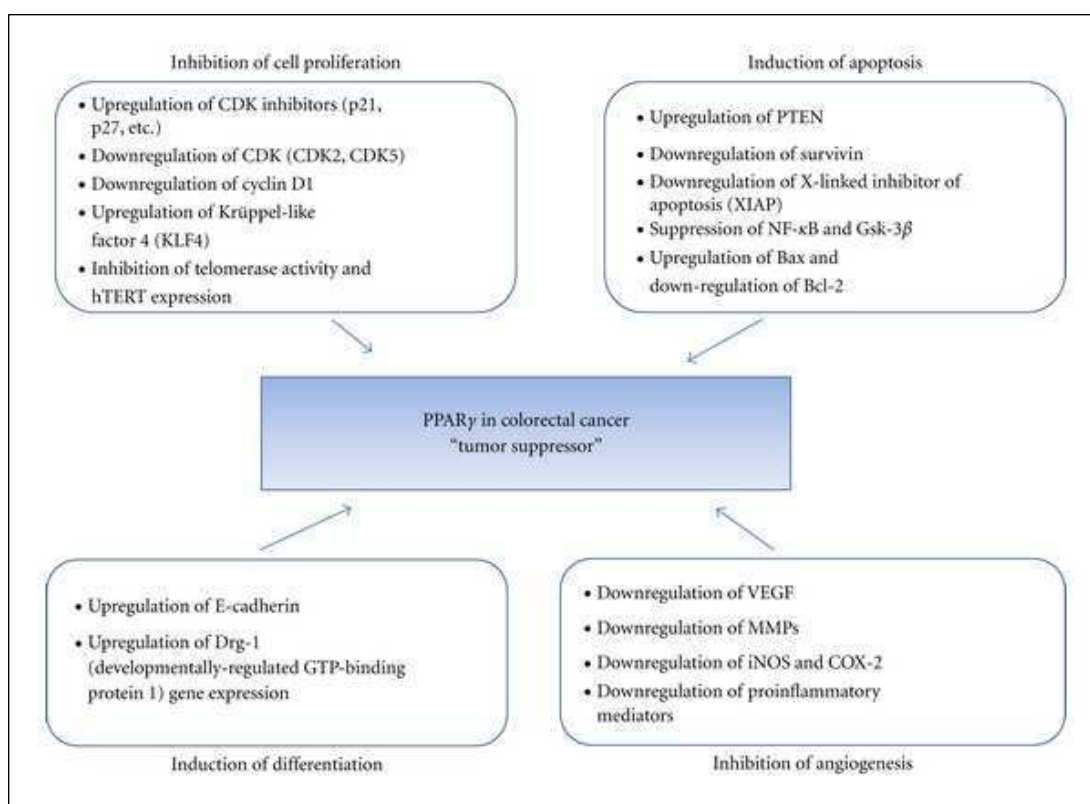


Figure 20. Molecular mechanisms for PPAR γ as tumor suppressor in colorectal cancer [15].

a) Inhibition of cell proliferation and induction of apoptosis

The mechanisms by which PPAR γ was shown to inhibit cell proliferation and induce apoptosis were numerous. The majority of the studies were performed in colon cancer cell lines using specific PPAR γ agonists.

(1) Some studies have demonstrated that rosiglitazone upregulates the Phosphatase and Tensin Homolog (PTEN) expression in Caco2 colorectal cancer cells [518]. PTEN is a tumor suppressor gene that modulates several cellular functions, including cell migration, survival, and proliferation by inhibiting phosphatidylinositol 3-kinase (PI-3K)-mediated signaling cascades [519]. *Dai et al.* also show that treatment of colon cancer cells with rosiglitazone stimulates expression of tumor suppressor gene PTEN. This effect is probably mediated through the binding of PPAR γ on PPRE in the promoter of PTEN [520]. Inhibition of the PI-3K/Akt pathway by increased PTEN expression is believed to underlie this effect of the PPAR γ ligand.

(2) PPAR γ agonist GW7845 induced cell death through downregulation of survivin in colorectal cancer cells [521]. Survivin is one of the inhibitors of apoptosis protein (IAP) family since it is overexpressed in almost every human tumor that has been studied, but is barely detectable in most normal adult tissues [522]. Overexpression of survivin is associated with poor clinical outcome with reduced tumor cell apoptosis in patients with colorectal cancer [523, 524].

(3) *Qiao et al.* showed that 15d-PGJ2 and troglitazone mediate downregulation of X-Linked Inhibitor of Apoptosis (XIAP) downregulation in colon cancer cells by facilitating ubiquitination and proteasomal degradation [525]. XIAP can inhibit apoptosis by binding and thereby inactivating caspases including caspase-9 and the effector caspases (-3 and -7) [526]. In addition, *Lee et al.* demonstrated that pioglitazone induces apoptosis through downregulation of XIAP via unknown mechanism in colorectal cancer cell lines [527].

(4) *Ban et al.* showed that PPAR γ agonist, troglitazone, inhibits colon cancer cell growth via inactivation of NF- κ B by suppressing glycogen synthase kinase-3 (GSK-3 β) activity

[528]. In fact, the transcription factor NF- κ B is involved in the regulation of various genes, including inflammatory response genes and glycogen synthase kinase-3 (GSK-3) [529]. Its activation is also associated with cell proliferation, cell cycle progression, promotion of tumor growth, angiogenesis, and metastasis through the expression of genes participating in malignant conversion and tumor promotion [397, 530, 531].

(5) Ciglitazone was shown to inhibit CDK5 (Cyclin-Dependent Kinase 5) protein expression and kinase activity which was associated with decreased proliferation in colon cancer HT-29 cells [532]. PPAR γ activation in intestinal epithelial cells results also in the inhibition of cell cycle and S-phase entry through a decrease in cyclin D1 expression which is involved in G1/S progression and increased proliferation [533, 534]. Furthermore, PPAR γ ligand treatment not only decreases the protein level of cyclin D1, but also increases the CDK inhibitors p21CIP and p27KIP1 through both increased transcriptional activity and inhibition of proteasome degradation in colorectal cancer cells [535, 536]. Ciglitazone also inhibited G1/S cell cycle progression through upregulation of p27 and inhibition of Cdk2 activity in HT-29 cells [535]. *Lee et al.* also showed that pioglitazone treatment leads to G2/M block through downregulation of cyclin B1 and cdc2 and upregulation of p21 in RB-deficient human colorectal cancer SNU-C4 and SNU-C2A cells [527]. Thus, these studies suggest that the antiproliferative or proapoptotic effects of PPAR γ agonist are associated with its ability to regulate the expression of various genes which are involved in controlling the cell cycle and cell survival/death.

(6) It has been shown that pioglitazone induces apoptosis through the downregulation of COX-2, activation of caspase-3, downregulation of Bcl-2 and upregulation of Bax in human colorectal cancer cells [527]. In fact, COX-2 contributes to tumorigenesis through various mechanisms and overexpression of COX-2 can stimulate tumor growth, invasion, and metastasis [537, 538].

(7) Zhi and Tseng demonstrated that 15d-PGJ2 inhibits proliferation of HT-29 human colon cancer cells and induces upregulation of Krüppel-Like Factor (KLF4) mRNA and protein through the activation of MEK/ERK and STAT-dependent pathway [539]. KLF4 is a member of the Krüppel-like zinc finger transcription factor family. It is extensively expressed in the

epithelial cells of the gastrointestinal tract [540, 541]. Over-expression of KLF4 in colon cancer cells caused inhibition of DNA synthesis and cell growth [542, 543]. It has been also demonstrated that rosiglitazone treatment of colorectal cancer cells caused to G1 arrest because increased expression of KLF4 by rosiglitazone leads to increased expression of p21 and decreased expression of cyclin D1 [544]. These data suggest that KLF4 is a nodal player in a network of PPAR γ -regulated genes.

(8) In another mechanism in colon cancer cells, treatment of the PPAR γ ligands (pioglitazone, troglitazone) induces apoptosis through upregulation of the proapoptotic protein Bax and downregulation of the antiapoptotic protein Bcl-2 [545, 546]. Alternative expression of Bax and Bcl-2 causes apoptosis by the release of cytochrome c and subsequent activation of several effector caspases.

(9) Finally, It has been reported that 15d-PGJ2 and rosiglitazone inhibit Caco-2 colon cancer cell proliferation through the inhibition of telomerase activity and human telomerase reverse transcriptase (hTERT) expression [547].

b) Induction of cellular differentiation

PPAR γ has been demonstrated to induce differentiation in solid tumors both *in vitro* and *in vivo* [548]. In colon cancer cells, activation of PPAR γ by troglitazone treatment inhibits growth and metastasis through differentiation-promoting effects, such as the marked increase in p21Waf-1, developmentally regulated GTP-binding protein 1 (DRG-1), and E-cadherin in human colon cancer cells [549].

c) Inhibition of angiogenesis

Angiogenesis, a formation of new capillaries from the preexisting vessels, is a complex process involved in the degradation of the basement membrane by cellular proteases, the penetration and migration of endothelial cells into the extracellular matrix (ECM), endothelial cell proliferation, tube formation, and vessel stabilization [550]. Inhibition of angiogenesis may contribute to the mechanism by which PPAR γ agonists halt

the cancer process. Several studies demonstrated that PPAR γ agonist inhibits angiogenesis through the following mechanisms.

(1) 15d-PGJ2 has been reported to have inhibitory effects on the proliferation and invasiveness of colon cancer cell lines which are associated with G1 cell cycle arrest and downregulation of matrix metalloproteinases (MMP)-7 synthesis [551]. MMPs are a family of proteases cleaving several macromolecules of the ECM and the process of cancer cell invasion is dependent on the degradation of the ECM by MMPs [552].

(2) It has been shown that both COX-2 and inducible nitric oxide synthase (iNOS) are overexpressed in various human cancers [553]. It was reported that iNOS is associated with altered expression of important modulators of angiogenesis [553]. 15d-PGJ2 downregulates iNOS [554-556] and COX-2 [557-559]. The expression of COX-2 and iNOS is regulated by NF- κ B. The recent several studies have demonstrated that 15d-PGJ2 can act as a negative regulator of proinflammatory signaling through blocking the NF- κ B activation pathway at multiple levels via covalent modification of NF- κ B or its regulators [560]. Thus, antiangiogenic effects of 15d-PGJ2 might be associated with disruption of NF- κ B and subsequent blockade of iNOS and COX-2 expression.

(3) The potential mechanism of angiogenesis inhibition by 15d-PGJ2 may also involve downregulation of pro-inflammatory mediators. Both physiological and pathological angiogenesis can be stimulated by pro-inflammatory cytokines, such as IL-1 and TNF- α . Certain cytokines (e.g., IL-6 and CSF-1) can influence the phenotype and the function of tumor-associated macrophages and indirectly stimulate tumor invasiveness and angiogenesis [561]. Tumor-associated macrophages play an important role in tumor progression due to production of several angiogenic factors, such as VEGF, IL-8, inflammatory cytokines (IL-1) and proteases (MMP-2 and MMP-9) [561]. Thus, 15d-PGJ2 inhibits angiogenesis through suppression of such pro-inflammatory cytokines [562]. Induction of several pro-inflammatory cytokines, such as TNF- α , IL-1, and IL-8, is regulated at the transcription level by NF- κ B. It is still unclear whether 15d-PGJ2 exerts an anti-angiogenic effect through inhibition of NF- κ B-dependent induction of pro-inflammatory mediators or through downregulation of cancer cell-derived proinflammatory cytokine

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release which is NF- κ B-independent. Hence, further investigations are necessary to clarify the signaling pathways that delineate the anti-angiogenic effects of 15d-PGJ2.

In conclusion, it has been clear enough that 5-ASA and PPAR γ not only have common therapeutic function in preventing colon cancer but also converge into the single mode of action through the activation of similar anti-carcinogenic pathways.

(C) AIM OF THE WORK

Patients with inflammatory bowel disease (IBD) display an increased risk of developing colorectal cancer (CRC), probably due to chronic inflammation. Surveillance colonoscopies provide limited protection against CRC because of the difficulty in recognizing dysplastic lesions in healthy-appearing mucosa and in maintaining patient compliance to routine colonoscopies. Therefore chemopreventive strategies have emerged as an additional approach that might provide enhanced protection against CRC in IBD patients. For instance, it has been reported that IBD patients who were as well regular users of 5-aminosalicylic acid (5-ASA) displayed decreased risk of developing CRC in longstanding colitis, as compared to non 5-ASA users. This is because 5-ASA was reported to display unique chemopreventive properties in addition to its efficacy as an anti-inflammatory of choice in IBD.

Nevertheless, the mechanisms underlying the anti-neoplastic properties of 5-ASA remain largely unknown. Some studies have dissected the signaling pathways sustaining the anti-tumorigenic role of 5-ASA. It has been shown that 5-ASA inhibited colon cancer cell growth and survival, improved DNA fidelity replication and suppressed inflammatory signaling pathways. Most importantly, 5-ASA was demonstrated to induce the expression and the activation of peroxisome proliferator-activated receptor gamma (PPAR γ). PPAR γ has been already demonstrated to mediate the anti-inflammatory effects of 5-ASA in the colon of different mouse models and in human colonic biopsies. The aim of this work is to investigate whether PPAR γ would be also capable of mediating the well-recognized anti-neoplastic effects of 5-ASA in the colon. To reach this aim, the anti-neoplastic properties of 5-ASA were examined *in vitro* on human colon cancer cell lines, as well as *in vivo* in two models of colon carcinogenesis: xenograft tumor model and azoxymethane-induced carcinogenesis. The involvement of PPAR γ in mediating 5-ASA effects was assessed by the use of a specific inhibitor for the receptor.

(D) PERSONAL WORK

Carcinogenesis– Favorable reviewing

5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

The 5-aminosalicylic acid (5-ASA) anti-neoplastic effect in the intestine is mediated by PPAR γ

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RUNNING HEAD: 5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

ABSTRACT

BACKGROUND: Epidemiological evidences suggested that 5-ASA therapy may prevent the development of colorectal cancer in inflammatory bowel disease (IBD) patients. Our aim is to investigate whether PPAR γ mediates the anti-neoplastic effects of 5-ASA. **METHODS:** HT-29 and Caco-2 cells were treated by 5-ASA, rosiglitazone (PPAR γ ligand), or etoposide (anti-carcinogenic drug, topoisomerase II inhibitor). Epithelial cell growth, proliferation and apoptosis were assessed by cell count, Ki-67 staining and TUNEL assay respectively. The anti-neoplastic effect of 5-ASA was evaluated in a xenograft tumor model in SCID mice and in azoxymethane (AOM)-induced colon carcinogenesis in A/J01aHsd mice. The role of PPAR γ was examined by administration of PPAR γ antagonist, GW9662. **RESULTS:** Compared to untreated cells, treatment of HT-29 cells by 5-ASA inhibited significantly cell growth and cell proliferation (respectively 60% and 63%) and induced apoptosis in 75% of cells. These effects were abolished by a co-treatment with GW9662. In contrast with etoposide, similar inhibitory effects of GW9662 were obtained in HT-29 cells treated with rosiglitazone. In the xenograft model, GW9662 abolished the therapeutic effect of 5-ASA which decreased tumor weight and volume by 80% in SCID mice compared to untreated mice. In A/J01aHsd mice, 5-ASA suppressed colon carcinogenesis by decreasing the number of aberrant crypt foci (75%) and aberrant crypts (22%) induced by AOM treatment with an absence of 5-ASA response after administration of GW9662. **CONCLUSION:** 5-ASA exerts potent anti-neoplastic effects which are mediated through PPAR γ . These data provide new rational for further designing more effective and safe anti-neoplastic PPAR γ ligands with topical effects.

KEY WORDS: 5-aminosalicylic acid, PPAR γ , inflammatory bowel disease, colorectal cancer, aberrant crypt foci

INTRODUCTION

One of the most serious complications of colonic inflammatory bowel disease (IBD) is colorectal cancer (CRC). Two prevention strategies of CRC are available: regular surveillance colonoscopy with random biopsies, and chemoprevention with 5-aminosalicylic acid (5-ASA) treatment. Surveillance colonoscopies are limited strategies during which only 20% - 50% of colonic neoplasms is detected (1). Epidemiological studies have shown that the chronic use of 5-ASA in IBD has chemopreventive effects on the development of CRC. A meta-analysis has estimated that 5-ASA halved the risk of developing CRC or dysplasia in ulcerative colitis (UC) patients, and showed a positive correlation between the protection and the treatment duration (2). Also, Eaden *et al.* in a case control study showed that mesalazine reduced the risk of CRC by 81% in UC patients (3). Furthermore, functional studies in rodents have shown potent anti-carcinogenic effects for 5-ASA in models of sporadic and colitis-associated cancer (4-6).

The mechanisms sustaining the putative anti-neoplastic property of 5-ASA are still under investigation. However, several *in vitro* studies have demonstrated that the anti-neoplastic effects of 5-ASA are mediated via inflammatory-dependent and -independent mechanisms including the inhibition of NF- κ B (7, 8) the Wnt/ β -catenin pathway (9), regulation of DNA replication checkpoints (10, 11), and disruption of TGF β pathway (12, 13). Another mechanism sustaining the effect of 5-ASA is the induction and activation of Peroxisome proliferator-activated receptor- γ (PPAR γ) (14). PPARs are nuclear receptors that function as transcription factors regulating the expression of genes involved in cellular differentiation, development, metabolism, and tumorigenesis. In the gut, PPAR γ is significantly expressed in colonic epithelial cells and exhibits anti-inflammatory and anti-carcinogenic effects, notably by interacting with the β -catenin pathway (15-17).

Our laboratory has already demonstrated that, PPAR γ mediates 5-ASA anti-inflammatory effects in the colon epithelium in mice and in human cultures colonic biopsies (15, 18, 19). The aim of the present study was to test the hypothesis that the anti-neoplastic effects of 5-ASA were mediated via PPAR γ . We studied the anti-neoplastic effect of 5-ASA first *in vitro* on the proliferation, growth and apoptosis of HT-29 and Caco-2 colon epithelial cell lines and *in vivo* in a mouse model of colon cancer cell xenograft and in Azoxymethane

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(AOM)-induced colon carcinogenesis. The involvement of PPAR γ in 5-ASA-induced anti-neoplastic effect was assessed by the use of PPAR γ antagonist GW9662.

MATERIALS AND METHODS

Chemicals

5-ASA, AOM and GW9662 were purchased at Sigma-Aldrich (St Quentin Fallavier, France). Rosiglitazone was ordered at Spi Bio (Massy, France). Etoposide was purchased at (TCI EUROPE N.V., Belgium). For in vivo studies in A/J01aHsd mice, ethyl cellulose granules (Pentasa, Ferring Switzerland) leading to a 5-ASA ileo-colonic release were used.

Cell lines

HT-29 (ATCC HTB-38) and Caco-2 (ATCC HTB-39) colon carcinoma cell lines were grown in DMEM supplemented with 10 and 20% fetal calf serum (FCS), respectively, antibiotics and 1x essential amino acids for Caco-2 cell line

Assessment of cell growth

HT-29 and Caco-2 cells were treated either with 5-ASA (30 mM) or rosiglitazone (10^{-5} M) with or without GW9662 (10^{-6} M) for 12, 24, or 36h. Etoposide 50 mM was used as a positive control. These doses of 5-ASA and rosiglitazone were chosen because in previous publications, they have shown to induce an anti-inflammatory effect on cultured cells (15) and are clinically relevant (20, 21). Cells were detached with the trypsin/EDTA solution before counting. Results were expressed as the mean number of cells counted blindly in 4 different experiments when a coefficient of variation <10% was obtained. The viability of cells was determined by the trypan blue exclusion test.

Analysis of cell proliferation by Ki-67 immunostaining

After 24h of culture, HT-29 and Caco-2 cells were treated for 48h with 5-ASA (30 mM) with or without GW9662 (10^{-6} M). Rosiglitazone (10^{-5} M) treatment was used as control. Cells were fixed in PFA 4%, permeabilized in PBS containing 0.1 % triton X-100 at 4°C and then incubated with 1.5% normal goat normal and blocking buffer (1% BSA in PBS). Cell proliferation was assessed by a nuclear Ki-67 staining using a mouse monoclonal primary antibody directed against Ki-67 (dilution 1:50 overnight; ZYMED, Clinisciences, Montrouge, France). An Alexa 594 donkey anti-mouse IgG was used as secondary antibody (dilution 1:100, Molecular Probes, Invitrogen, Cergy Pontoise, France). Nuclei were stained with Hoescht 33342 solution (0.125 mg/ml) (Sigma-Aldrich) and visualized under a fluorescence

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microscope (Leica, Bensheim, Germany). An irrelevant mouse serum was used as a negative control. At least 500 cells/sample were counted in four different experiments. Results were expressed as the mean \pm SEM percentage of stained cells.

Detection of apoptosis

After 24h of culture, HT-29 and Caco-2 cells were treated for 24 hours with 5-ASA (30 mM) with or without GW9662 (10^{-6} M). Rosiglitazone (10^{-5} M) was used as control ligand of PPAR γ and etoposide (50 mM) as an apoptosis inductor. Cells undergoing apoptosis were identified using a terminal transferase dUTP nick end labelling assay (TUNEL assay, Roche Diagnostics, Meylan, France). At least 500 cells/sample were counted in four different experiments. Results were expressed as the mean \pm SEM percentage of stained cells.

Xenograft tumor model

Six to seven weeks old pathogen-free BALB/c *SCID* mice (Institut Pasteur, Lille, France) were used. Animals were housed five per cage in barrier environment and had free access to sterile water and food. Human colon cancer cells (10^7 HT-29 cells) pre-treated or not with GW9662 for 24h were implanted subcutaneously in the flank of animals. Two days after cell inoculation, mice were treated with 5-ASA (5 or 50 mM) administered daily by peri-tumoral injection for 10 or 21 days. The effect of PPAR γ during 5-ASA treatment was evaluated by daily intra-peritoneal injection of GW9662 (1 mg/kg/day). The control group has received saline instead of 5-ASA. Mice were checked 3 times a week for tumor development. After sacrifice at 10 or 21 days, tumor size and volume were calculated as previously described with the formula $(L \times W^2) \times 0.5$, where L is length and W is width (22). Tumors were weighted before paraffin embedding for histological examination.

Aberrant crypt model

A/JOl^aHsd mice have been used in this experiment given the high susceptibility of this genetic background to the carcinogen AOM (23). A/JOl^aHsd mice aged of 5 weeks, were randomized into 4 groups each of 10 mice and treated for 7 weeks as follows: Control group fed with standard diet, 5-ASA group fed with 5-ASA ethyl-cellulose granules-containing chaw at 200mg/kg/day, 5-ASA+GW9662 group fed with 5-ASA ethyl-cellulose granules-containing chaw at 200mg/kg/day and receiving daily intra-peritoneal injection of GW9662

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at 2mg/kg/day, GW9662 group fed with standard diet and receiving daily intra-peritoneal injection of GW9662 at 2mg/kg/day. Control and 5-ASA mice groups have received daily intra-peritoneal injection of 3% DMSO/PBS (GW9662 vehicle). One week after 5-ASA and/or GW9662 treatment, all received two intra-peritoneal injections of AOM, at 10 mg/kg/day dissolved in isotonic saline, with one week interval between doses. At the end of the seventh week, mice were euthanized and the colon was removed via a midline laparotomy. The colons were opened along the mesenteric border, washed with phosphate buffered saline and pinned flat. The colon was fixed in 4% neutral buffered formalin for 24 hrs, and then washed with phosphate buffered saline. To identify aberrant crypt foci (ACF), colons were stained with 0.2% methylene blue in water for 3minutes. The entire mucosal surface was examined using stereomicroscope. ACF were distinguished from normal colonic crypts if they were at least two times greater than normal size, had an irregular crypt cavity, and had a densely staining pericryptal zone. The number of aberrant crypt foci per colon and the number of aberrant crypts per focus were double blindly recorded (N El-J and LD).

RESULTS

5-ASA regulates epithelial cell growth in a PPAR γ -dependent manner

HT-29 and Caco-2 cell lines were used to investigate the cellular mechanisms underlying 5-ASA-induced cell growth arrest (Figure 1). Compared to untreated cells, incubation of HT-29 cells for 24, 36 and 48h with 5-ASA (30 mM) resulted in a significant inhibition of cell growth to 45, 54 and 85% respectively ($p < 0.01$). Similar results were obtained in cells treated with the two positive controls, *i.e.*, rosiglitazone (10^{-5} M) and etoposide (50 mM) ($p < 0.01$). As expected, GW9662 was not capable of significantly modulating etoposide-induced cell growth inhibition, while it abolished this effect on cells treated with 5-ASA or rosiglitazone. Similar results were observed in Caco-2 cells (Data not shown).

5-ASA regulates epithelial cell proliferation through a PPAR γ -dependent mechanism

To assess the involvement of PPAR γ in the potential anti-proliferative effect of 5-ASA, we performed immunohistochemistry assays using an antibody directed to the nuclear protein Ki-67. We first verified that HT-29, colonic adenocarcinoma cell lines, were characterized by a high rate of proliferation under basal conditions (94% \pm 1 of stained cells). In HT-29 cells, 5-ASA treatment induced a dramatic decrease in the proliferative index (35 \pm 4% vs 94 \pm 1%, $p < 0.001$). Similar results were obtained in rosiglitazone-treated cells, with less than 10% of cells stained. Furthermore, the proliferation of HT-29 cells was decreased by 95% ($p < 0.0001$) when treated with Etoposide (Figure 2 A-B). Treatment with GW9662 significantly abolished the anti-proliferative effect of 5-ASA and rosiglitazone (38% vs 75%, $p < 0.05$ of stained cells, and 10% vs 84%, $p < 0.05$, respectively). The proliferative potential of HT-29 was restored by 40% in cells co-treated with Etoposide and GW9662 ($p < 0.05$). Similar results were observed in Caco-2 cells (Data not shown).

5-ASA regulates epithelial cell apoptosis through a PPAR γ -dependent mechanism

Apoptosis is another major mechanism involved in the regulation of cell growth. In HT-29, no apoptosis was detected by the Tunel method, which labeled DNA strand breaks. However, Etoposide and rosiglitazone induced a high rate of apoptosis (95% and 62% respectively). As expected, similar results were obtained with 5-ASA treatment (30mM), with 83 \pm 1% stained cells (Figure 3 A-B). Co-treatment with 5-ASA and GW9662 suppressed the

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induction of apoptosis ($83\pm 1\%$ vs $35\pm 0,1\%$ $p < 0.001$) indicating that 5-ASA pro-apoptotic effect is at least in part, dependent of the PPAR γ signaling pathway. Similar inhibition of HT-29 apoptosis was obtained in cells co-treated by rosiglitazone and GW9662 but not in etoposide treated cells (Figure 3). Likewise, the pro-apoptotic effect of 5-ASA in Caco-2 cells (97%) was completely abolished by the addition of GW9662 (3%) (Data not shown).

Taken together, these observations suggest that the regulation of epithelial cell growth, proliferation and apoptosis by 5-ASA are mediated, at least partially, via PPAR γ signaling pathway.

5-ASA has an anti-neoplastic effect in a xenograft tumor model

To evaluate the *in vivo* anti-neoplastic effect of 5-ASA, SCID mice engrafted with HT-29 colon cancer cells were treated daily for 21 consecutive days with 5-ASA at 50 mM (Figure 4). At the end of the treatment, a reduction of 80 to 86% of tumor weight and volume was observed in SCID mice receiving 5-ASA compared to control mice or mice treated with GW9662 alone (Figure 4A-C). The anti-neoplastic effect of 5-ASA was already detectable after 10 days of 5-ASA treatment. Similar results were obtained with mice treated with 5-ASA at 5 mM (data not shown). Anti-tumorigenic effect of 5-ASA was completely abolished at 21 days by simultaneous intraperitoneal administration of GW9662. Thus, the observed anti-neoplastic effect of 5-ASA is at least partially dependent on PPAR γ .

Anti-neoplastic effect of 5-ASA *in vivo*

In order to examine the direct anti-neoplastic effect of 5-ASA on mice, a model of azoxymethane (AOM)-carcinogenesis was employed (Figure 5 A,B). Mice were treated with 5-ASA and/or GW9662 for 7 weeks as described above. To induce carcinogenesis, mice have received two intra-peritoneal injections of AOM at one week interval. After 7 weeks, mice were harvested and the formation of aberrant crypts (ACs) and aberrant crypt foci (ACF) was recorded. A significant decrease in the number of aberrant crypts/focus (22%, $p = 0.038$) and aberrant crypt foci/colon (75%, $p = 0.017$) was observed under 5-ASA compared to control mice treated with AOM only (Figure 5 C-D). The co-treatment of mice with GW9662 suppressed almost completely the anti-neoplastic effect of 5-ASA. For instance,

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mice treated with 5-ASA+GW9662 had increased number of ACF (5.6 ± 0.5) compared to mice treated with 5-ASA only (1.75 ± 0.25), revealing a 68% increase in the number of ACF ($p = 0.04$). Interestingly, GW9662-treated mice had even higher ACF count compared to mice treated with 5-ASA+GW9662 (8.5 ± 0.64 vs 5.6 ± 0.5 , $p = 0.003$) and even to control mice (8.5 ± 0.64 vs 7 ± 1.22) (not significant).

Taken together, these data support the anti-neoplastic properties of 5-ASA *in vivo* and reveal that PPAR γ signaling is required for mediating 5-ASA anti-neoplastic effects.

DISCUSSION

Patients suffering from Crohn's disease and ulcerative colitis displayed an increased risk of developing colorectal cancer. Surveillance colonoscopies provided limited protection against CRC due to the technical limitations of detecting dysplastic lesions in normal-appearing mucosa (1, 24). However retrospective studies have shown that chemopreventive strategies, employing aminosalicylates, provided enhanced protection for IBD patients against CRC development (25, 26). 5-ASA was opted since decades as the treatment of choice of active mild to moderate of IBD, notably ulcerative colitis. In addition to its efficacy and safety as an anti-inflammatory therapy, 5-ASA was also believed to have unique chemopreventive properties. Several studies have shown that regular usage of 5-ASA prevented the development of CRC in IBD patients by decreasing cell proliferation and promoting apoptosis (25, 27). However, the mechanisms sustaining 5-ASA chemopreventive properties remain largely unknown. In this work, we demonstrated that the well recognized anti-neoplastic effects of 5-ASA are mediated via PPAR γ . Using clinically-relevant concentrations (20, 21), we first showed by *in vitro* cell count, Ki67 immunostaining, and Tunel assay that 5-ASA displayed both anti-proliferating and pro-apoptotic properties. Subsequent to GW9662 application, evidence that PPAR γ mediated the chemopreventive effects of 5-ASA was established. In an *in vivo* model of xenograft tumor, we furthermore showed a strong reduction in tumor development due to topic effect of 5-ASA through a PPAR γ -dependent mechanism. Finally, in a model of AOM-induced colon carcinogenesis, 5-ASA significantly suppressed the number of both aberrant crypt foci and aberrant crypts via PPAR γ signaling pathway.

The anti-proliferative and pro-apoptotic effects of 5-ASA on several tumor-derived cell lines have been previously reported and different mechanisms have been proposed namely, inhibition of NF- κ B, prostaglandins (7, 8), and cyclo-oxygenase (28), activation of caspase-3 (29, 30), inhibition of the Wnt/ β -catenin pathway (9), disruption of the EGFR signaling (13), regulation of DNA replication checkpoints (10, 11), suppression of oncogenes (31), and disruption of TFG β pathway (12, 13). In fact, the anti-neoplastic role of PPAR γ was already recognized, given that PPAR- γ ligands inhibited formation of aberrant crypts foci and development of CRC in mice (17, 32). In our work, we further established, thanks to the specific effect of GW9662, that the anti-proliferative and pro-apoptotic actions of 5-ASA

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were at least partially PPAR γ -dependent. Regarding the *in vivo* anti-neoplastic effects of 5-ASA, data from rodent models of CRC were contradictory. Several studies have shown that using chemical CRC animal models, 5-ASA reduced the number of ACF, as well as the rate of tumor cell proliferation, and increased tumor apoptosis (4-6). On the other side, in APC mouse model of multiple intestinal neoplasia (Min), not only 5-ASA was found without any anti-neoplastic properties (33), but also PPAR γ ligands were shown to enhance colon carcinogenesis (34, 35). A possible explanation for such discrepancies is that colon carcinogenesis in APC^{Min} mice was driven by germ line mutations in the *Apc* gene. It has been shown that mice with preexisting damage to *APC*, a regulator of β -catenin, develop tumors in a manner insensitive to the status of PPAR γ . Thus, PPAR γ can suppress colon carcinogenesis only before damage to the APC/ β -catenin pathway (36). In this work, the results are in good agreement with previous reports showing anti-tumorigenic role for 5-ASA, but we demonstrated for the first time the link between the role of 5-ASA in suppressing colon carcinogenesis and its signaling through PPAR γ . Interestingly, blockage of PPAR γ with GW9662 induced ACF formation in the colon of mice reflecting the important endogenous anti-neoplastic role of PPAR γ (Figure 5).

Several clinical studies have evaluated the potential chemopreventive effect of PPAR γ ligands, including 5-ASA. For example, in patients with chemotherapy-resistant metastatic colorectal cancer, troglitazone treatment did not induce any objective tumor response and all patients had progressive disease as their best response to therapy (37). In fact, troglitazone, as all thiazolidinediones, has a wide systemic distribution due to broad expression of PPAR γ . This might explain, from one side, its inefficiency in tumor prevention and highlights, from the other side, the need for further development of ligands targeting tumor cells. What adds a further level of complexity is that UC patients were shown to have impaired colonic expression of PPAR γ which might also reduce drug efficiency (38). In this perspective, 5-ASA galenic presentations of delayed release have been developed so that to act locally in the colon and to be absorbed by colonic epithelial cells. Therefore, the efficiency of this drug is related to its mucosal concentration and systemic dosages remain low after oral or rectal administration (20). Thus, the availability of 5-ASA at the cellular level could explain its superior efficacy in preventing colorectal cancer among IBD subjects compared to other PPAR γ ligands. Notably, Rubin *et al.* observed a 3.4-fold risk reduction of

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dysplasia and CRC in UC patients treated with $\geq 1.2\text{g/day}$ of 5-ASA (26). In a case-control study comparing 102 patients with UC and CRC to matched controls, it was shown that CRC risk was reduced by 75% under 5-ASA regular treatment compared with no 5-ASA use (39). Likewise, a nested case-control study involving 18,969 patients with IBD in the UK General Practice Research Database (1987–2001) showed that regular 5-ASA users had a significantly reduced risk of CRC (adjusted OR 0.6) compared with irregular 5-ASA users (40). On the other hand, some retrospective studies did not support 5-ASA as protective in preventing CRC in IBD patients (41-43) potentially due to the heterogeneity of the populations analyzed and the short period of exposure to 5-ASA. Furthermore, because of the design of retrospective studies, some key areas have not been sufficiently addressed such as concurrent use of other medications and accurate data on dosing.

In our experimental model, 5-ASA treatment (200mg/kg/day) was started one week before the injection of the carcinogenic AOM and maintained for six weeks afterward. The treatment consisted of 5-ASA granules, usually used in IBD therapy, administered orally in mouse chaw in order to mimic tablets medication in humans. Whether or not this protocol reflects the chemoprevention strategy in patients with IBD, is arguable. In case control studies, regular users, having decreased risk of CRC, were defined as having at least six months of non-interrupted 5-ASA treatment at doses ranging between 1.2 and 2g/day (26, 39, 40). Nevertheless, the accurate dosage and duration of 5-ASA pharmacological therapy targeting CRC prevention are not yet well-established. Moreover, little information is available regarding the potential chemopreventive effect of other IBD medications, and it is currently unclear whether a patient would benefit from enhanced chemoprevention when combining for example 5-ASA and another treatment (e.g., azathioprine). Finally, despite its well characterized anti-neoplastic activities, it remains to be determined the step at which 5-ASA would interfere in the carcinogenesis process. While there is complete absence of data from humans, studies in rodent models of colon cancer have shown that 5-ASA was able to prevent tumor formation and further to reduce the number of developed tumors (5, 6, 33). Although these observations warrant further investigation since they were detected in various animal models, using different dosages and administration routes, they may reveal that 5-ASA would act at multiple stages of the carcinogenesis.

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5-ASA was originally developed without any knowledge of its molecular targets. However, in the past years, our knowledge of its role as a chemopreventive agent in IBD-related CRC has increased. Our piece of work, establishing the key role of PPAR γ in mediating 5-ASA anti-neoplastic prevention, may lead to the development of more effective PPAR γ ligands with higher efficiency or for use in association with agents of additive or synergic effects.

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REFERENCE LIST

1. Leung K, Pinsky P, Laiyemo AO, et al. Ongoing colorectal cancer risk despite surveillance colonoscopy: the Polyp Prevention Trial Continued Follow-up Study. *Gastrointestinal endoscopy*.2010;71:111-117.
2. Velayos FS, Terdiman JP, Walsh JM. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. *The American journal of gastroenterology*.2005;100:1345-1353.
3. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut*.2001;48:526-535.
4. Brown WA, Farmer KC, Skinner SA, et al. 5-aminosalicylic acid and olsalazine inhibit tumor growth in a rodent model of colorectal cancer. *Digestive diseases and sciences*.2000;45:1578-1584.
5. Ikeda I, Tomimoto A, Wada K, et al. 5-aminosalicylic acid given in the remission stage of colitis suppresses colitis-associated cancer in a mouse colitis model. *Clinical cancer research : an official journal of the American Association for Cancer Research*.2007;13:6527-6531.
6. Clapper ML, Gary MA, Coudry RA, et al. 5-aminosalicylic acid inhibits colitis-associated colorectal dysplasias in the mouse model of azoxymethane/dextran sulfate sodium-induced colitis. *Inflammatory bowel diseases*.2008;14:1341-1347.
7. Bantel H, Berg C, Vieth M, et al. Mesalazine inhibits activation of transcription factor NF-kappaB in inflamed mucosa of patients with ulcerative colitis. *The American journal of gastroenterology*.2000;95:3452-3457.
8. Wahl C, Liptay S, Adler G, et al. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. *The Journal of clinical investigation*.1998;101:1163-1174.

5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

9. Bos CL, Diks SH, Hardwick JC, et al. Protein phosphatase 2A is required for mesalazine-dependent inhibition of Wnt/beta-catenin pathway activity. *Carcinogenesis*.2006;27:2371-2382.
10. Campregher C, Honeder C, Chung H, et al. Mesalazine reduces mutations in transforming growth factor beta receptor II and activin type II receptor by improvement of replication fidelity in mononucleotide repeats. *Clinical cancer research : an official journal of the American Association for Cancer Research*.2010;16:1950-1956.
11. Stolfi C, Fina D, Caruso R, et al. Mesalazine negatively regulates CDC25A protein expression and promotes accumulation of colon cancer cells in S phase. *Carcinogenesis*.2008;29:1258-1266.
12. Koelink PJ, Hawinkels LJ, Wiercinska E, et al. 5-Aminosalicylic acid inhibits TGF-beta1 signalling in colorectal cancer cells. *Cancer letters*.2010;287:82-90.
13. Monteleone G, Franchi L, Fina D, et al. Silencing of SH-PTP2 defines a crucial role in the inactivation of epidermal growth factor receptor by 5-aminosalicylic acid in colon cancer cells. *Cell death and differentiation*.2006;13:202-211.
14. Sarraf P, Mueller E, Jones D, et al. Differentiation and reversal of malignant changes in colon cancer through PPAR γ . *Nature medicine*.1998;4:1046-1052.
15. Rousseaux C, Lefebvre B, Dubuquoy L, et al. Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator-activated receptor-gamma. *The Journal of experimental medicine*.2005;201:1205-1215.
16. Grommes C, Landreth GE, Heneka MT. Antineoplastic effects of peroxisome proliferator-activated receptor gamma agonists. *The lancet oncology*.2004;5:419-429.

5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

17. Osawa E, Nakajima A, Wada K, et al. Peroxisome proliferator-activated receptor gamma ligands suppress colon carcinogenesis induced by azoxymethane in mice. *Gastroenterology*.2003;124:361-367.
18. Dubuquoy L, Bourdon C, Peuchmaur M, et al. [Peroxisome proliferator-activated receptor (PPAR) gamma: a new target for the treatment of inflammatory bowel disease]. *Gastroenterologie clinique et biologique*.2000;24:719-724.
19. Dubuquoy L, Dharancy S, Nutten S, et al. Role of peroxisome proliferator-activated receptor gamma and retinoid X receptor heterodimer in hepatogastroenterological diseases. *Lancet*.2002;360:1410-1418.
20. Staerk Laursen L, Stokholm M, Bukhave K, et al. Disposition of 5-aminosalicylic acid by olsalazine and three mesalazine preparations in patients with ulcerative colitis: comparison of intraluminal colonic concentrations, serum values, and urinary excretion. *Gut*.1990;31:1271-1276.
21. Frieri G, Pimpo MT, Palumbo GC, et al. Rectal and colonic mesalazine concentration in ulcerative colitis: oral vs. oral plus topical treatment. *Alimentary pharmacology & therapeutics*.1999;13:1413-1417.
22. Euhus DM, Hudd C, LaRegina MC, et al. Tumor measurement in the nude mouse. *Journal of surgical oncology*.1986;31:229-234.
23. Papanikolaou A, Wang QS, Delker DA, et al. Azoxymethane-induced colon tumors and aberrant crypt foci in mice of different genetic susceptibility. *Cancer letters*.1998;130:29-34.
24. Ahmadi A, Polyak S, Draganov PV. Colorectal cancer surveillance in inflammatory bowel disease: the search continues. *World journal of gastroenterology : WJG*.2009;15:61-66.

5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

25. Rubin DT, Cruz-Correa MR, Gasche C, et al. Colorectal cancer prevention in inflammatory bowel disease and the role of 5-aminosalicylic acid: a clinical review and update. *Inflammatory bowel diseases*.2008;14:265-274.
26. Rubin DT, LoSavio A, Yadron N, et al. Aminosalicylate therapy in the prevention of dysplasia and colorectal cancer in ulcerative colitis. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*.2006;4:1346-1350.
27. Stolfi C, Pallone F, Monteleone G. Colorectal cancer chemoprevention by mesalazine and its derivatives. *Journal of biomedicine & biotechnology*.2012;2012:980458.
28. Stolfi C, Fina D, Caruso R, et al. Cyclooxygenase-2-dependent and -independent inhibition of proliferation of colon cancer cells by 5-aminosalicylic acid. *Biochemical pharmacology*.2008;75:668-676.
29. Reinacher-Schick A, Schoeneck A, Graeven U, et al. Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells. *Carcinogenesis*.2003;24:443-451.
30. Chan TA, Morin PJ, Vogelstein B, et al. Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*.1998;95:681-686.
31. Chu EC, Chai J, Ahluwalia A, et al. Mesalazine downregulates c-Myc in human colon cancer cells. A key to its chemopreventive action? *Alimentary pharmacology & therapeutics*.2007;25:1443-1449.
32. Tanaka T, Kohno H, Yoshitani S, et al. Ligands for peroxisome proliferator-activated receptors alpha and gamma inhibit chemically induced colitis and formation of aberrant crypt foci in rats. *Cancer research*.2001;61:2424-2428.

5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

33. Koelink PJ, Robanus-Maandag EC, Devilee P, et al. 5-Aminosalicylic acid inhibits colitis-associated but not sporadic colorectal neoplasia in a novel conditional Apc mouse model. *Carcinogenesis*.2009;30:1217-1224.
34. Lefebvre AM, Chen I, Desreumaux P, et al. Activation of the peroxisome proliferator-activated receptor gamma promotes the development of colon tumors in C57BL/6J-APCMin/+ mice. *Nature medicine*.1998;4:1053-1057.
35. Saez E, Tontonoz P, Nelson MC, et al. Activators of the nuclear receptor PPARgamma enhance colon polyp formation. *Nature medicine*.1998;4:1058-1061.
36. Girnun GD, Smith WM, Drori S, et al. APC-dependent suppression of colon carcinogenesis by PPARgamma. *Proceedings of the National Academy of Sciences of the United States of America*.2002;99:13771-13776.
37. Kulke MH, Demetri GD, Sharpless NE, et al. A phase II study of troglitazone, an activator of the PPARgamma receptor, in patients with chemotherapy-resistant metastatic colorectal cancer. *Cancer J*.2002;8:395-399.
38. Dubuquoy L, Jansson EA, Deeb S, et al. Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. *Gastroenterology*.2003;124:1265-1276.
39. Eaden J, Abrams K, Ekbom A, et al. Colorectal cancer prevention in ulcerative colitis: a case-control study. *Alimentary pharmacology & therapeutics*.2000;14:145-153.
40. van Staa TP, Card T, Logan RF, et al. 5-Aminosalicylate use and colorectal cancer risk in inflammatory bowel disease: a large epidemiological study. *Gut*.2005;54:1573-1578.
41. Nguyen GC, Gulamhusein A, Bernstein CN. 5-Aminosalicylic Acid Is Not Protective Against Colorectal Cancer in Inflammatory Bowel Disease: A Meta-Analysis of Non-Referral Populations. *The American journal of gastroenterology*.2012.

5-aminosalicylic acid suppresses colon carcinogenesis via PPAR γ

42. Bernstein CN, Blanchard JF, Metge C, et al. Does the use of 5-aminosalicylates in inflammatory bowel disease prevent the development of colorectal cancer? *The American journal of gastroenterology*.2003;98:2784-2788.

43. Terdiman JP, Steinbuch M, Blumentals WA, et al. 5-Aminosalicylic acid therapy and the risk of colorectal cancer among patients with inflammatory bowel disease. *Inflammatory bowel diseases*.2007;13:367-371.

FIGURES

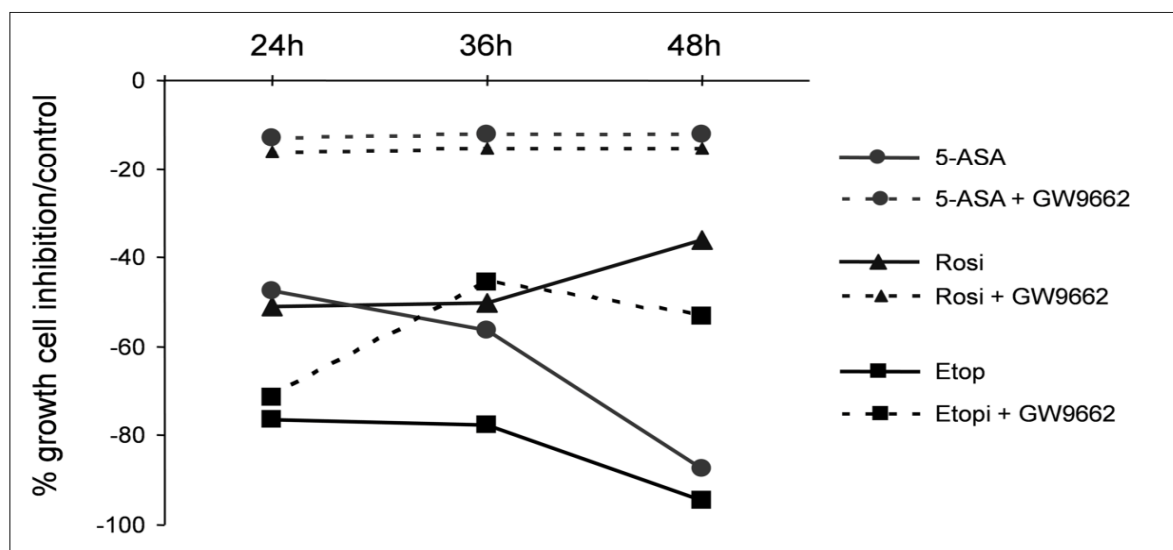


FIGURE 1: 5-ASA inhibits cell growth in a PPAR γ -dependent way

Incubation of HT-29 cells with 5-ASA (30 mM) resulted in a significant inhibition of cell growth compared to controls. Similar results were obtained in cells treated with the rosiglitazone (rosi, 10^{-5} M) and etoposide (etop, 50 mM). Addition of GW9662 (10^{-6} M) completely abolished the growth inhibitory activities of 5-ASA and rosiglitazone. Results were expressed as the mean number of 500 cells counted blindly in 4 different experiments.

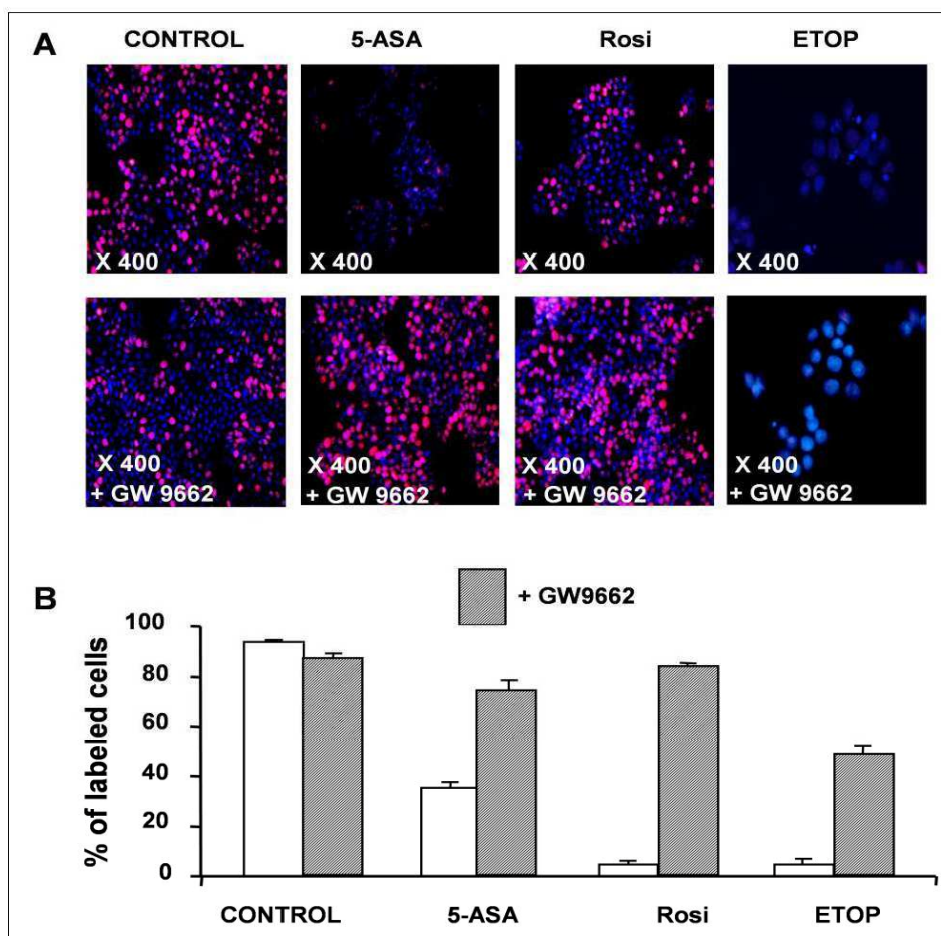


FIGURE 2: 5-ASA inhibits proliferation through PPAR γ

A, 5-ASA (30 μ M) and rosiglitazone (rosi, 10^{-5} M) inhibited HT-29 cell proliferation assessed by the nuclear Ki-67 staining (pink) compared to cells incubated with the medium alone (control). Nuclei were stained in blue with Hoescht 33342 solution. Addition of GW9662 (10^{-6} M) for 24h blocked the anti-proliferative effects of 5-ASA and rosiglitazone. **B**, In comparison to untreated HT-29 cells, 5-ASA treatment for 48h inhibited by 63% cell proliferation ($35\pm 4\%$ stained cells vs $94\pm 1\%$ stained cells, $p < 0.001$). Co-treatment with GW9662 blocked the anti-proliferative effects of 5-ASA. Results expressed as the mean number of 500 cells counted blindly in 4 different experiments.

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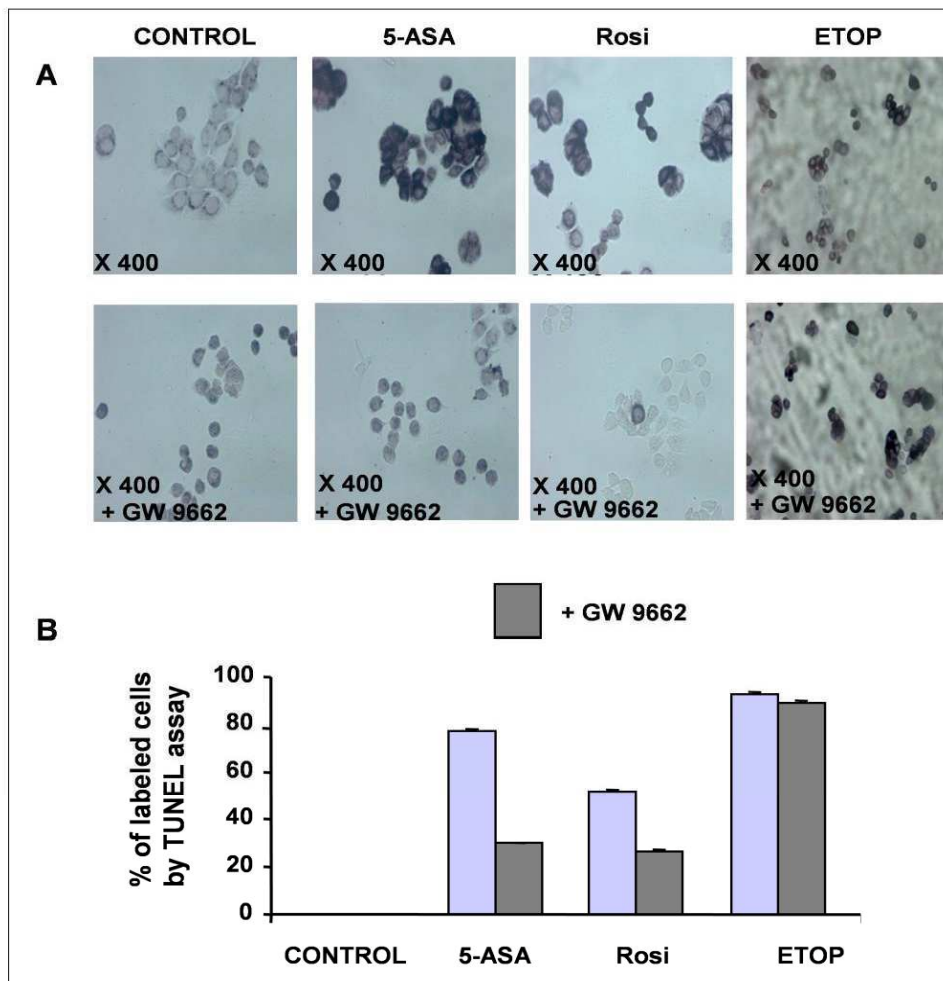


FIGURE 3: 5-ASA induces apoptosis through PPAR γ

A, Treatment of either 5-ASA (30 mM) or rosiglitazone (10^{-5} M) induced apoptosis measured by TUNEL assay in HT-29 cells. Addition of GW9662 (10^{-6} M) for 24h completely abolished the pro-apoptotic effects of 5-ASA and rosiglitazone. **B**, 5-ASA and rosiglitazone treatment for 48h induced apoptosis in 83 and 62% of cells, respectively. Co-treatment with GW9662 blocked the pro-apoptotic effect of 5-ASA and rosiglitazone. Results were expressed as the mean number of 500 cells counted blindly in 4 different experiments.

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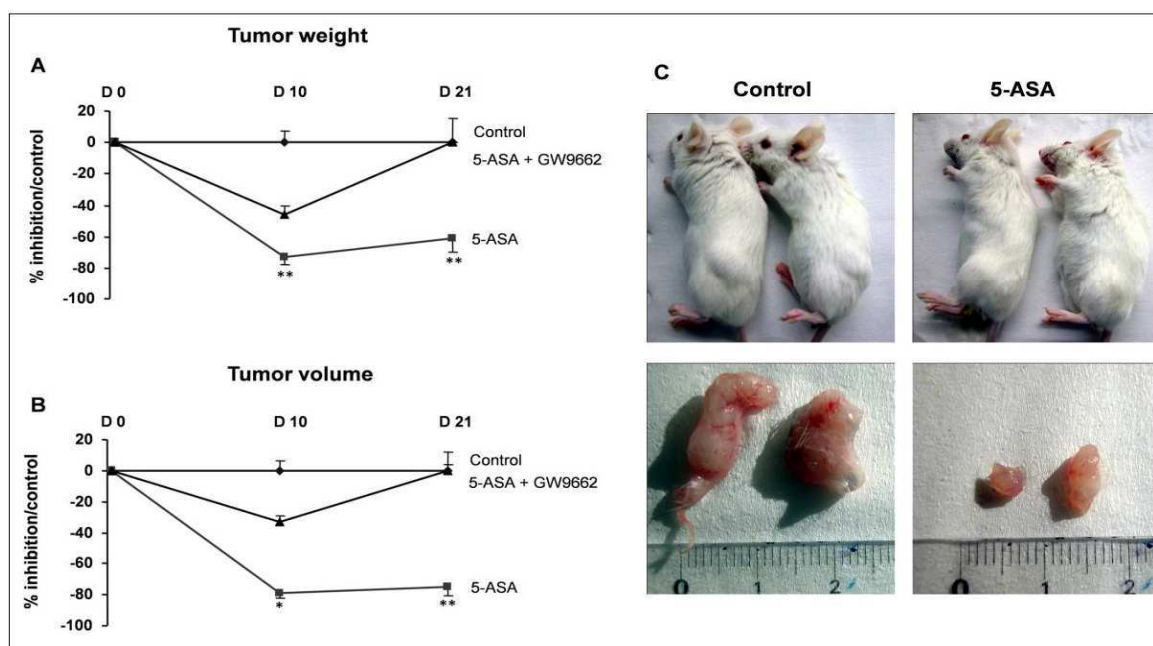


FIGURE 4: xenograft tumor model in SCID mice

10^7 HT-29 cells pretreated or not with GW9662 were injected subcutaneously in 6-7 weeks old SCID mice. Mice were treated for 21 days at the injection point of HT-29 with 5-ASA (50mM) or PBS (control). One group of mice was co-treated with a local injection of 5-ASA and an intraperitoneal injection of GW9662 (1mg/kg/day). Following injection of HT-29 cells, tumor development was monitored at days 10 and 21 and evaluated by tumor weight (A) and volume (B) measurement. Results are expressed as percentage of inhibition compared to control mice. Topical subcutaneous treatment of 5-ASA (50 mM) reduced the decreased by 60% tumor weight and by 83% volume compared to controls after 10 days of treatment. Injection of PPAR γ antagonist GW9662 (1mg/kg/day) significantly abolished the inhibition of tumor growth induced by 5-ASA. C, Illustrative photo of the tumor mass in control and 5-ASA groups taken upon animal harvest and showing the effect of 5-ASA on tumor size.

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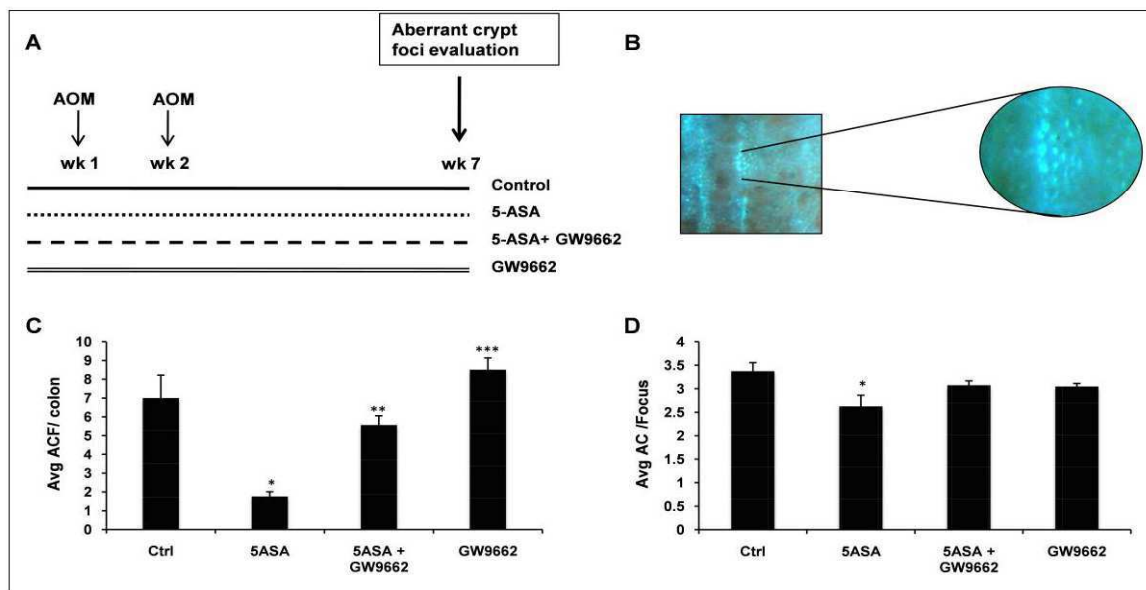


FIGURE 5: 5-ASA exhibits a potent anti-carcinogenic effect *in vivo*

A, A/J01aHsd mice were randomly divided into four groups: Control group (Ctrl) fed with standard diet, 5-ASA group fed with 5-ASA granules-containing chaw at 200mg/kg/day, 5-ASA+GW9662 group fed with 5-ASA granules-containing chaw at 200mg/kg/day and receiving daily intra-peritoneal injection of GW9662 at 2mg/kg/day, GW9662 group fed with standard diet and receiving daily intra-peritoneal injection of GW9662 at 2mg/kg/day. Control and 5-ASA mice groups have received daily intra-peritoneal injection of 3% DMSO/PBS (GW9662 vehicle). To induce aberrant crypts (**B**), mice have received intra-peritoneally two doses of AOM (10mg/kg/day) at weeks 2 and 3. At the end of the protocol (week 7), mice were harvested and the formation of aberrant crypts (ACs) and aberrant crypt foci (ACF) was blindly recorded. **C-D** 5-ASA treatment induced a significant reduction of aberrant crypt foci (75%) and aberrant crypts (22%) compared to mice fed with standard diet. Co-administration of GW9662 suppressed the anti-tumoral effect of 5-ASA. GW9662-treated group displayed higher number of ACF compared to 5-ASA+GW9662 group. $p < 0.05$.

(E) CONCLUSION AND PERSPECTIVES

Although multiple clinical studies have suggested a role for 5-ASA in prevention of CRC in patients with IBD, less is known about how 5-ASA exerts a chemopreventive effect. Functional studies have proposed several signaling pathways and downstream mediators as involved in the anti-neoplastic properties of 5-ASA. These studies have shown that 5-ASA would be able to suppress colon carcinogenesis via inflammatory dependent and independent mechanisms. However, the key receptor mediating all these anti-tumorigenic properties of 5-ASA was not identified. In this work, we showed, thanks to the specific PPAR γ antagonist, that 5-ASA suppressed colon carcinogenesis at least partially via PPAR γ . Given the well-recognized anti-neoplastic properties of PPAR γ , this study supports the therapeutic potential of 5-ASA in preventing CRC. Therefore, the therapeutic utility of 5-ASA in IBD resides in both its anti-inflammatory and anti-neoplastic effects, since CD and UC patients suffer from chronic inflammation and display an increased risk of developing CRC. Although the parameters of therapeutic usage of 5-ASA (dose, duration) as an anti-inflammatory therapy in IBD are well established, the standards of 5-ASA pharmacological therapy targeting CRC prevention are not yet known.

The anti-inflammatory characteristics of 5-ASA suggest the possible use of other drugs with similar properties in the chemopreventive therapy. Some of these compounds, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and selective inhibitors of cyclooxygenase (COX)-2 are effective in attenuating the growth and diffusion of CRC cells [409, 563]. However, their use in the chemopreventive programs of IBD-related CRC is not justified because the administration of NSAIDs and COX2-inhibitors in IBD patients associates with high risk of disease flare-ups [564, 565]. 5-ASA is structurally related to NSAIDs, but unlike these compounds, it is safe and free of serious adverse effects. In fact, 5-ASA has been developed so that to act locally in the colon and to be absorbed by colonic epithelial cells leading to low systemic dosages after oral or rectal administration. This particular formulation would limit potential adverse effects and provide superiority over other PPAR γ ligands or NSAIDs in attenuating intestinal inflammation or preventing CRC.

Even with the data supporting the chemopreventive role of 5-ASA, several questions still need to be addressed. First, should all IBD patients be taking 5-ASA to reduce the risk of cancer? or particular patients with even increased risks should be targeted? Second, is one 5-ASA therapy better than another? As already stated, some studies have shown a difference between the delayed-release formulations of sulfasalazine and mesalamine in CRC risk reduction [343, 454, 455]. It is possible, however, that socio-demographic factors may have had a bearing on this effect. For example, sulfasalazine is cheaper than most mesalamine formulations. Hence, cost may influence prescribing decisions. Also, sulfasalazine has been available for more than 60 years, whereas the newer mesalamine formulations were only introduced in the early 1990s. Sulfasalazine users may therefore be older than mesalamine users and have other factors that may make them at higher risk of developing CRC. Because sulfasalazine inhibits the absorption of folate and folate deficiency may be associated with a higher risk of sporadic colon cancer, it is also possible that the lack of a chemopreventive effect with sulfasalazine may be a result of reduced folate [566, 567]. Third, what is the optimal 5-ASA dose for chemoprevention? Two studies have found a dose–response in which the odds of CRC or dysplasia/CRC decreased as the total dose of 5-ASA increased [453, 454]. In both studies, 5-ASA doses equivalent to mesalamine of at least 1.2 g/day provided the greatest risk reduction (72%–81%). However, these studies assessed dose–response in different ways, making it difficult to interpret the data as whole. Fourth, are systemic 5-ASA therapies more effective than topical formulations? Fifth, what is the minimal treatment time of 5-ASA that would provide efficient chemoprevention for IBD patients? In case control studies, regular users, having decreased risk of CRC, were defined as having at least six months of non-interrupted 5-ASA treatment [454, 455]. Thus, further studies need to address the dose, the duration and the formulation of 5-ASA therapy. The ideal study would be a prospective, placebo-controlled study examining the incidence of CRC in the general IBD population. Sixth, at present, less information is available regarding chemopreventive action of other IBD therapies compared with that of 5-ASA, and it is currently unclear whether a patient receiving another treatment (e.g., azathioprine) would benefit from additional 5-ASA in terms of chemoprevention

Although data on 5-ASA chemopreventive properties increase exponentially for the recent years, still a long time is required before establishing a 5-ASA-based

chemopreventive therapy. Whether or not, such therapy would substitute for surveillance colonoscopies remains unknown. Currently, colonoscopic surveillance is recommended, the essential aim of which, along with the detection of macroscopic lesions, is the identification of precancerous dysplastic regions, with the help of systematic multilevel biopsies. Therefore, until now despite the extensive studies showing a chemopreventive effect of 5-aminosalicylates, rigorous colonoscopic surveillance could not be avoided. However, recent improvement of imaging technologies, especially the development of chromoendoscopy, increases the yield of dysplasia, thus raising the question of the relevance of systematic random biopsies [568].

5-ASA was originally developed without any knowledge of its molecular targets. However, in the past years, our knowledge of its role as a chemopreventive agent in IBD-related CRC has increased. However, the step at which 5-ASA would interfere in the carcinogenesis process is not yet identified. Functional studies in rodent models of colon cancer have shown that 5-ASA was able to prevent tumor formation and further to reduce the number of developed tumors [462, 463, 569]. These observations suggest that 5-ASA would interfere at multiple steps of the carcinogenesis. However, data from these studies require further verification since they were generated using different 5-ASA dosages and administration routes and also in different CRC models. Furthermore, data from APC^{Min} mice model suggest that PPAR γ can suppress colon carcinogenesis only before damage to the APC/ β -catenin pathway [570]. Thus, further studies should aim at defining whether 5-ASA exhibits only preventive or addition curative properties against CRC.

Overall, this study, provides, from one side, rational for further development of more effective and safe anti-neoplastic PPAR γ ligands with topical effects and from the other side, reinforces the therapeutic potential of 5-ASA in view of the tumor-suppressing properties of PPAR γ . Besides, while different 5-ASA formulations have been developed since decades with the aim of optimizing the anti-inflammatory profile of 5-ASA; the synthesis of new 5-ASA-derivatives with potent anti-carcinogenic properties would represent a novelty in the chemoprevention of colitis-associated cancer.

SKIN HOMEOSTASIS

In Vivo Imaging Reveals Selective PPAR Activity in the Skin of Peroxisome Proliferator-Activated Receptor Responsive Element-Luciferase Reporter Mice

PREFACE

The biological significance of PPAR γ , and more generally PPARs is not restricted to the gastrointestinal tract. PPARs have been shown to mediate important regulatory functions in different physiological systems. In the skin, PPARs have been demonstrated to contribute to epidermal development and homeostasis and to regulate important cellular functions, including cell proliferation and differentiation. Various cell culture and *in vivo* approaches suggest that PPAR α contributes to fetal skin development, to epidermal barrier maturation and to sebocyte activity. PPAR β/δ regulates sebocyte differentiation, promotes hair follicle growth and has pro-differentiating effects in keratinocytes in normal and inflammatory conditions. In contrast, the role of PPAR γ appears to be rather minor in keratinocytes, whereas its activity is required for sebaceous gland differentiation. An important regulatory role for PPARs has also been demonstrated in pathological states. It has been shown that in inflammatory skin disorders, including hyperproliferative psoriatic epidermis and the skin of patients with atopic dermatitis, the expression of both PPAR α and PPAR γ is decreased. This observation suggests the possibility that PPAR α and PPAR γ activators, or compounds that positively regulate PPAR gene expression, may represent novel NSAIDs for the topical or systemic treatment of common inflammatory skin diseases such as atopic dermatitis, psoriasis, and allergic contact dermatitis. Studies in non-diabetic patients suggest that oral thiazolidinediones, which are synthetic ligands of PPAR γ , not only exert an antidiabetic effect but also may be beneficial for moderate chronic plaque psoriasis by suppressing proliferation and inducing differentiation of keratinocytes; furthermore, they may even induce cell growth arrest, apoptosis, and terminal differentiation in various human malignant tumors. Also, PPAR δ has been demonstrated to have an anti-apoptotic role and to maintain survival and differentiation of epithelial cells. In melanoma, the growth inhibitory effect of PPAR γ activation is independent of apoptosis and seems to occur primarily through induction of cell cycle arrest or induction of re-differentiation. In clinical

trials of gemfibrozil, a PPAR α ligand, significantly fewer patients treated with this lipid-lowering drug were diagnosed with melanoma as compared to those in the control group. Thus, an increasing body of evidence indicates that PPAR signaling pathways may represent interesting therapeutic targets for a broad variety of skin disorders, including inflammatory diseases and skin malignancies. Major efforts should be therefore made to generate valid models for the characterization and development of new PPAR ligands in the context of drug management of skin diseases.

Nevertheless, the development of novel drugs is a long process requiring years of preclinical research and many steps indispensable to ensure that the molecule of interest can be administered to humans with a minimal risk of toxic effects. Thus, pharmaceutical companies are eager for the development of novel methodologies that shorten the time required for pharmacodynamic, pharmacokinetic and toxicological studies to be carried out *in vitro* and in animal systems. Currently, quantitative analysis of molecular events in living organisms is done with the combined application of imaging and genetic engineering technologies leading to possibility of live imaging of specific event in living animals. This technique would dramatically improve the identification of new drug candidates and speed up the research at preclinical stages.

We have recently developed a reporter mouse for the ubiquitous and quantitative measurement of the transcriptional activity of PPARs. These mice (PPRE-luc) were generated by genomic integration of a transgene flanked by insulators and carrying a reporter gene, the *Firefly luciferase* gene, under the control of PPAR responsive regulatory element. In the present study, we reported a novel usage for PPRE-luc mice for assessing the transcriptional activation of PPARs *in vivo* in mice skin. We demonstrated that PPRE-luc reporter mouse is a non-invasive powerful tool for pharmacological screening of novel molecules targeting PPARs.

(A) ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) have been revealed as key regulators of several skin disorders such as atopic dermatitis and psoriasis. This has led to a growing interest in the pharmacological development of drugs targeting PPARs as therapeutics for skin diseases. To evaluate skin PPAR activity, we have made novel usage of PPRE-luc mice, a mice model in which the luciferase gene expression is under the control of a PPAR-inducible promoter in all organs. Our aim was to define and validate experimental conditions to establish PPRE-luc mice as a valuable tool for *in vivo* non invasive evaluation of PPARs activation in the skin. We demonstrated by optical imaging that topical application of 40mM of Luciferin for 10 minutes was enough to reveal the optimal luciferase activity in mice skin. The treatment of mice skin with the PPAR γ and PPAR α agonists, pioglitazone and WY14643, was associated with significant increase in photons emission reaching maximal signaling at 6 hours. We have performed dose response studies by testing a large range of pioglitazone and WY14643 concentrations on mouse skin. The specificity of bioluminescence signal induced pioglitazone and WY14643 was assessed using PPAR γ and PPAR α antagonists, GW9662 and GW6471, respectively. This approach revealed that the isoform specificity of PPARs agonists decreased when high ligand concentrations were applied on mouse skin. These results were further confirmed by *in vitro* measurement of luciferase activity in skin extracts. Taken together, our results demonstrated that PPRE-luc mice represent a valuable reporter mouse model for the *in vivo* pharmacological profiling of drugs targeting PPARs in the skin.

(B) BACKGROUND

I- Peroxisome Proliferator-Activated Receptors (PPARs): A General Overview

I-A. Origin and Expression Pattern

The first peroxisome proliferator-activated receptor PPAR (PPAR α) was cloned originally from a mouse-liver complementary-DNA library as the nuclear receptor that mediates the effects of a group of endogenous and xenobiotic compounds known as peroxisome proliferators. Peroxisomes are subcellular organelles found in most plant and animal cells that perform diverse metabolic functions including H₂O₂-based respiration, β -oxidation of fatty acids (FAs), and cholesterol metabolism. Peroxisome proliferators were named for their common property of increasing both the number and activity of liver peroxisomes after chronic high-dose administration to rodents. However, PPARs were not associated with hepatic peroxisome proliferation in humans [571].

PPARs are currently ligand-activated transcription factors which belong to a nuclear hormone receptor superfamily. The three PPAR isoforms, PPAR α , PPAR β/δ (also known as PPAR β or PPAR δ) and PPAR γ , are found in all the mammalian species. Since the identification of the PPARs more than 20 years ago, numerous studies have revealed that PPARs influence many important biological functions, including inflammation and carcinogenesis. Although they are encoded by distinct genes on different chromosomes, the PPAR isoforms have a high degree of sequence and structural homology. However, the isoforms are unique in their quantitative patterns of tissue distribution (Table 1), have important differences in their regulatory activities and modulate specific responses after activation.

Table 1. Tissue expression and functions of peroxisome proliferator-activated receptors [17].

SUBTYPE	TISSUE OR CELL TYPE
PPAR α	Liver, skeletal muscle, brown adipose tissue, heart, monocytes/macrophages, endothelial cells, smooth muscle cells, lymphocytes
PPAR β/δ	Ubiquitously expressed
PPAR γ	Brown and white adipose tissues, colon, spleen, retina vascular, monocytes/macrophages, endothelial cells, smooth muscle cells, lymphocytes, dendritic cells; platelets; megakaryocytes.

I-B. Structure and Ligands

The PPARs possess the canonical domain structure common to other nuclear receptor family members with functional domains called A/B (ligand-independent domain), C (DNA binding domain), D (hinge domain) and E-F (ligand binding domain) (Figure 1) [572]. The DNA binding domain of the PPARs is formed by two zinc finger-like motifs, which fold to form a globular three dimensional structure. This domain is highly conserved between PPAR isoforms, which is consistent with the fact that each isoform has restricted binding to similar PPRE sequences [573].

In mice, only two PPAR γ isoforms have been described [574], whereas in humans four PPAR γ messenger RNA (mRNA) isoforms have been identified, i.e. PPAR γ 1, γ 2, γ 3, and γ 4 generated by alternative splicing and promoter usage and differing at their 5'-end [575-577]. Some studies have also demonstrated the presence of PPAR- γ 5 and - γ 7 transcripts in human THP-1 macrophages [578]. The PPAR γ 1, γ 3 and γ 4 mRNAs give rise to an identical protein product (PPAR γ 1 protein), whereas PPAR γ 2 gives rise to a protein with 28 additional amino acids at its NH₂ terminus. The differences in PPAR γ protein isoforms are restricted to the NH₂-terminal region of the protein [575], but they may alter protein folding and thereby ligand specificity and affinity. Current information about isoform-specific functions of PPAR γ s is limited to the role of PPAR γ 2 in adipocyte differentiation and carbohydrate metabolism

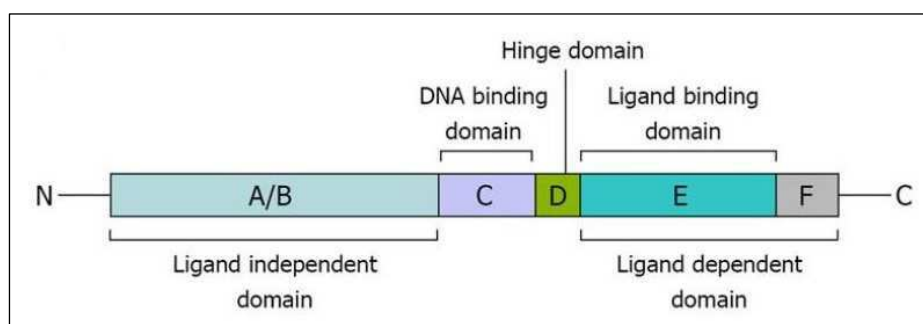


Figure 1. Structure and transcriptional activation of PPARs. Adapted from [14].

Although the nature of true endogenous PPAR ligands is still not fully known [579], PPARs are activated by a wide variety of endogenous ligands that are derived from the metabolism of fatty acids with some degree of isoform specificity. Exogenous ligands are mainly pharmacologically produced as therapeutics for inflammatory and autoimmune diseases (Table 2). The therapeutic utility of PPARs ligands is discussed in details in the section I.D.2, I.D.3 and I.D.4). Exogenous ligands for PPARs may also derive from the diet, which is consistent with the fact that PPARs regulate the expression of many genes involved in glucose and lipid metabolism [580]. PPAR α activators include variety of long-chain fatty acids and, in particular, polyunsaturated fatty acids such as palmitic acid, oleic acid, linoleic acid, and arachidonic acid [581]. The PPAR α isotype is the cellular target for Leukotriene (LTB)-4, fibric acid derivatives such as gemfibrozil, bezafibrate, and fenofibrate, which are hypolipidemic drugs widely used for reducing triglyceride levels, a risk factor for cardiovascular disease. Similarly, PPAR γ can be activated by a number of ligands, including 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2) and the anti-diabetic glitazones (rosiglitazone, pioglitazone, ciglitazone), used as insulin sensitizers. PPAR γ ligands include as well as a number of lipids, including oxidized LDL (i.e. 9- and 13 hydroxyoctadecadienoic acid (HODE)), eicosanoids, and prostaglandins. Dyslipidemia and insulin-dependent diabetes are commonly found existing together as part of the metabolic X syndrome. Because PPAR α and PPAR γ ligands independently are useful clinical drugs in the treatment of these respective disorders, synthetic dual PPAR α/γ ligands have recently been developed (Table 2) and show a combined clinical efficacy [582]. PPAR β/δ activators include fatty acids and prostacyclin and synthetic compounds L-165041 and GW501516 [579, 583-585]. Unlike PPAR α or- γ , there are no PPAR β/δ drugs in the clinic. Indeed, part of the challenge in determining the function of PPAR β/δ has been the identification and availability of new ligands with more potency and selectivity for use as pharmacological tools.

Table 2. The pharmacological ligands of PPARs. Abbreviations: 2Br-C16, 2-bromopalmitate; 15d-PGJ2, 15-deoxy-D12,14-PGJ2; BADGE, bisphenol A diglycidyl ether; CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; CDDO-Me, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid methyl ester; ETYA, eicosatetraenoic acid; GW1929, (2S)-((2-benzoylphenyl)amino-3-[4-[2-(methylpyridin-2-ylamino)ethoxy]phenyl]-propionic acid, GW501516, 2-methyl-4(((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)methyl)sulfanyl)phenoxy)acetic acid, GW7647, 2-([4-{2-([cyclohexylamino]carbonyl)[4-cyclohexylbutyl]amino)ethyl}-phenyl]thio)-2-methylpropanoic acid; GW9662, 2-chloro-5-nitro-N-phenylbenzamide; HEET, hydroxyepoxyeicosatrienoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HpODE, hydroperoxyoctadecadienoic acid; L165041, (4-[3-{4-acetyl-3-hydroxy-2-propylphenoxy}propoxyl]phenoxy) acetic acid; LDL, low density lipoprotein; MK886, 3-(1-[p-chlorobenzyl]-5-[isopropyl]-3-tert-butylthioindol-2-yl)-2,2-dimethylpropanoic acid methyl ester; oxoODE, oxidized octadecadienoic acid; T0070907, 2-chloro-5-nitro-N-(4-yrityl)benzamide; WY14643, 4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid [17].

Ligand class	PPAR α	PPAR β	PPAR γ
Synthetic agonists	GW7647	L165041	Rosiglitazone
	WY14643	GW501516	Ciglitazone
	Clofibrate	GW0742	Troglitazone
	Fenofibrate		Pioglitazone
	Bezafibrate		CDDO
	Ciprofibrate		GW1929
	Gemfibrozil		
designed dual antagonists	Muraglitazar		Muraglitazar
	Tesaglitazar		Tesaglitazar
	Farglitazar		Farglitazar
	Ragaglitazar		Ragaglitazar
Fatty acids	Docahexanoic acid	Docahexanoic acid	Docahexanoic acid
	Arachidonic acid	Arachidonic acid	Arachidonic acid
	Linoleic acid	Linoleic acid	
	C6-C18	C6-C8	
	ETYA		

(Continued Table 2)

Ligand class	PPARα	PPARβ	PPARγ
Eicosanoids	15d-PGJ ₂	15d-PGJ ₂	15d-PGJ ₂
	PGJ ₂	PGJ ₂	PGJ ₂
	Prostacyclin (PGI ₂)	Prostacyclin (PGI ₂)	
	PGA _{1/2}	PGA _{1/2}	PGA _{1/2}
	PGB ₂	PGB ₂	PGB ₂
	8-HEPE		
	8-(R)HETE		8-(R)HETE
	8-(S)HETE		8-(S)HETE
	12-HETE		15-HETE
	LTB ₄		9-(R/S)HODE
	9-(R/S)HODE		13-(R/S)HODE
	13-(R/S)HODE		13-(S)HpODE
	20,8,9-HEET		9-oxoODE
	20,11,12-HEET		13-oxoODE
20,14,15-HEET			
Synthetic antagonists	MK886	Sulindac	CDDO-Me
			GW9662
			Diclofenac
			BADGE
			T0070907
Fatty acid inhibitors	2Br-C16	2Br-C16	
	Tetradecylglycidic acid	Tetradecylglycidic acid	
	Nonythioacetic acid		
	Tetradecythioacetat		

I-C. PPARs-Mediated Regulation of Gene Expression

Substantial progress has been made in delineating the molecular mechanisms that mediate PPAR-regulated gene expression and associated cellular functions (Figure 2).

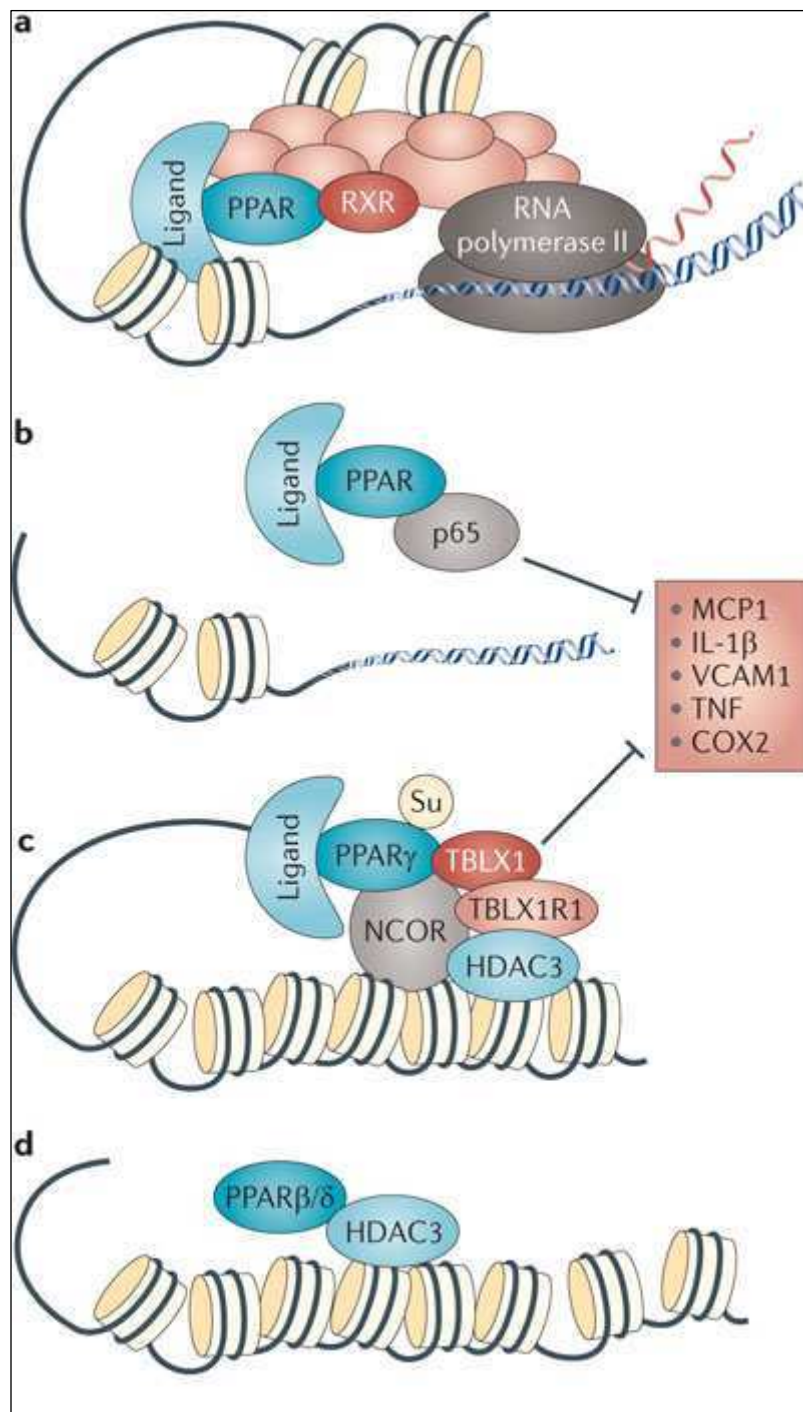


Figure 2. Molecular regulation of transcription by PPARs. a. The transcriptional upregulation of target gene expression. b. The repression of pro-inflammatory gene expression. c. The repression of pro-inflammatory gene expression by PPAR γ . d. Repression of gene expression by PPAR β/δ [22].

Mechanistically, they form heterodimers with the retinoid X receptor (RXR) and activate transcription by binding to a specific DNA element, termed the peroxisome proliferator response element (PPRE), in the regulatory region of a variety of genes. PPREs are direct repetitions, consisting of two hexanucleotides with the consensus sequence AGGTCA separated by one or two nucleotide spacers named direct repeat (DR) DR-1 or DR-2. Such a sequence, or a similar one, has been found in numerous PPAR-inducible genes. Several ways of PPARs-mediated regulation of transcription have been identified (Figure 2). More specifically, binding of agonists within the ligand-binding site of PPARs causes a conformational change promoting binding to transcriptional coactivators. Conversely, binding of antagonists results in a conformation that favors the binding of corepressors [586, 587]. Following ligand binding, PPARs undergo a conformational change that causes the release of histone deacetylase (HDAC) co-repressors, thus enabling PPARs to heterodimerize with retinoid X receptor (RXR). RNA polymerase II and co-activators with histone acetyl transferase (HAT) activity are then recruited to this complex, which binds to response elements in target genes, leading to chromatin remodeling and ultimately to increased transcription (Figure 2a) [588]. PPARs can also downregulate gene expression by interfering with other proteins and transcription factors through a 'trans-repression' mechanism (Figure 2b). For example, PPAR α and PPAR β/δ can sequester the p65 subunit of the NF- κ B complex and can prevent NF- κ B-dependent regulation of genes that are involved in pro-inflammatory responses [589-591]. Alternatively, trans-repression by PPAR γ can involve its sumoylation (Figure 2c), through which ligand activation leads to the conjugation of PPAR γ with SUMO. Sumoylated PPAR γ then binds to a nuclear receptor co-repressor (NCOR)-containing complex that is bound to a pro-inflammatory target gene. This prevents degradation of the NCOR complex, thereby maintaining active repression of pro-inflammatory gene expression [592]. Sumoylation-dependent trans-repression might also be relevant for PPAR α and PPAR β/δ because the amino acid (Lysine) that is sumoylated is conserved between all three PPARs [2]. PPAR β/δ can interact with histone deacetylases (HDACs), independently of ligand binding, and can maintain chromatin in a compact structure preventing gene expression (Figure 2d) [593].

Trans-repression of pro-inflammatory signaling pathways (Figure 2b & 2c) is thought to be central to the well-documented anti-inflammatory activities that are associated with

PPARs [2, 589]. More recently, it has been shown that the beneficial effects of PPAR γ activation in diabetics can be modulated by 'non-agonist' PPAR γ ligands that inhibit the phosphorylation of PPAR γ , and so are independent of the classic receptor-mediated modulation of gene transcription [594]. Thus, there are multiple levels of regulation that can be targeted to selectively alter PPAR-dependent activities. It is interesting to note that there is an overlap in target genes that are regulated by each PPAR, but the physiological effects induced by selective PPAR agonists are unique owing to the complexity of the PPAR-dependent and the PPAR-independent effects that each agonist induces.

I-D. The Role of PPARs in Health and Disease

I-D.1. The Physiological Functions of PPARs

The cellular and systemic roles that have been attributed to PPARs extend far beyond the control of hepatic peroxisome proliferation in rodents after which these receptors were initially named [571]. Given the broad distribution of PPARs isoforms in different tissues and the diversity of ligands that could activate them, it seems logical that research progress would reveal several regulatory roles for PPARs in physiological and pathophysiological conditions.

a) PPAR α

PPAR α , which was the first PPAR to be identified [571], is expressed in many tissues, particularly those that require fatty acid oxidation as a source of energy (Figure 3) [595]. PPAR α is central for the maintenance of lipid homeostasis:

a primary role of PPAR α is to increase the cellular capacity to mobilize and to catabolize fatty acids, particularly in the liver during starvation, where the oxidation of fatty acids is

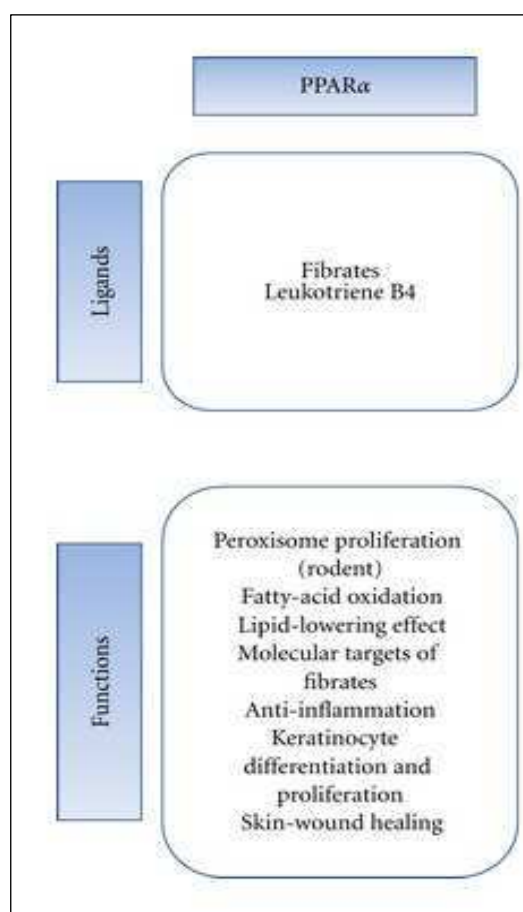


Figure 3. Summary of ligands and functions of PPAR α . Updated from [15].

essential for energy production (Figure 4A) [596]. Under these conditions, PPAR α is probably activated by endogenous fatty acids and fatty acid derivatives [596]. In fasted PPAR α -null mice, its absence is associated with pronounced hepatic steatosis, decreased levels of plasma glucose and ketone bodies, elevated plasma free fatty acid levels and hypothermia [597-599]. These severe metabolic disturbances are the result of the decreased expression of a large number of genes involved in hepatic lipid metabolism, many of which have been identified as direct PPAR α target genes [600, 601]. PPAR α can also improve insulin resistance in high-fat and genetic models of diabetes through pleiotropic changes in gene expression that prevent weight gain and adiposity [602]. In addition, PPAR α is a target of hypothalamic hormone signaling as it plays an important role in the anti-inflammatory action of glucocorticoids [603]. In fact, during fasting, as well as in situations of physical and physiological stress, the hypothalamic adrenocorticotrophic hormone (ACTH) induces the release of glucocorticoids by the adrenal glands, which stimulates the hepatic expression of PPAR α . Moreover, it accumulates according to a daily rhythm with highest levels at the beginning of feeding time (Figure 4A) [604]. The availability and production of physiologically relevant ligands of PPAR α is central for coordinating its multiple functions in response to the amount and type of food available (Figure 4B) [605, 606].

Interestingly, PPAR α is involved in embryonic development and the viability of pups. Although PPAR α -null mice are viable, they present an increased risk of maternal abortion and neonatal mortality [607]. In fact, roughly 50% of diabetic PPAR α -deficient mice abort between day 12 and 16 of gestation and approximately 80% of their offspring die at birth [607]. Several reasons have been suggested for this increased mortality. Gemfibrozil and clofibrate, two PPAR α agonists, down-regulate human chorionic gonadotrophin and up-regulate progesterone secretions in human trophoblast cells, suggesting that a lack of PPAR α may be deleterious for the secretion of these hormones, essential for maintaining pregnancy [608]. PPAR α is expressed in the placenta in late gestation and all three PPARs (α , β/δ and γ) are expressed in junctional (endocrine functional) and labyrinth (barrier transport) zones of the rat placenta, from day 13–21 of gestation [609, 610]. PPAR α has also been observed in the human term placenta, suggesting a potential role in placental fatty acid transfer to meet fetal requirements [610]. These observations show that even if PPAR α -null mothers are able to accumulate lipids in the placenta labyrinth, due to the

Evaluation of skin PPAR activity by in vivo imaging

presence of PPAR γ , the lack of PPAR α may affect fetal-maternal nutrient exchange, which may explain, at least in part, the lethality mentioned above. This placental dysfunction may be more pronounced in diabetes, which increases energy demands. In fact, PPAR α -null diabetic mice are hypoinsulinaemic, with a reduced ability to use glucose and lipids as energy sources [609]. Therefore, in addition to hormonal dysfunctions and the high proinflammatory Th1 cytokine levels observed in pregnant PPAR α -null diabetic mice and their offspring, the high rate of abortion and mortality rate of pups could also be related to the defect in energy utilization.

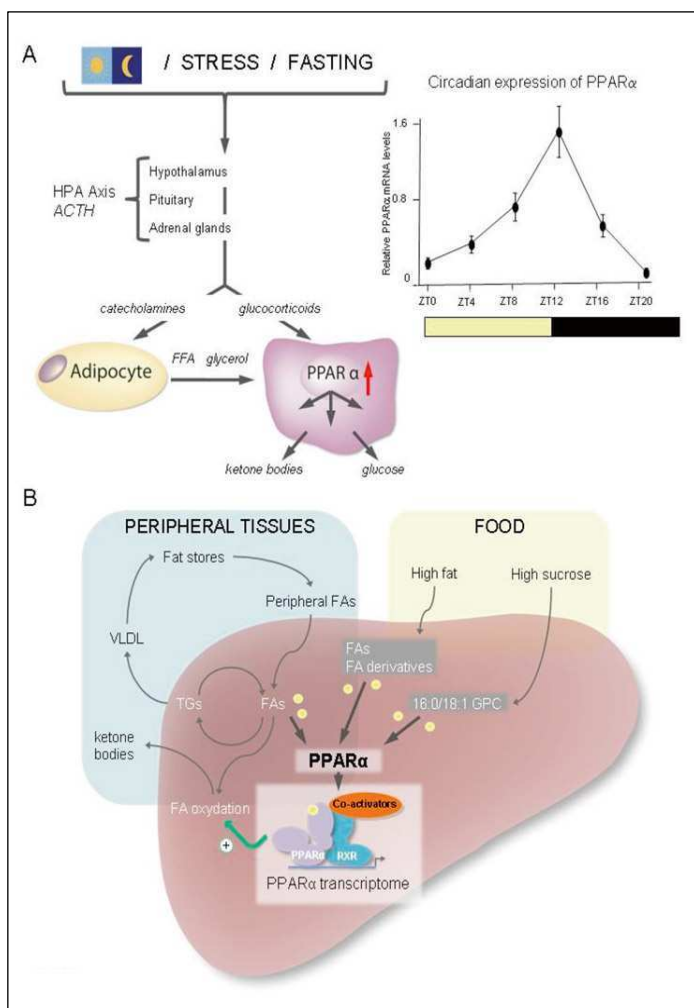


Figure 4. PPAR α regulation and activation in the liver. A. PPAR α expression is stimulated by glucocorticoids and follows a circadian rhythm with the highest expression at the end of the light phase. B. Dietary fatty acid derivatives activate PPAR α . On a high sucrose, the production of 16:0/18:1 GPC activates PPAR α . Activation of PPAR α results in the activation of PPAR α transcriptome, which contributes to maintaining the energy balance in part through the promotion of mitochondrial and peroxisomal β -oxidation of fatty acids and many other metabolic pathways. Abbreviations: FA, fatty acids; 16:0/18:1 GPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; TG, triglycerides; VLDL, very low density lipoprotein [10].

Therapeutically, PPAR α is also the molecular target of fibrates (hypolidaemic drugs), which are widely used drugs that reduce serum lipids through the increased oxidation of lipids, thus treating hypertriglyceridaemia [582, 611]. The number of direct PPAR α -target

genes includes many that encode enzymes that are involved in glucose, lipid and amino acid metabolism [612, 613].

b) PPAR β/δ

This isotype is ubiquitously expressed and is implicated in diverse processes ranging from the regulation of energy homeostasis, thermogenesis, to keratinocyte proliferation and differentiation during wound healing (Figure 5) [595, 614]. As with PPAR α , polyunsaturated fatty acids have a relatively high affinity for PPAR β/δ ; and a number of eicosanoids were shown to activate PPAR β/δ [615]. Activation of PPAR β/δ increases serum high-density lipoprotein cholesterol levels in rats, non-human primates and humans [616-618]. This is probably mediated by PPAR β/δ -dependent expression of the reverse cholesterol transporter ATP-binding cassette A1 and by increased apolipoprotein A1-specific cholesterol efflux [617]. Ligand activation of PPAR β/δ can also decrease serum triglycerides, prevent high-fat diet-induced

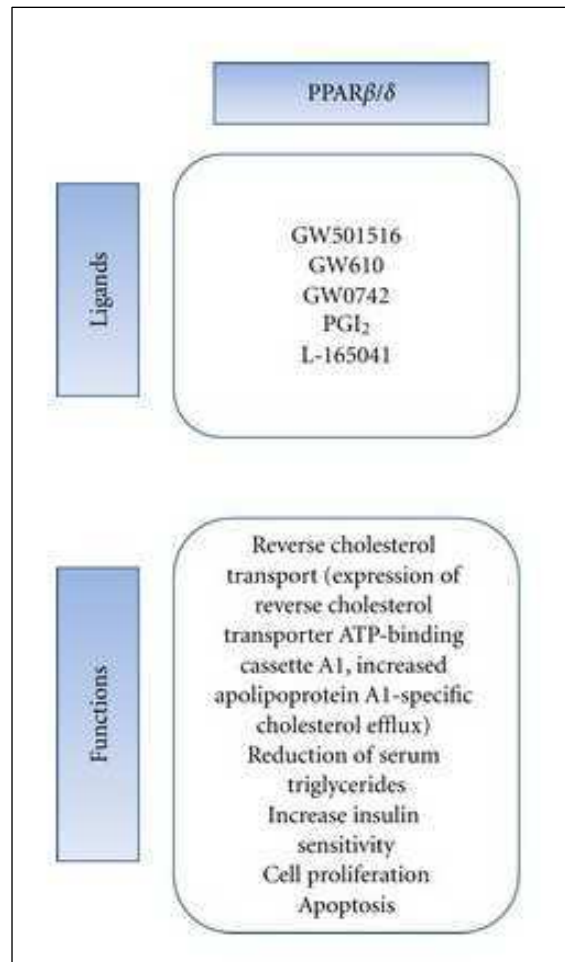


Figure 5. Summary of ligands and functions of PPAR β/δ . Updated from [15].

obesity, increase insulin sensitivity and improve symptoms that are associated with metabolic syndrome through the regulation of genes encoding fatty acid-metabolizing enzymes in skeletal muscle and genes encoding lipogenic proteins in the liver [617, 619-621]. In fact, after selective deletion of PPAR β/δ in skeletal muscle, mice exhibit a switch in muscle fibre types toward a lower oxidative capacity that precedes the development of obesity and diabetes [622]. Furthermore, its multiple implications in lipid metabolism comprise preadipocyte clonal expansion [623] and lipoprotein homeostasis [617]. These metabolic effects have led to the proposal of PPAR β/δ ligands as potential drugs for treating

metabolic syndrome [624]. PPAR β/δ also inhibits hepatic inflammation that is caused by genetic, dietary and chemical stimuli in part by the trans-repression of NF- κ B-dependent signaling, which results in reduced expression of cytokines such as TNF- α , IL-1 β and IL-6 [625-629]. While various physiological functions of PPAR β/δ are still being investigated, roles are already documented in embryo implantation [630], myelination in the brain [631] and osteoclastic bone resorption [632]. Activating PPAR β/δ can also promote terminal differentiation in keratinocytes, intestinal epithelium, oligodendrocytes and osteoblasts, and this function might have important consequences for tumor development [590, 633].

c) PPAR γ

The physiological effects of PPAR γ activation are primarily mediated by PPAR γ 1 and PPAR γ 2, which are derived from four different mRNAs (*PPARG1*, *PPARG2*, *PPARG3* and *PPARG4*) [574, 576]. Polyunsaturated fatty acids, fatty acid derivatives such as 15-deoxy- Δ -12,14-prostaglandin J2 (15d-PGJ2), 9-hydroxyoctadecadienoic acid (9-HODE), 13-HODE and nitrated fatty acids can activate PPAR γ and may be endogenous ligands (Figure 6) [580]. While PPAR α controls lipid catabolism and homeostasis in the liver, PPAR γ promotes the storage of lipids in adipose tissues. PPAR γ is abundantly expressed in adipocytes and plays a pivotal role in adipocyte differentiation [634]. Despite its relatively low expression levels in healthy liver, PPAR γ is critical for the development of hepatic steatosis [635]. The functions of PPAR γ extend far beyond fat storage and adipocyte differentiation. For instance, it has a recently

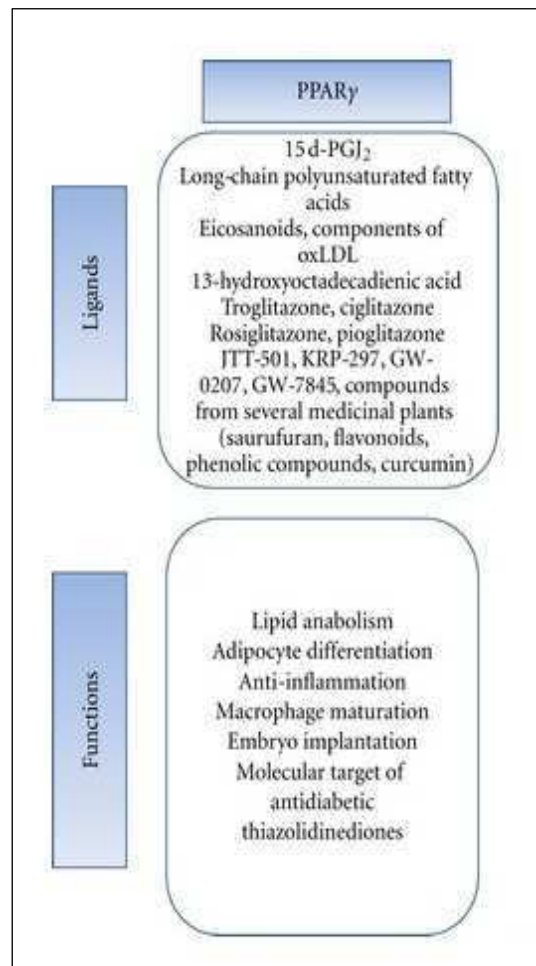


Figure 6. Summary of ligands and functions of PPAR γ . Updated from [15].

recognized role in promoting osteoclast differentiation and bone resorption when activated by rosiglitazone [636]. Its deletion in mouse osteoclast precursors leads to increased bone

mass and density and extramedullary haematopoiesis. This phenotype corresponds to the clinical syndrome called osteopetrosis, associated with impaired osteoclast differentiation from haematopoietic stem cells. Conversely, PPAR γ downregulates osteogenesis. In fact, it influences the competition between adipogenic and osteoblastic differentiation of bone marrow progenitors in favor of adipogenesis [637, 638]. Furthermore, PPAR γ is crucial for development, in particular of the placenta and heart [639] and all PPAR γ -null embryos die at an early developmental stage because of placental defects including impaired vascularisation [640].

Therapeutically, white adipose tissue is the primary target of the PPAR γ agonists, the thiazolidinediones, widely used for the treatment of type 2 diabetes. Thiazolidinediones decrease serum lipids by increasing adipogenesis and lipid storage, and which increase the expression of various adipokines, such as adiponectin and resistin, which collectively increase insulin sensitivity [641].

I-D.2. PPARs in Diseases

The great diversity of functions in which PPARs are implicated parallels the large panel of ligands that can be accommodated in the PPAR ligand-binding pocket and the broad tissue distribution of these receptors. In light of these observations, PPARs appear to be targets for the treatment of various diseases including metabolic disorders, autoimmune and inflammatory diseases, as well as carcinogenesis. For instance, important regulatory roles for PPARs subtypes have been reported in pathogenesis of cardiovascular diseases, metabolic syndrome, inflammatory bowel disease, pulmonary diseases, obesity and several cancer types. In fact, PPAR α and PPAR γ are already therapeutic targets for the treatment of hypertriglyceridaemia and insulin resistance, respectively. Evidence is now emerging that PPAR β/δ may also be a potential pharmacological target for the treatment of various metabolic disorders (Figure 7). Of particular importance to us, are the biological function and the therapeutic utility of PPARs in inflammatory states and in tumorigenesis. This particular focus does not aim to underestimate the importance of PPARs in other diseases such as metabolic syndrome, but rather to emphasize on “inflammation” and “carcinogenesis”, the main research themes of this work.

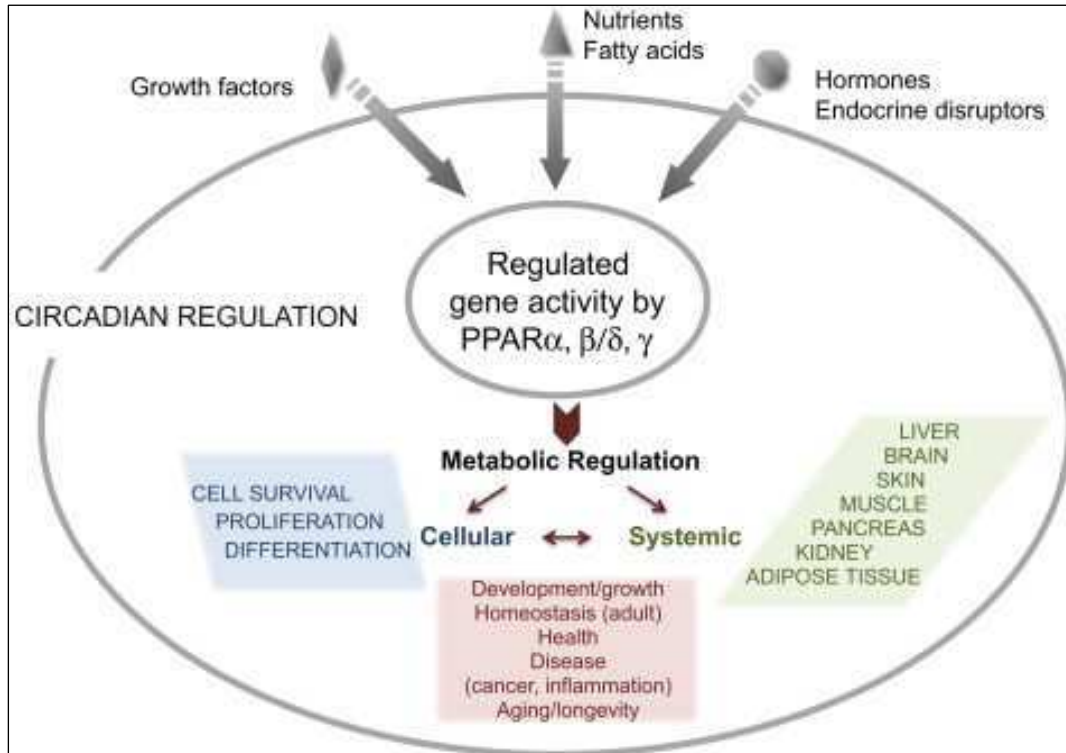


Figure 7. PPARs regulate cellular and systemic metabolic pathways. Energy homeostasis depends on a complex web of interconnected pathways forming a coherent ensemble of integrated processes. PPARs are key regulators that are activated by fatty acids and fatty acid derivatives and, alternatively, by endocrine disruptors (peroxisome proliferators). PPAR activity is also modulated by growth factors activating kinase signaling cascades [8].

a) PPARs-Mediated Immune Response in Inflammation

PPARs are expressed in dendritic cells, macrophages, and B and T lymphocytes, suggesting a role in immunity. They are also expressed in epithelial cells, which have an essential function in the mucosal immune response. For instance, an anti-inflammatory role for PPAR α and PPAR γ has been well documented in the literature while the role of PPAR β/δ remains less characterized. In fact, PPAR α is found in endothelial cells where it regulates the expression of leukocyte adhesion molecules. In mutant mice, PPAR α deficiency shifts the Th1/Th2 balance towards the pro-inflammatory Th1 phenotype [607]. In fact, upon ligand activation, PPAR α down-regulates diverse components of the pro-inflammatory response such as chemokines and cytokines by decreasing the expression of the Th1 transcription

factor T-bet (T-box expressed in T cells) and increasing the expression of GATA-3 (guanosine adenosine thymidine adenosine 3), known as a positive regulator of Th2 cytokines [642]. PPAR α agonists inhibit the transcriptional activity of NF- κ B, AP-1 and NFAT (nuclear factor of activated T-cells), which mediate the induction of genes responsible for the development of inflammation and cardiac hypertrophy [643, 644]. In chronic inflammation, ligand-bound PPAR α represses the production of pro-inflammatory IFN- γ and IL-17 by CD4 $^{+}$ T cells and PPAR γ ligands modulate the function of dendritic cells to elicit the development of anergic CD4 $^{+}$ T cells [2]. The PPAR α and γ ligands also repress the expression of cell adhesion molecules on endothelial cells and the secretion of chemokines by epithelial and other cells, decreasing the recruitment of leukocytes to the site of inflammation [2]. Interestingly, the increased severity of inflammatory diseases in PPAR-deficient mice suggests an anti-inflammatory role for either unliganded PPAR or PPAR activated by endogenous ligands [645, 646] (Figure 8).

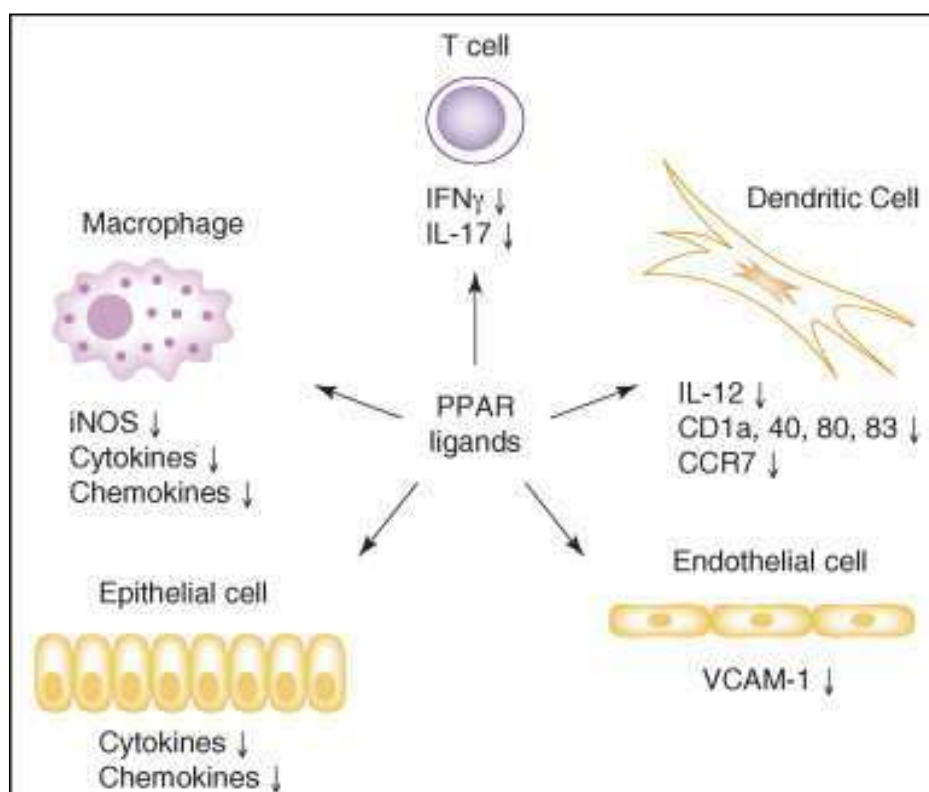


Figure 8. Cellular targets for anti-inflammatory actions of PPARs [2].

In line with these observations, many studies have confirmed a therapeutic activity of PPAR ligands in several rodent models of inflammatory and autoimmune diseases including autoimmune encephalomyelitis, multiple sclerosis, metabolic syndrome, and inflammatory bowel disease (Table 3) [647-649]. For instance, activation of PPAR γ in colonic epithelial cells was shown to protect against experimental colitis by decreasing inflammation severity and decreasing inflammatory cytokines production [650].

Table 3. Therapeutic activity of Ligands for PPAR γ and PPAR α in rodent models for human inflammatory and autoimmune diseases. GI262570, 2(S)-(2-Benzoylphenylamino)-3-[4-[2-(5-methyl-2-phenyloxazol-4-yl)ethoxy]phenyl]propionic acid; 15d-PGJ2, 15-deoxy-D12,14-prostaglandin J2; SB219994, 3-[4-[2-[N-(2-Benzoxazolyl)-N-methylamino]ethoxy]phenyl]-2(S)-(2,2,2-trifluoroethoxy)propionic acid; WY14643, 4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid. Updated from [2].

Rodent model	Ligands for PPARγ	Ligands for PPARα
Experimental autoimmune encephalomyelitis	Troglitazone, Pioglitazone	Gemfibrozil
	Ciglitazone, 15d-PGJ2	Fenofibrate
Inflammatory bowel disease	Troglitazone, Pioglitazone	WY14643
	Ciglitazone, 15d-PGJ2	Fenofibrate
Airway inflammation	Rosiglitazone, Pioglitazone	
	Ciglitazone, 15d-PGJ2 GI262570, SB219994	Fenofibrate
Arthritis	Rosiglitazone, Pioglitazone	Fenofibrate
Acute inflammation (Carrageenan-induced edema)	Rosiglitazone	Palmitoylethanolamine
		Fenofibrate
Pancreatitis (caerulein-induced)	Rosiglitazone	No data
Acute phase response (liver)	No data	Fenofibrate

The anti-inflammatory activity of PPAR ligands in mouse models encourages the evaluation of the possibility of PPARs as targets for the treatment of human inflammatory and autoimmune diseases. In fact, PPAR α agonists have already been suggested against cardiovascular inflammatory responses. Along the same line of thought, the PPAR α agonist fenofibrate is a potent anti-inflammatory drug used in the treatment of patients with rheumatoid arthritis [651]. When used as a lipid-lowering agent in patients with

atherosclerosis, fenofibrate induces a decrease in circulating TNF- α , IL-1 β and IFN- γ [652]. Finally, the PPAR γ agonists thiazolidinediones efficiently normalise skin homeostasis when orally administered to patients suffering from psoriasis, suggesting that their beneficial effects are most likely due to systemic anti-inflammatory functions of PPAR γ [653]. These reports underscore a promising therapeutic benefit of PPAR ligands in the treatment of inflammatory diseases.

b) PPARs and Cancer Development

Whether PPARs function as tumor suppressors or as oncogenes in cancer is still unclear. The complexity of the pathways that are regulated by PPARs, and the propensity of these pathways to be altered in cancer, offers some explanation for the disparate functions of PPARs in different tumor types. However, as targeting PPARs can improve the clinical consequences of metabolic disorders that are known to be associated with increased cancer risk (such as diabetes, obesity, dyslipidemias and chronic inflammation); modulating the activities of the PPARs is an attractive approach for the treatment and prevention of cancer. The challenge is to elucidate the molecular mechanisms of action of PPAR agonists in different tissues and tumor types, and to identify and characterize effective PPAR agonists that have acceptable safety profiles.

Concerning PPAR α , it has been shown that long-term administration of PPAR α agonists causes liver cancer in rodents [654], an effect that is dependent on PPAR α , as PPAR α -null mice are resistant to the hepatocarcinogenic effects of PPAR α agonists [655, 656]. The cascade of molecular events leading to liver cancer in rodents involves hepatocyte proliferation and oxidative stress, but the PPAR α target genes that mediate this response are unknown and, interestingly, this mechanism is not evident in humans [657, 658]. On the other hand, there are studies suggesting that activating PPAR α could be useful for the prevention or the treatment of different cancers. Oral administration of different PPAR α agonists inhibited the growth of tumors that were derived from melanoma, Lewis lung carcinoma, glioblastoma and fibrosarcoma cell lines [659], and xenografts from A549 human lung cancer cells [660]. PPAR α agonists also inhibited angiogenesis in these models [659, 660]. These inhibitory effects are mediated by the PPAR α dependent inhibition of

endothelial cell proliferation, and downregulation of cytochrome P450 CYP2C, an enzyme that catalyses the epoxidation of arachidonic acid to epoxyeicosatrienoic acids that promote angiogenesis [660]. As these effects are not evident in *PPAR α* -null mice, they are *PPAR α* -dependent, and thus *PPAR α* agonists could be used to prevent multiple tumor types [659, 660]. There are two other potential *PPAR α* -dependent pathways that could inhibit tumorigenesis. First, *PPAR α* inhibits inflammatory signaling through repressive mechanisms that are mediated by interacting with the p65 subunit of NF- κ B. Because inhibiting NF- κ B-dependent signals, such as TNF, can effectively inhibit the growth of multiple tumor types, targeting this *PPAR α* -dependent activity may be useful [661]. Second, *PPAR α* agonists also negatively influence the Warburg effect by interfering with metabolic pathways. Ligand activation of *PPAR α* can increase mitochondrial oxidation of fatty acids [662], and can inhibit expression of glutaminase [613], which decreases glutamine levels and limits cancer cell growth. As fatty acids and glutamine are enzymatically produced by the Warburg effect, and are substrates required for cell proliferation [663], targeting *PPAR α* to potentially inhibit tumor cell proliferation by interfering with the Warburg effect should be examined.

As for *PPAR β/δ* , there is no broad consensus on the role of *PPAR β/δ* in cancer, owing to contradictory studies in the literature [590]. However, two hypotheses have emerged: that *PPAR β/δ* is overexpressed in tumors and promotes anti-apoptotic activities and increased cell proliferation; and that *PPAR β/δ* promotes terminal differentiation and inhibits pro-inflammatory signaling, thereby attenuating tumorigenesis. Further studies in rodents and humans would better establish whether *PPAR β/δ* displays a cancer preventing or promoting role.

The function of *PPAR γ* in tumor development is also controversial but more convincing toward a preventive role (Figure 9). Indeed, most studies to date show that *PPAR γ* agonists can promote terminal differentiation, inhibit cell growth and increase apoptosis of human cancer cell lines, as well as inhibit tumorigenesis in animal models of cancer [664, 665]. Ligand activation of *PPAR γ* in cancer cell lines is associated with the induction of cell cycle arrest, the increased expression of mRNAs and proteins required for terminal differentiation, including keratins, carcinoembryonic antigen, E-cadherin, alkaline phosphatase and developmentally-regulated GTP-binding protein 1 (DRG1), as well as with

the changes to cell morphology that are consistent with a differentiated phenotype [509, 666-668]. PPAR γ agonists modulate the expression of different cell cycle regulators, including decreasing the expression of cyclin D1 [669-672], increasing the expression of the cyclin-dependent kinase inhibitors p21 [673] and p27 [673] and increasing the turnover of β -catenin [674]. PPAR γ agonists can also inhibit cell proliferation by inactivating eukaryotic initiation factor 2, which leads to the inhibition of translation initiation [675]. Increased apoptotic signaling is another mechanism that mediates the growth inhibitory effects of PPAR γ agonists. PPAR γ agonists can increase the expression of pro-apoptotic BAX and BAD [676, 677], inhibit BCL-X_L and BCL-2 function [676, 678], increase the expression of PTEN [518], inhibit PI3K activity and AKT phosphorylation [679], and inhibit activation of JUN N-terminal protein kinase [676]. Many of these changes increase caspase activity and apoptosis.

Chronic inflammation that is associated with many cancers, including colorectal, liver and lung cancer, is typically associated with increased NF- κ B activity and is causally linked with tumor promotion [680]. PPAR γ agonists can inhibit the production of pro-inflammatory signaling proteins, such as TNF, IL-6 and monocyte chemoattractant protein (MCP)-1, and these changes are mediated through trans-repression mechanisms, including directly interfering with NF- κ B activity and/or through receptor sumoylation. PPAR γ is expressed in tumor cells and infiltrating immune cells, and there is evidence that anti-inflammatory activities are mediated by PPAR γ in many cell types [681]. Indeed, PPAR γ expressed in intestinal epithelial cells [646] and macrophages [645] inhibits inflammation that is associated with experimentally induced colitis, and inflammation is known to be required for colon carcinogenesis (Figure 9) [397].

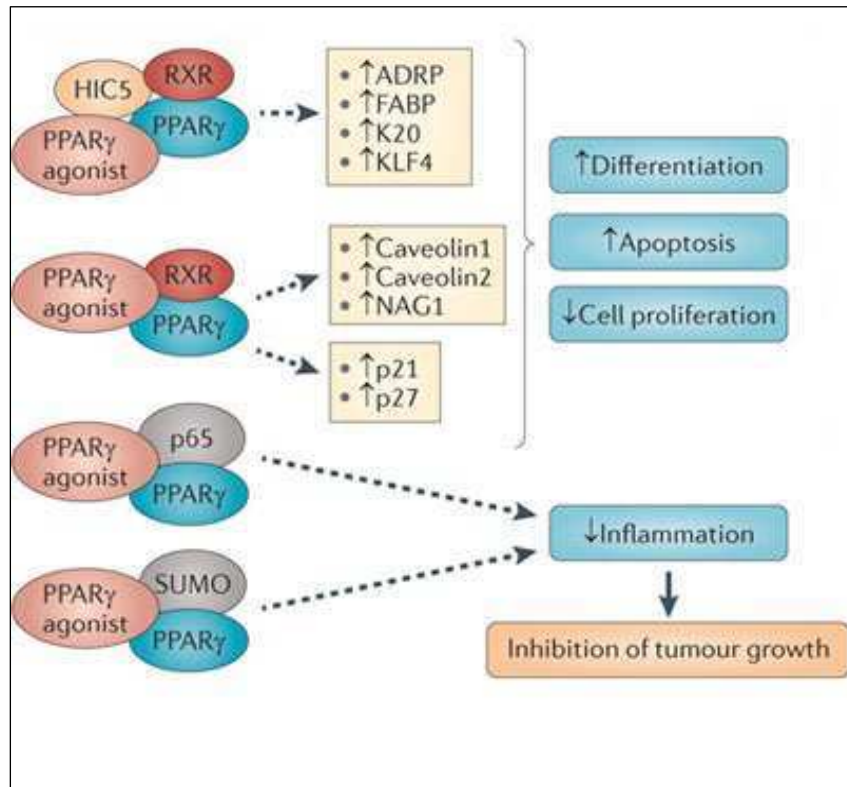


Figure 9. PPAR γ -mediated inhibition of tumor growth. Following ligand activation, HIC5 can act as a transcriptional co-activator of the receptor complex and can increase the expression of genes required for the induction of terminal differentiation. Ligand activation of PPAR γ can also cause increased expression of caveolin, non-steroidal anti-inflammatory drug activated gene 1 (NAG1), p21 and p27 through an undefined mechanism that requires PPAR γ . Ligand activation of PPAR γ can attenuate inflammation by interfering with nuclear NF- κ B signaling and through a SUMO-dependent mechanism. ADRP, adipose differentiation related protein; FABP, fatty acid binding protein; K20, keratin 20; KLF4, Kruppel-like factor 4. Updated from [22].

Clinically, there are many published studies showing that activating PPAR γ prevents cancer in tissues such as colon, breast, prostate and lung [664]. Increased differentiation of liposarcoma cells was observed in patients treated with troglitazone [682], and another clinical trial indicated that treatment with rosiglitazone increased the mean time to progression (which was defined as a doubling in tumour volume) [683]. However, no effect of rosiglitazone treatment was found in a larger cohort of patients with prostate cancer [684]. Troglitazone has been tested in patients with prostate cancer with variable results on

prostate-specific antigen levels [685, 686], and administration of LY293111 to patients with prostate cancer had no clinical effect [687]. In two Phase II studies, troglitazone had no effect in patients with either breast cancer or colorectal cancer [688, 689]. Some clinical trials examining the effect of PPAR γ ligands combined with other therapeutics revealed positive results for patients with thyroid carcinoma and glioma [690-692]. Thus, the clinical trials carried out to date have yielded evidence suggesting that PPAR γ might be suitable for targeting pre-cancerous and cancer cells in selected tumor types. Despite this evidence suggesting that activating PPAR γ inhibits tumorigenesis, doubts persist because some studies indicate that activating PPAR γ promotes carcinogenesis [693, 694]. Indeed, increased bladder cancer incidence is reported to be associated with the clinical use of rosiglitazone or pioglitazone, although there is evidence that this might reflect off-target effects of these PPAR γ agonists [695, 696]. No definitive mechanisms have yet been elucidated that explain these pro-carcinogenic effects.

Overall, beside their well established therapeutic role in hypertriglyceridaemia and insulin resistance, it has been clear that PPAR targeting, specifically PPAR α and PPAR γ , would be clinically significant in the therapeutic management of inflammatory and cancerous diseases. Therefore, the development of valid tools and animal models that contribute to the pharmacological profiling of drugs targeting PPARs is necessary. Hence, the importance and the utility of PPRE-luc mice. In PPRE-luc mice the expression of the *luciferase* gene is under the control of PPARs-inducible promoter in all target organs. This will allow for the evaluation and the modulation of PPARs activity in any PPARs-expressing organ in living animals under normal and pathological states. These mice represent therefore a valuable biological tool for pharmacodynamic and toxicological studies for novel PPARs ligands (discussed in details in section-III (Reporter mice for PPARs: A Novel Reporter Mouse Technology))

II- The Role of Peroxisome Proliferator-Activated Receptors in the Skin

II-A. The development and maturation of the skin

During epidermal maturation in the latest stages of fetal development, the epidermis evolves from a single layer of epithelial cells to a fully stratified and differentiated epithelium. After birth, the epidermis is renewed continuously. The basal layer contains undifferentiated keratinocytes, which undergo a vectorial differentiation program as they migrate from the basal to the uppermost layer. Keratinocyte differentiation includes the sequential expression of structural proteins (keratins, involucrin, loricrin and filaggrin), the processing and reorganization of lipids and, cell death [697]. The outermost layer of the epidermis, the stratum corneum, is the end product of keratinocyte differentiation and consists of a layer of cross-linked proteins and lipids, which acts as an efficient barrier to water loss, mechanical damage, microorganism invasion, and xenobiotic poisoning (Figure 11). The epidermal homeostasis and the integrity of the barrier function depend on the tight coordination of keratinocyte proliferation, differentiation, and programmed death.

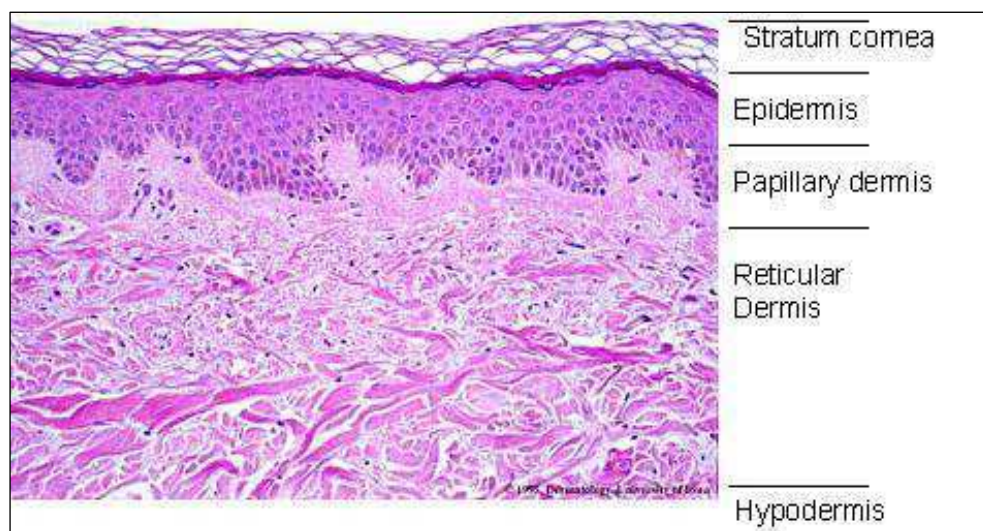


Figure 11. Histological image of skin layers (www.melanoma.com)

II-B. PPARs in Skin development and homeostasis

II-B.1. PPARs in epidermal development and differentiation

The expression of PPARs is associated with all major events of the fetal maturation of the murine epidermis, including the expression of differentiation markers, and changes in

lipid metabolism. After birth, the three PPARs progressively disappear from the interfollicular epidermis. In the adult animal, they are undetectable (in situ hybridization) in the interfollicular keratinocytes, while all three remain expressed in the hair follicles [609]. In contrast to the rodent situation, all three PPARs are expressed in the basal and suprabasal layers of the human adult epidermis [698, 699].

The functions of PPARs in keratinocyte proliferation, differentiation and death, and in epidermal barrier maturation were studied in these various cell cultures and animal models (Figure 12). More specifically, PPAR β/δ seems to play an important role in the differentiation of epithelial cells [698, 700]. Treatment with PPAR β agonists was reported to decrease the proliferation and to stimulate the differentiation of wild-type mouse primary keratinocytes; whereas these effects were not seen in PPAR β -null cells [701]. Moreover, administration of L165041 (PPAR β/δ ligand), results in a dramatic decrease in proliferation of human keratinocytes and a robust upregulation of expression of involucrin and transglutaminase, markers of melanocytes [699]. Similarly, in cultured human keratinocytes, treatment with PPAR α or PPAR γ ligands increases the expression of involucrin and transglutaminase, which are essential for formation of the stratum corneum [702, 703]. Also, *Kang et al.* have shown that PPAR α (WY14643) and PPAR γ (ciglitazone) activators inhibit human melanocyte growth and stimulate melanogenesis [704]. The effect of PPARs ligands on skin development and maturation has been also examined *in vivo*. For instance, the selective PPAR β agonist GW1514 stimulates mouse epidermal differentiation *in vivo* without affecting cell proliferation, as shown by an increased expression of the late differentiation markers filaggrin and loricrin [705]. PPAR α ligands also inhibit proliferation and induce differentiation of keratinocytes in the adult mouse epidermis *in vivo* [706, 707]. Topical treatments with the PPAR γ agonists troglitazone and ciglitazone increases as well the expression of differentiation markers and promote epidermal barrier recovery in hairless mice [708]. Although thiazolidinediones are known to have PPAR γ -independent activities, their effects on keratinocyte differentiation are PPAR γ -dependent in this model, as evidenced by the absence of effect in PPAR γ epidermal-null mice [708]. These data provide strong evidence that PPARs isoforms are involved in keratinocytes differentiation with differential effects according to the level and time of expression of PPARs isotypes in the skin. During differentiation of human keratinocytes *ex vivo*, the level of PPAR β/δ

expression remains high, whereas expression of PPAR α and PPAR γ increases significantly upon differentiation [698, 699, 709]. Interestingly, simultaneous addition of the PPAR β/δ -selective ligand L165041 and the PPAR γ -selective ligand BRL49653 resulted in a strong synergistic induction of involucrin [699] and had a synergistic effect on human keratinocyte differentiation [702].

On the other hand, several studies have reported important roles for PPARs in epidermal lipid metabolism and epidermal permeability barrier. For instance, the PPAR α ligand WY14643 increases the synthesis of ceramides and cholesterol derivatives in a skin equivalent model [710]. Consistent with these results, prolonged exposure to PPAR α (WY14643) or PPAR β (GW1514) agonists induces lipid accumulation in cultures of human keratinocytes [705]. Moreover, PPAR β activation by GW1514 induces the expression of the adipose differentiation related protein (ADRP) and of the fasting-induced adipose factor (FIAF), both of which play important roles in lipid metabolism [705]. Furthermore, PPAR α activators have been shown to greatly enhance (in skin equivalents) synthesis of membrane-coating granules, which are secreted into the extracellular space and constitute the structural components of the epidermal permeability barrier [710, 711]. Consistently, topical treatment of mice with GW1514 accelerates the restoration of the epidermal barrier functions following its disruption by tape stripping, solvent or detergent application [705].

Information on the role of PPARs in epidermal homeostasis also emerged from the study of PPAR-mutant mouse models. Although normal skin architecture is observed in adult PPAR α null mice [712], delayed fetal epidermis development, with defects in the formation of the stratum corneum, occurs in these mutant mice [713]. Furthermore, a detailed analysis of adult PPAR α -null epidermis reveals a thin stratum granulosum with focal parakeratosis, suggesting impaired keratinocyte differentiation [706]. Recently, overexpression of PPAR α in transgenic mice showed that PPAR α plays an important role in the development of several epithelia, including the epidermis [714]. The consequences of PPAR α overexpression during embryonic development are a thinner epidermis and fewer hair follicles than normal in perinatal pups, but there is no abnormality when PPAR α is overexpressed in the adult mouse skin. Primary keratinocytes isolated from these animals

exhibit slow proliferation and increased differentiation. Thus, PPAR α might be important for the development of the epidermis during late embryogenesis, but dispensable for renewal of the epidermis in the adult animal. No defect in epidermal maturation is observed in PPAR γ -heterozygous mice or in PPAR γ -null mice born after placental rescue [639, 715]. Consistent with these observations, mice chimeric for PPAR γ -null and -wild type cells show that the PPAR γ -null cells participate in epidermal differentiation, demonstrating that PPAR γ is most probably not required for this process [716]. Histological analysis of PPAR β -mutant skin does not reveal any defect in epidermal architecture, neither during fetal development nor in the adult animal. Accordingly, the expression patterns of keratinocyte differentiation markers are not altered in PPAR β -null animals [715]. However, epidermal hyperplasia in response to a treatment with tetradecanoylphorbol acetate (TPA) is enhanced in PPAR β -mutant animals, suggesting a role for PPAR β in the control of keratinocyte proliferation and differentiation [631, 715]. The slight increase in keratinocyte proliferation in the PPAR β -heterozygous animals also indicates the existence of such a control [715]. In agreement with these observations, PPAR β expression is rapidly upregulated following hair plucking or cutaneous injury, and skin wound-healing is altered in PPAR β -heterozygous mice. The skin wound-healing delay is largely due to a disrupted balance between proliferation and apoptosis [717, 718], as well as to defects in cell adhesion and migration of the PPAR β -mutant keratinocytes [719].

Altogether, these data suggest that the three PPAR isotypes are involved, through divergent ways and timings, in the induction or control of proliferation and differentiation of skin cells as well as in the enhancement of epidermal permeability barrier (Figure 12).

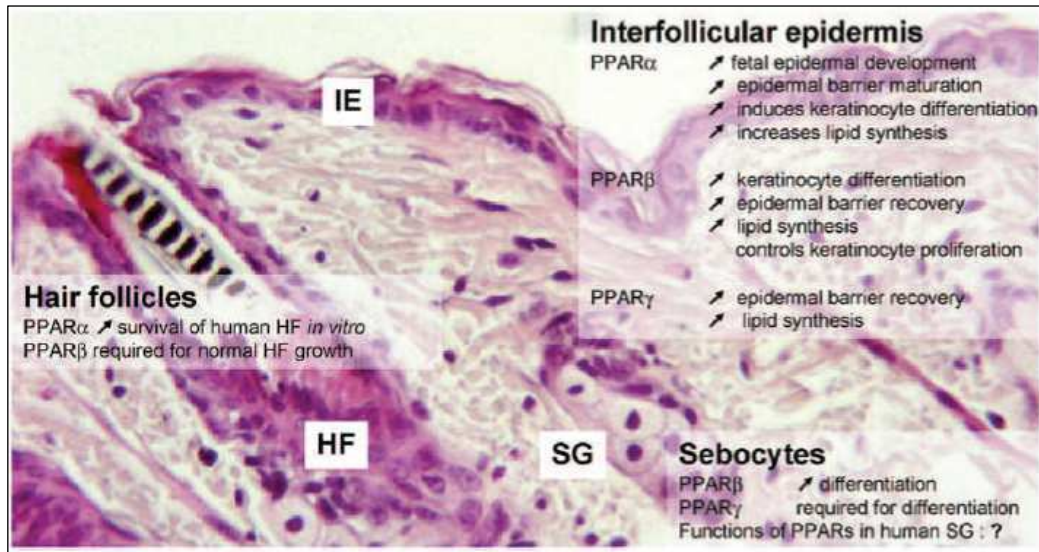


Figure 12. Consequences of PPAR activation in hair follicles, sebocytes and interfollicular epidermis. The hematoxylin/eosin staining of a mouse skin section shows the normal interfollicular epidermis (IE), a hair follicle (HF) and a sebaceous gland (SG). The figure summarizes the data obtained after activation of PPARs by their specific ligands in each of these skin compartments [7].

II-B.2. PPARs in the differentiation of skin appendages

During embryonic development, the skin epithelial progenitor cells give rise to the epidermis and its appendages, namely hair follicles and associated sebaceous glands. Hair follicle morphogenesis depends on complex bidirectional interactions between the keratinocytes and the underlying mesenchyme, and on a tightly controlled balance between keratinocyte proliferation and apoptosis. In rodents, the three PPARs are expressed in the hair follicles during embryonic skin development, and they remain highly expressed in postnatal and adult hair follicles. In humans, all PPARs isoforms are expressed in both dermal and epithelial human hair follicle cells [720]. Interestingly, the PPAR α agonist clofibrate has been shown to increase the survival of human hair follicles *in vitro* [720]. Moreover, it has demonstrated PPAR β activation protects hair peg keratinocytes against apoptosis, which allows normal hair follicle development in mice [721].

The lipid-loaded sebocyte is the last differentiated cell type to appear in the skin during its development. Sebocytes initially arise from cells at the surface of hair follicles, and eventually form a gland located at the periphery of the mature hair follicle, but remain associated with it. Sebaceous lipogenesis leads to the accumulation of lipids and to sebum secretion by the sebocytes, which represents a major step in their differentiation [722]. In brief, the three PPARs are expressed in adult rat sebocytes and in human sebaceous gland cell lines [700, 723]. PPAR γ is also present in mouse and human skin sebaceous glands *in vivo* [716, 724]. The activation of PPARs by their respective ligands or by the PPAR pan-agonist linoleic acid stimulates lipid droplet accumulation in primary rat sebocytes, but not in keratinocytes [722, 723]. Interestingly, PPAR β appears to be important in the late stages of rat sebocyte differentiation [700], whereas, and in contrast, it is involved in the early phase of adipocyte differentiation [725]. Thus, PPAR β may play different roles in two differentiation programs that involve lipogenesis in two different cell types (Figure 12).

So far, the role of PPARs in human sebocytes and sebaceous glands is unclear, due to several contradictory results obtained using various cellular experimental models or assays involving human volunteers. The activation of the three PPARs stimulates lipogenesis in a human sebocyte cell line [726], while treatment of whole human sebaceous gland cultures with PPAR α or PPAR γ ligands inhibits sebaceous gland lipogenesis [727]. This suggests that the activation of PPARs in primary sebocytes or in whole sebaceous glands may have different effects with regard to lipogenesis. In human volunteers too, the observations are contradictory, with reduced [728] or increased [726] sebum production after treatment with the PPAR α agonist ETYA, and with other PPAR α and γ agonists, respectively.

Despite the importance of PPARs in lipid metabolism and their high expression in sebocytes, their function has not been examined so far in sebaceous glands of either PPAR α or PPAR β -mutant mouse models. Importantly, mice chimeric for PPAR γ -null and wild-type cells showed little or no contribution of PPAR γ -null cells to the formation of adipose tissue and sebaceous glands [716]. Consistent with the well-known function of PPAR γ in lipid metabolism, this observation suggests that PPAR γ -null cells cannot differentiate into adipocytes or sebocytes.

Together, these observations definitively implicate PPARs in sebaceous gland differentiation and function, but clear specific roles cannot yet be attributed due to contradictory reports, with the exception that PPAR γ is definitively required for the sebocyte differentiation process (Figure 12).

II-C. PPARs in Skin Disease

As described above, PPARs play important roles in healthy skin homeostasis, in the epidermis, the hair follicles, and the sebaceous glands. Therefore, PPARs may be interesting targets for the treatment of various epidermal disorders, such as inflammatory hyperproliferative disorders and skin malignancies.

II-C.1. Inflammatory Hyperproliferative Disorders - Psoriasis

Psoriasis is an inflammatory and hyperproliferative skin disease characterized by keratinocyte proliferation and differentiation and cutaneous accumulation of inflammatory polymorphonuclear leukocytes capable of release of toxic oxygen and free radical species. Knowing that psoriasis is characterized by epidermal hyperproliferation and abnormal differentiation of keratinocytes accompanied by an inflammatory response, PPARs may be interesting targets for treatment. PPAR β expression was reported to be dramatically increased in the hyperproliferative lesional skin of psoriatic patients [709, 729]. PPAR β gene expression is known to be upregulated in mouse skin in response to inflammatory cytokines [717], suggesting that the increased expression in psoriatic lesions is most probably due to pro-inflammatory signals, a condition reminiscent of that following skin injury. The consequences of PPAR β activation in this pathological situation are unclear, the activation of PPAR β/δ in the epidermis was shown to be sufficient to trigger inflammatory changes, immune activation and gene dysregulation characteristic of psoriasis [717, 730]. These observations dampened the interest of PPAR β/δ agonist in the treatment of psoriasis.

Unlike PPAR β , cutaneous levels of PPAR α and PPAR γ are decreased in lesional psoriatic skin [709, 729], and there is no correlation between sequence variations in the genes encoding PPAR α and PPAR γ and psoriasis [731]. Nevertheless, the two PPAR α

ligands, clofibrate and WY14643, are able to restore epidermal homeostasis in acute and chronic mouse models of hyperproliferative epidermis [706], and to reduce inflammation in models of irritant and allergic dermatitis [732]. Consistently, transgenic mice overexpressing PPAR α in the epidermis show reduced hyperplasia upon topical application of tetradecanoylphorbol acetate (TPA), suggesting that PPAR α prevents hyperproliferation of keratinocytes in adult mouse skin [714]. Furthermore, the PPAR γ ligand troglitazone normalizes the morphology of psoriatic skin in organ culture, and reduces epidermal hyperplasia in a model of human psoriatic skin transplant [733]. *In vivo*, topical application of the PPAR γ agonists ciglitazone and troglitazone improves epidermal homeostasis in murine models of keratinocyte hyperproliferation [734].

However, in a pilot study with psoriatic patients and despite the above-mentioned promising animal studies, PPAR α (clofibrate), PPAR β (tetradecylthioacetic acid), and PPAR γ (rosiglitazone) agonists did not normalize skin homeostasis when topically applied on plaque psoriasis [735, 736]. Interestingly, several reports have described a therapeutic benefit of the PPAR γ agonists, thiazolidinediones, when orally administered to patients suffering from plaque psoriasis [737]. These observations indicate that systemic, but not local activation of PPAR γ is beneficial, maybe due to the general anti-inflammatory functions of PPAR γ and its possible impact on the immune system. The exact mechanism of this is still unknown since *Mao et al.* showed that PPAR γ -activated keratinocyte differentiation and decreased cutaneous inflammation by TZDs is not dependent on PPAR γ in keratinocytes [708]. Although all PPAR isotypes act to various degrees on the pathogenic factors for psoriasis, it is not presently possible to distinguish an isotype that would be most useful in the treatment of psoriasis.

II-C.2. Atopic Dermatitis

It was long thought that atopic dermatitis (AD) is solely attributable to immunological defects. Evidence is accumulating that primary keratinocyte abnormalities may underlie the pathogenesis of this skin disorder in many patients. It has been shown that PPARs reduce certain inflammatory mediators in the skin and regulate epidermal barrier homeostasis, alterations of which contribute to the inflammation associated with AD.

Studies have demonstrated that PPAR ligands inhibit T helper cell (Th) responses in terms of inhibition of IL-2 production by T cell clones, while not inhibiting proliferation of such clones [738]. Specifically, PPAR γ play a critical role in the regulation of genes that are involved in cellular proliferation, specific components of the Th2 inflammatory pathway and maintenance of the skin barrier [736]. This suggestion was supported by the observation that the PPAR γ ligand ciglitazone inhibits allergic immune response by inhibiting Th2-driven IgE production and also production of pro-inflammatory cytokines of the Th response *in vitro* and *in vivo* [739]. Recent analysis of skin lesions in patients with AD show increased PPAR γ expression not only in keratinocytes, but also in infiltrating T cells and monocytes [740]. The same group demonstrated that systemic ciglitazone administration inhibits not only systemic but also local inflammatory immune response in the skin by diminishing the severity of allergen-induced dermatitis in mice [741]. Systemic treatment with the PPAR γ agonist rosiglitazone, another member of the TZDs, lead to a decreased total body surface area involvement, severity of lesions and number of flares in patients with severe AD [742]. Beside this, topical treatment with a PPAR α agonist had a beneficial effect for childhood atopic dermatitis allowing to spare dermo-corticoids and improving the quality of life [743].

II-C.3. Skin Injury

Skin repair after an injury is a life-saving priority process that eventually leads to the covering of the wounded area with a newly differentiated protective epidermis. The initial inflammatory stage of repair is followed by the proliferation and migration of keratinocytes, a process called re-epithelialization. In parallel, dermal repair involves the recruitment and proliferation of fibroblasts and angiogenesis. As mentioned previously, PPAR α and PPAR β expression is undetectable in the interfollicular epidermis of healthy rodent skin. The expression of both of them is reactivated in keratinocytes at the edges of skin wounds, although with specific timing [715]. The upregulation of PPAR α is transient and parallels the inflammatory phase, while that of PPAR β lasts over the entire healing process with downregulated expression upon remodeling. The expression of PPAR β is increased via binding of the AP-1 transcription factor complex to its promoter, triggered by the activation of the stress associated protein kinase pathway by pro-inflammatory cytokines, such as TNF- α [717]. In parallel, the release of pro-inflammatory cytokines also induces the production of

an endogenous ligand for PPAR β , resulting in maximal PPAR β activation in the wounded epithelium [717]. During this process, PPAR β directly stimulates the genes coding for integrin-linked kinase (ILK) and 3-phosphoinositide-dependent kinase-1 (PDK1), with strong activation of the Protein kinase B alpha/protein kinase B (PKB α /Akt1) anti-apoptotic pathway as a consequence [719]. Once epithelialization is completed, TNF- α -induced PPAR β expression is repressed by TGF β -1 signaling, which inhibits AP-1 binding to the PPAR β promoter (Figure 13) [744].

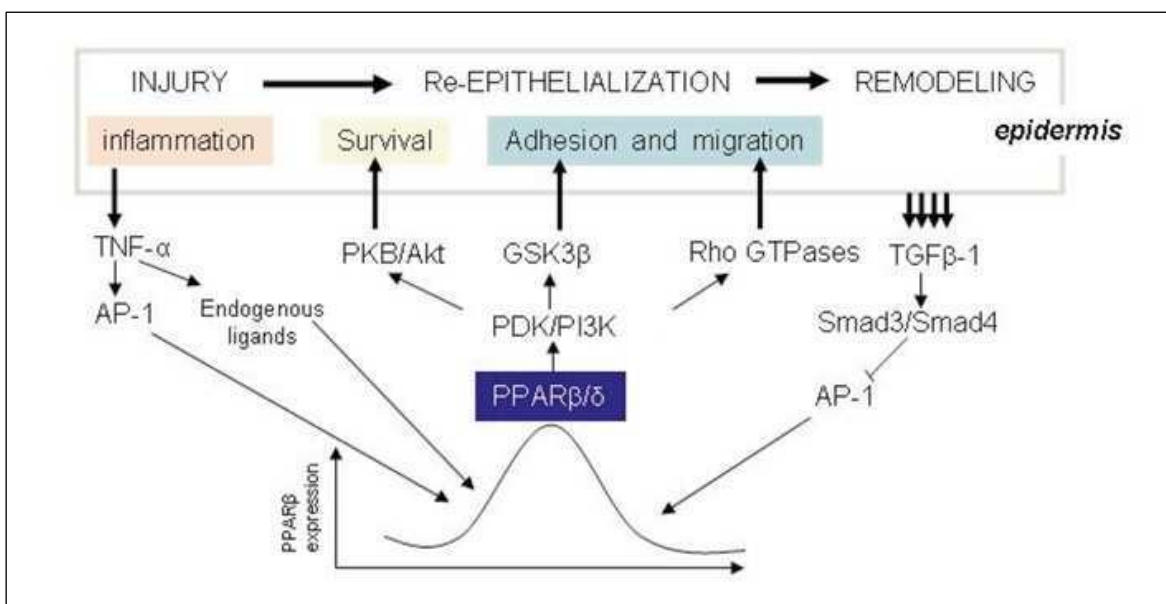


Figure 13. Roles of PPAR β/δ in skin wound healing The expression of the PPAR β gene is increased via binding of the AP-1 transcription factor complex to its promoter, which is triggered by activation of the stress-associated protein kinase pathway by pro-inflammatory cytokines such as TNF- α . In parallel, the release of pro-inflammatory cytokines also induces the production of PPAR β ligands in the wounded epithelium. The activated PPAR β protein stimulates the genes coding for integrin-linked kinase (ILK) and 3-phosphoinositide-dependent kinase-1 (PDK1), which results in activation of the PKB α /Akt1 anti-apoptotic pathway. Once epithelialisation is completed, TNF- α -induced PPAR β expression is repressed by TGF β -1 signaling which inhibits AP-1 binding to the PPAR β promoter. Updated from [10].

An important role for TGF β -1 and IL-1 were reported during the process of skin repair following injury via PPAR β . It has been shown that prolonged expression of PPAR β , obtained through down-regulation of TGF β -1 activity (Smad3-null mice) [745], or through early treatment of the wounds with recombinant TGF β -1 (at the time of injury), is paralleled with a prolonged increase of PKB α /Akt1 activity and accelerated skin wound closure [746]. Conversely, exogenous application of TGF β -1 on a skin wound at day 2 following the injury has opposite effects, with PPAR β expression and PKB α /Akt1 activity prematurely downregulated, and a transient delay in wound closure [746]. These data suggest that the described paradoxical actions of exogenous TGF- β 1 on wound healing most likely reside in its dual role. As a chemoattractant of immune cells, it stimulates PPAR β / δ expression via the production of inflammatory cytokines. As a repressor, it reduces the inflammation-induced PPAR β / δ expression via Smad3 signaling.

On the other hand, it has been shown that keratinocyte proliferation following wounding is fine tuned by a PPAR β -dependent interaction between epidermal keratinocytes and dermal fibroblasts. It has been demonstrated that IL-1 produced by keratinocytes stimulates the activity of the AP-1 (Jun/Fos) transcription complex in dermal fibroblasts, resulting in an increased production of mitogenic cytokines that enhance keratinocyte proliferation (Figure 14). In parallel, increased levels of PPAR β in fibroblasts stimulate the production of the secreted IL-1 receptor antagonist (sIL-1ra), which results in an autocrine down-regulation of the IL-1 signaling pathway. As a consequence, there is reduced production of secreted mitogenic factors by the IL-1-stimulated fibroblasts and thus reduced keratinocyte proliferation. Together, these findings show that the regulation of PPAR β by IL-1 in fibroblasts contributes to the homeostatic control of keratinocyte proliferation.

In summary, PPAR α and PPAR β both play important nonredundant functions in the wounded epidermis, with isotype-specific timing. PPAR α favors skin healing via modulation of the inflammatory phase, while PPAR β is an important player of keratinocyte survival and migration.

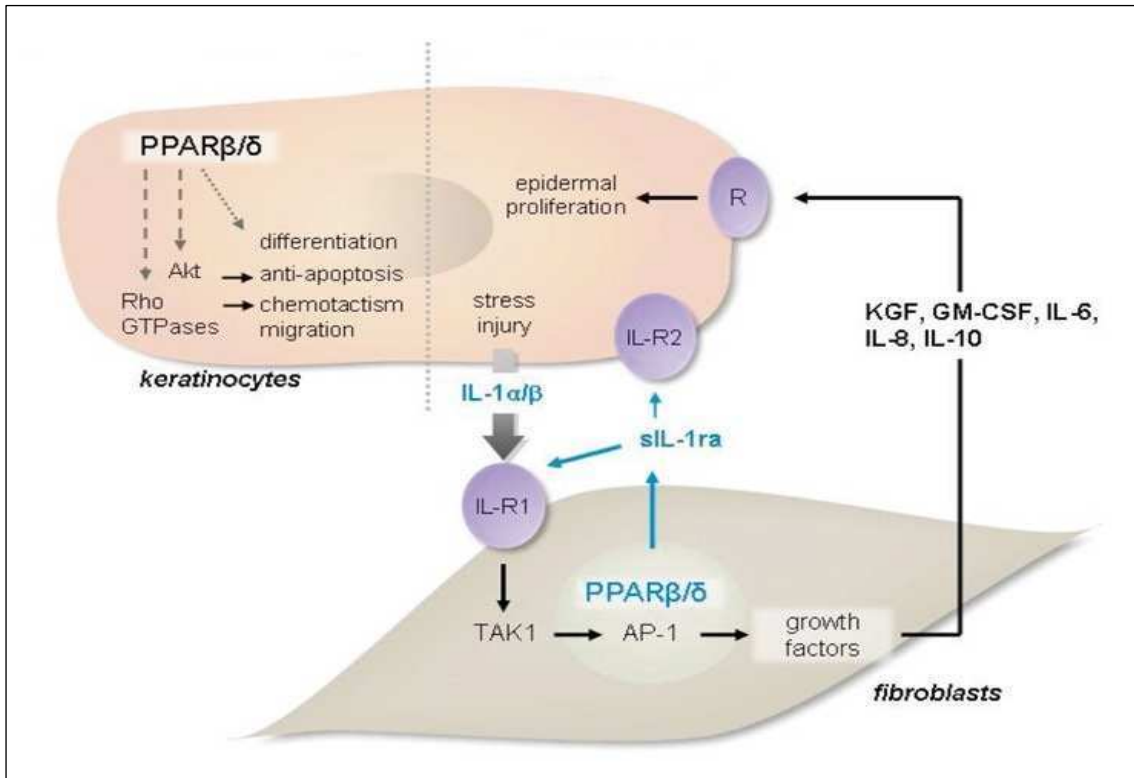


Figure 14. Keratinocytes proliferation is regulated via a paracrine mechanism involving IL-1. IL-1, produced by keratinocytes, activates c-Jun in fibroblasts leading to stimulation of the expression of mitogenic factors. In fibroblasts, PPAR β/δ attenuates IL-1 signaling via the production of sIL-1ra. The sIL-1ra has little affinity for IL-1R2, expressed in keratinocytes. But, it binds with high affinity to IL-1R1 expressed by the fibroblasts. The binding of sIL-1ra to IL-1R1 down-regulates IL-1-mediated signaling events. Consequently, the production of mitogenic factors is down-regulated, leading to reduced keratinocyte proliferation. Updated from [10].

II-C.4. Inflammation

It is well recognized that PPAR expression is modulated by inflammation both in the epidermis and in other organs, such as the liver, heart, and adipose tissue. In the skin, anti-inflammatory activities have been attributed to PPARs. *Kippenberger et al.* showed that topically applied PPAR α agonists increased the minimal erythema dose in ultraviolet B (UV-B)-irradiated skin [747]. Topical or systemic application of PPAR α activators or compounds that positively regulate PPAR α gene expression may therefore help to counteract exaggerated inflammatory processes. In fact, UV-B irradiation led to a decrease of all three

peroxisome proliferator-activated receptor subsets at the mRNA level, which may be consistent to an exaggerated and prolonged inflammation. Moreover, it has been suggested that PPAR α mediates the anti-allergic and anti-inflammatory effects of palmitoylethanolamide, a natural fatty acid ethanolamide found in mouse skin [748]. Likewise, in a model of TPA-induced irritant contact dermatitis and in an oxalozone-induced allergic contact dermatitis, PPAR α agonists reduced ear swelling, the magnitude of the inflammatory infiltrate, and the expression of TNF- α and IL-1 β [732]. Moreover, PPAR α activation inhibited cytokine secretion, maturation, and migration and the T-cell-stimulatory activity of the epidermal antigen-presenting cell, the Langerhans cell. This was associated with decreased levels of NF- κ B [749]. The effects of PPAR α agonists were abolished in keratinocytes and Langerhans cells in PPAR α -deficient mice, indicating that they are receptor-mediated [732, 748, 749].

In contrast to PPAR α , the effects of PPAR β/δ activators on cutaneous inflammation are less certain. In the mouse model of TPA-induced irritant contact dermatitis, topical GW1514 (PPAR β/δ agonist) decreased the degree of inflammation, as determined by ear thickness, ear weight, histology, and myeloperoxidase quantification [701, 705]. However, the anti-inflammatory effect did not require PPAR β/δ , because pharmacologic PPAR β/δ activation also reduced myeloperoxidase in the ears of PPAR β/δ -deficient mice, raising the possibility that the observed effects on inflammation are not attributable to PPAR β/δ activation. Additionally, the anti-inflammatory effects required doses much higher than those necessary to induce keratinocyte differentiation [701]. On the other hand, PPAR β/δ - deficient mice displayed an increased inflammatory response to TPA treatment [631, 708], suggesting that PPAR β/δ in fact plays a role in downregulating inflammation. Clearly, further studies are required to determine whether activators of PPAR β/δ are anti-inflammatory in the skin.

Overall, PPAR γ can interfere with many components of the inflammatory response by altering expression of cytokines, receptors and adhesion molecules by T cells, monocyte/macrophages, vascular smooth muscle cells and endothelial cells [750]. Production by macrophages of pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, RANTES and MCP-1 is suppressed by PPAR γ agonists [751, 752]. Beside, studies of PPAR γ activators have

also failed to definitively demonstrate an anti-inflammatory effect in mouse skin. Although the PPAR γ agonists ciglitazone and troglitazone have anti-inflammatory properties in both irritant and allergic contact dermatitis mouse models, reducing the production of proinflammatory cytokines, such as TNF- α and IL-1 β [708], similar anti-inflammatory properties are seen in PPAR γ -deficient mice. These results indicate that the inhibition of cutaneous inflammation by ciglitazone and troglitazone is not necessarily mediated by epidermal PPAR γ . This observation is similar to reports in macrophages, in which glitazones also had anti-inflammatory effects independent of PPAR γ [753].

These data provide clear evidences that all PPARs isoforms are involved at different levels in the regulation of the inflammatory process.

II-C.5. Non-Melanoma Skin Cancer

As the first barrier between the body and the environment, the skin is exposed to various kinds of aggression. Among them, exposure to xenobiotics or UV may provoke damage, with subsequent proliferation of damaged keratinocytes and promotion of skin carcinogenesis. As discussed above, studies have shown that PPAR activation can regulate proliferation and differentiation of different cell types and therefore induce growth arrest and apoptosis in a variety of cancer types [664, 754]. In skin cancer, the role of PPAR β was explored using a model of 7,12-dimethylbenz[α]anthracene/tetradecanoylphorbol acetate (DMBA/TPA)-induced carcinogenesis. In this context, the development of tumors is more severe in PPAR β -null than in wild-type animals, suggesting that the activation of PPAR β may attenuate the development of chemically-induced skin cancers [755]. Further investigation using the same mouse model suggests that this anti-carcinogenic effect of PPAR β is due to the inhibition of keratinocyte proliferation, mainly via modulation of the Protein Kinase C α (PKC α) activity [756]. In human cell lines, UV treatment of keratinocytes induces the induction of PPAR γ -agonistic activity [757], but the consequences of an increased activity of PPAR γ in these cells remain unclear. Although reduced PPAR γ activity in PPAR γ +/- animals increases the susceptibility of these mice to DMBA-mediated carcinogenesis in the skin and other organs [758], the activation of PPAR γ does not prevent the development of UV- or DMBA/TPA-induced skin tumors [759]. As for PPAR α , its activation of reduces ultraviolet

(UV)-induced inflammation in human skin, which may have a beneficial effect with regard to UV-induced skin cancers [747]. Topical treatment of mouse skin with PPAR α activators also showed a protective effect against DMBA/TPA-induced carcinogenesis [760]. Moreover, and consistent with their overall anti-inflammatory function, PPAR γ and α activators may directly downregulate or inhibit cyclooxygenase-2 (COX-2) expression which is increased in cutaneous squamous cell carcinoma (SCC) [761, 762]. Moreover, the combination of PPAR agonists and COX inhibitors may have synergistic effects [763]. Also, PPAR γ and PPAR α would limit cancer progression by inhibiting angiogenesis [764].

Overall, these observations underscore an involvement of PPARs in skin carcinogenesis, but further work using different models is awaited, which should shed additional light on this rather important process given the prevalence of UV-induced skin cancer.

II-C.6. Melanoma

Over the past three decades the incidence of malignant melanoma has significantly risen worldwide as a consequence of excessive exposure to sunlight [765]. Despite advancements in early diagnosis and treatment, metastasized malignant melanoma has a very poor prognosis [766]. One reason could be that molecular mechanisms involved in skin repair, skin carcinogenesis and melanoma growth are still poorly understood. So far there are only little available therapies for metastatic melanoma. In addition to active prevention and early detection of melanomas, it appears necessary to develop new therapeutic substances to improve the outcome of patients with metastatic melanoma.

In addition to non-melanoma skin cancer, evidence is accumulating that PPAR ligands also had mild antiproliferative effects in melanocytes and human melanoma cells [704, 767, 768]. Whether this inhibition of cell proliferation is induced by apoptosis or through specific PPAR-dependent pathways is not clear. Correlating to the antiproliferative effects, the vitamin D receptor (VDR) expression was increased in the melanoma cell line MeWo by some PPAR ligands at the same time-point [769]. This gave an indication of an interconnection of the PPAR and VDR signaling pathways at the level of cross-regulation of

their respective transcription factor mRNA levels. The complete mechanisms and the physiological and pathophysiological relevance of this cross-talk are not yet known, but may open new perspectives for treatment and/or prevention of melanoma.

In addition to the observed antiproliferative effects, PPAR α activation by fenofibrate, inhibited metastatic potential of both B16F10 mouse melanoma cells and human SkMel188 cells *in vitro* caused by inhibition of migration and anchorage-independent growth [770]. In Bomirski hamsters with melanoma subcutaneous tumors oral administration of fenofibrate led to significantly fewer metastatic foci in the lungs [771]. However, primary tumor growth remained unaltered. In clinical trials significantly fewer melanoma were diagnosed in patients treated with lipid-lowering drugs (i.e., gemfibrozil) compared with the control group [772]. Nevertheless a recently published metaanalysis could not confirm this observation [773]. On the other hand, studies with thiazolidinediones (PPAR γ ligands) demonstrated inhibition of colony forming by arresting melanoma cells in the G1 phase, leading to inhibition of tumorigenesis in nude mice [774]. Especially for ciglitazone the antiproliferative effects were mediated by cell cycle arrest through a PPAR γ -dependent pathway at low concentrations. At higher concentrations a major part of the antitumoral activity is mediated through the induction of apoptosis independently of PPAR γ [764, 767].

In addition to the synthetic PPAR ligands it has been shown that naturally PPAR agonists like fatty acids inhibit melanoma cells. Especially, dietary ω -3 polyunsaturated fatty acids (PUFA) like for example eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have shown to decrease the COX-2 mRNA expression and in consequence invasion in brain-metastatic melanoma, whereas ω -6 PUFA such as arachidonic acid promote the growth of tumor cells [775]. *Cario-André et al.* suggested that an ω -6 PUFA rich-diet may increase oxidative damage in melanocytes without inducing apoptosis, the long-term net outcome could be cumulated mutations and an increased risk of skin cancer, especially melanoma, whereas ω -3 PUFA are rather protective [776]. Although the relationship between dietary ω -3 PUFA and carcinogenic risk factors is inconclusive, the evidence suggests that increasing dietary ω -3 PUFA is beneficial.

First clinical studies using PPAR ligands as a supplementary agent in melanoma treatment show promising results. A combined treatment with PPAR agonists and COX-2 inhibitors seems to have not only synergistic effects in non-melanoma skin cancer prevention, but may also increase the susceptibility of malignant cells to pulsatile chemotherapy by upregulating proapoptotic cellular mechanisms [777]. Administration of pioglitazone (PPAR γ ligand) and rofecoxib (COX-2 inhibitor) combined with low-dose metronomic trofosfamide prolonged the progression-free survival of patients with treatment-resistant metastatic melanoma compared to treatment with trofosfamide alone [778]. The same group showed that patients with PPAR γ -positive metastases and biomodulatory metronomic chemotherapy alone or combined with COX-2/PPAR γ -targeting showed a significantly prolonged progression-free survival [778].

In order to avoid the side-effect of chemotherapeutic drugs *Wang et al.* synthesized a conjugate of the PPAR ligand DHA and the anticancer agent doxorubicin, which deliver the chemotherapeutic agent specifically to tumor tissue [779]. *In vitro* and in experimental animal tumor models the created DHA-doxorubicin conjugate was significantly more efficacious than free doxorubicin. Another group analyzed in a phase I study the maximum tolerated dose, dose-limiting toxicity and pharmacokinetics of weekly DHA-paclitaxel (anticancer agent) in resistant solid tumor malignancies [780]. DHA-paclitaxel administered weekly to a maximum dose of 600 mg/m² was well-tolerated and provided stable disease for 16 weeks in a patient with melanoma. In addition to this, compared with the respectively chemotherapeutic agent alone, the combined conjugates DHA-doxorubicin and DHA-paclitaxel were much less toxic [779-781].

Taken together, further investigations have to identify which PPAR ligands might be a new anti-melanoma drug and might provide the initial impetus for coming clinical trials in melanoma treatment.

II-C.7. Acne

The pathophysiology of acne centers on the interplay of follicular hyperkeratinization, inflammation induced by *Acne Vulgaris* and the production of sebum

that serves as a nutrient source for bacteria [782-784]. Because of the many similarities that exist between adipogenesis and sebaceous lipogenesis PPARs may be important in the regulation of human sebum production and the development of acne [785, 786]. Studies in sebocytes and human sebaceous glands indicate that PPAR α agonist-GW7647, PPAR α/δ agonist-GW2433, PPAR γ agonist rosiglitazone, increased lipogenesis. Also, patients treated with thiazolidinediones or fibrates had significant increases in sebum production (37 and 77%, respectively) when compared to age-, disease-, and sex-matched controls. [726]. In addition to this, PPAR regulation can modulate the tissue inflammation in acne lesions by inhibiting the expression of proinflammatory genes [787]. Recent evidence indicates a role for lipoxygenase products, such as leukotrienes B (LTB) 4, in the development of inflammatory acne lesions [788, 789]. Interestingly, LTB4 is also a natural ligand for peroxisome proliferator-activated receptor PPAR α [787, 790]. Recently Zouboulis reported that systemic treatment with the lipoxygenase inhibitor Zileuton reduces the inflammatory lesions in acne patients with the concomitant decrease of sebum hydroperoxides amount, indicating that these compounds have a role in the pathogenesis of acne exerting proinflammatory activity [791]. The exact mechanism is still unknown. However in agreement with the ability of Zileuton to inhibit LTB4 formation, an effect on PPARs can be implicated.

II-C.8. Scleroderma

The hallmark of systemic sclerosis is the excessive collagen accumulation in the skin and the lungs leading to organ dysfunction, failure and death [792]. The pathogenesis of fibrosis remains incompletely understood [793]. Recently the paradigm that inflammation leads to fibrosis has been supplanted by the concept that inflammation and fibrosis may be independent of each other [794, 795]. Therefore the focus on developing new treatments for fibrosis has shifted to target both the anti-inflammatory and fibrogenic process. It has been recognized that PPAR γ agonists have antifibrotic properties too, characterized by inhibition of pulmonary myofibroblast differentiation and type I collagen protein production through mechanisms dependent and independent of PPAR γ [796, 797]. These effects are in part induced by reduction of TGF- β , leading to decreased activation of fibroblasts and collagen production, downregulation of expression of cell surface receptors for growth

factors, reduced secretion of cytokines and chemokines, and suppression of myofibroblast differentiation [798]. Deletion of PPAR γ resulted in enhanced susceptibility to bleomycin-induced skin fibrosis, as indicated by increases in all measures of skin fibrosis and enhanced sensitivity of fibroblasts to TGF β in PPAR γ -deficient mice [799]. In a murine model of subcutaneous bleomycin-induced scleroderma systemic administration of the PPAR γ agonist rosiglitazone inhibited early inflammation responses and abrogated skin fibrosis, local collagen accumulation, lipoatrophy and reduced tissue accumulation of myofibroblasts [800]. *In vitro* rosiglitazone alleviated the persistent fibrotic phenotype of skin scleroderma fibroblasts and may be therefore considered as a possible new treatment for scleroderma [801].

Overall, targeting PPARs would seem an efficient strategy in the management of several skin diseases (Table 4). However, pharmacological profiling of drugs targeting PPARs and the pharmacokinetics and pharmacodynamics studies paralleling drug development are long processes requiring years of preclinical research. Thus, the development of new technologies that would shorten the research at preclinical stages seems necessary. Hence, the rationale of the following section addressing the utility of reporter mouse technology and more specifically peroxisome proliferator response element-luciferase (PPRE-luc) mice.

Table 4. Biologic effects of PPAR ligands on skin cells and potential therapeutic applications. Updated from [25]

Cell type	PPAR ligand	Biological effect	Potential application
Fibroblasts	15d-PGJ2 Troglitazone (PPAR γ ligand)	- Modest suppression of collagen expression	Cutaneous fibrosis sclerodema
Keratinocytes	tetradecylthioacetic acid (non specific PPAR ligand)	- Decrease in proliferation - Upregulation of expression of involucrin and transglutaminase - Keratinocyte differentiation	Psoriasis
Keratinocytes	Clofibrate (PPAR α ligand)	- Upregulation of expression of involucrin and transglutaminase - Keratinocyte differentiation	Psoriasis
Keratinocytes	L165041 (PPAR β/δ ligand)	- Upregulation of expression of involucrin and transglutaminase - Keratinocyte differentiation	Psoriasis
Keratinocytes	L165041 (PPAR β/δ ligand) BRL49653 (PPAR γ ligand)	- Strong synergistic induction of involucrin	Psoriasis
Keratinocytes	TZDs (PPAR γ ligands)	- Suppression of proliferation of normal and psoriatic keratinocytes	Psoriasis
Keratinocytes	LTB4 (PPAR α ligand)	- Increased epidermal differentiation - Synergistic anti-inflammatory effects	Psoriasis
Keratinocytes	WY14643 (PPAR α ligand)	- Increased minimal erythema dose in UVB-irradiated skin	Chemoprevention of skin cancer
Keratinocytes	WY14643, Clofibrate (PPAR α ligand) TZDs (PPAR γ ligands)	- Induction of differentiation - Inhibition of proliferation	Chemoprevention of skin cancer
Melanocytes	WY14643 (PPAR α ligand)	- Inhibition of proliferation - Accelerated melanin synthesis	Post-inflammatory hyperpigmentation
Melanocytes	PPAR α ligands PPAR γ ligands	- Modulation of melanin synthesis	Post-inflammatory hyperpigmentation
Melanoma cells	15d-PGJ2 Troglitazone (PPAR γ ligand)	- Inhibition of proliferation - Induction of re-differentiation	Post-inflammatory hyperpigmentation
Melanoma cells	Fenofibrate (PPAR α ligand)	- Inhibition of migration and anchorage-independent growth	chemoprevention for melanoma
Sebocytes	Clofibrate (PPAR α ligand)	- Increased differentiation - Induced apoptosis	Follicular comedogenesis
Sebocytes	WY14643 (PPAR α ligand) BRL-49653 (PPAR γ agonist)	- Differentiation of sebocytes	Acne

III- Peroxisome Proliferator Response Element-Luciferase (PPRE-luc) Reporter Mice

III-A. Rational for Reporter Mouse Technology

New drug approval is a lengthy process employing a time frame of several years and spans a vast array of disciplines. Minor delays within individual disciplines may easily add to the cost of drug development and widen the gap between the discovery and the request of valuable patent exclusivity. Pharmaceutical companies work hard to improve the decision making process and to make project phases more efficient by applying novel technologies. However, the demand for safety has limited the flexibility in testing procedures and discouraged the application of novel methodologies for pharmaco-toxicological studies, which are now carried out following fixed protocols suggested by the drug approval authorities. Needless to say, the rigidity of drug development protocols with its intrinsic costs has limited research, into the full range of pharmacological actions of new chemical entities. The consequence is that the number of novel drugs has decreased, while the number of withdrawals from the US market for safety reasons has grown. In fact, according to a Food and Drug Administration (FDA) report, a new medical compound entering Phase I testing in recent years is estimated to have almost the half success rate of reaching the market compared with the historical success rate. On the other hand, during the last 20 years, around 40 compounds have been withdrawn from the market owing to stricter safeguards [802].

A boost to the entire drug discovery process may come from the combined application of imaging technologies and animal engineering, with the development of reporter animals in which selected molecular events (e.g. expression of a gene or intra-molecular interactions) can be visualized in real time in living animals [19, 803]. Recently, imaging technologies have been approved by the FDA as a means to provide secondary endpoints in clinical trials for some drugs directed to slow progress diseases like cancer and acquired immunodeficiency syndrome (AIDS). Nowadays, imaging modalities are also applied to provide secondary endpoints in therapeutic areas other than cancer [804-806], such as rheumatoid arthritis [807] and osteoporosis [808]. It would be advisable now to extend the use of imaging modalities as valid end-points to preclinical investigation by generating animal models engineered to report the activity of selected biomarkers that are

detectable by one or more imaging methods. Such an approach would represent a useful tool to rapidly verify concrete benefits of the drug of interest in living organisms [809]. Hence, the introduction of surrogate imaging endpoints in the preclinical phase would greatly accelerate the release of innovative drugs onto the market, with concurrent price decrease.

III-B. Reporter Gene Imaging

Biological discovery has moved at an accelerated pace in recent years, with considerable focus on the transition from *in vitro* to *in vivo* models. As such, there has been a greater need to adapt clinical imaging methods for noninvasive assays of biochemical processes. Considerable efforts have been directed in recent years toward the development of noninvasive, high-resolution, small animal molecular imaging technologies (Figure 15). The term “molecular imaging” refers to the visual representation, characterization, and quantification of biological processes at the cellular and subcellular levels within intact living organisms [23]. Dissecting the functioning, advantages and disadvantages of all the *in vivo* molecular imaging techniques available nowadays is far beyond the objective of this section. We would rather analyze and compare the technologies using the reporter system technology, thus **reporter gene imaging**.

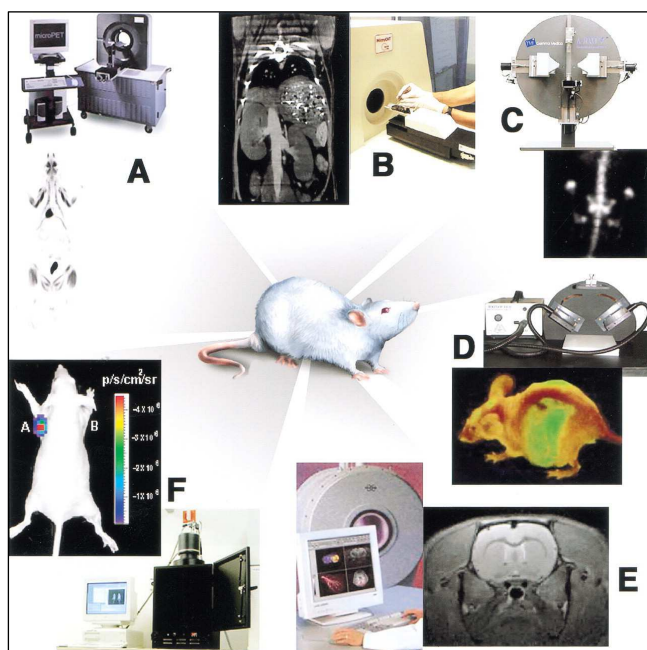


Figure 15. Multiple imaging modalities available for small-animal molecular imaging. (A) Positron emission tomography image of a rat. **(B)** Computed tomography image of a mouse abdomen. **(C)** Single photon emission computed tomography image of a mouse abdomen and pelvis. **(D)** Optical green fluorescent protein fluorescence image of a mouse. **(E)** Magnetic resonance imaging image of a mouse brain. **(F)** Optical bioluminescence image of a mouse [23].

III-B.1. General Features of Reporter Imaging Systems – Ideal Reporter Gene

Reporter genes are used to study promoter/enhancer elements involved in gene expression, inducible promoters to look at the induction of gene expression, and endogenous gene expression through the use of transgenes containing endogenous promoters fused to the reporter [810]. In all these cases, transcription of the reporter gene can be tracked, and therefore gene expression can be studied. The reporter gene driven by a promoter of choice must first be introduced into the cells of interest. This is a common feature for all delivery vectors in a reporter gene-imaging paradigm; that is, a complementary DNA expression cassette (an imaging cassette) containing the reporter gene of interest must be used. The promoter can be constitutive or inducible; it can also be cell-specific. If the reporter gene is transcribed, an enzyme or receptor product is made, thus trapping the imaging reporter probe, which may be a substrate for an enzyme or a ligand for a receptor. The trapping of the probe leads to an imaging signal, be it from a radioisotope, a photochemical reaction, or a magnetic resonance metal cation, depending on the exact nature of the probe itself (Figure 16).

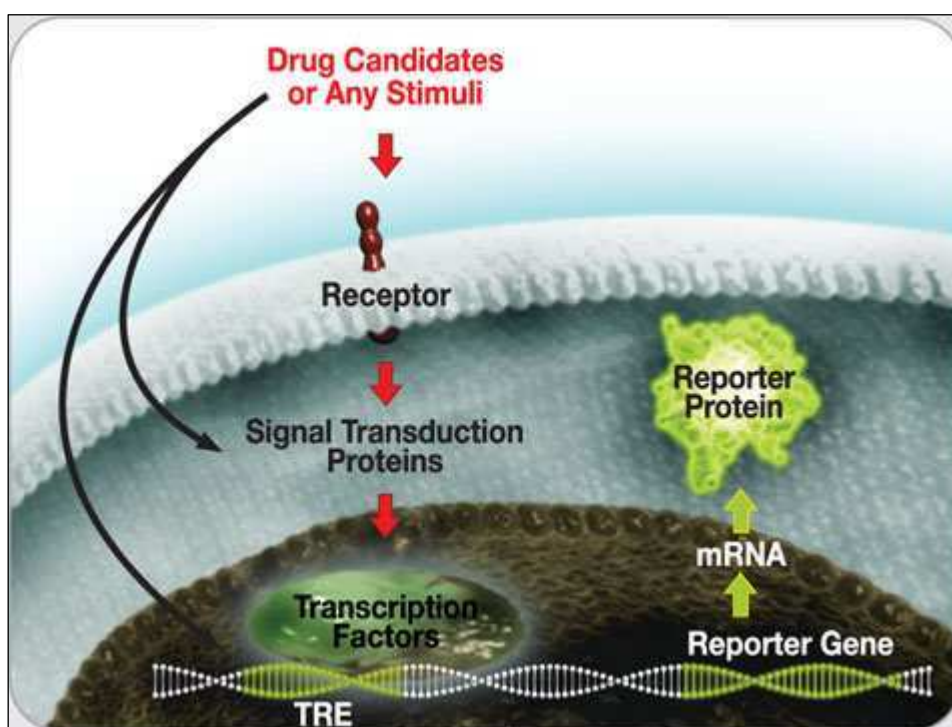


Figure 16. Reporter gene technology (www.sabiosciences.com).

The ideal reporter gene/probe would have the following characteristics [810]:

1. To prevent an immune response, the reporter gene should be present in mammalian cells, but not expressed
2. Specific reporter probe should accumulate only where reporter gene is expressed.
3. No reporter probe should accumulate when the reporter gene is not expressed.
4. The product of the reporter gene should also be non-immunogenic.
5. The reporter probe should be stable *in vivo* and not be metabolized before reaching its target.
6. The reporter probe should rapidly clear from the circulation and not interfere with detection of specific signal.
7. The reporter probe or its metabolites should not be cytotoxic.
8. The size of the reporter gene and its driving promoter should be small enough to fit into a delivery vehicle (plasmids, viruses), except for transgenic applications.
9. Natural biological barriers must not prevent the reporter probe from reaching its destination.
10. The image signal should correlate well with levels of reporter gene mRNA and protein *in vivo*.

No single reporter gene/reporter probe system meets all these criteria at present. Therefore, the development of multiple systems provides a choice based on the application of interest. The availability of multiple reporter gene/reporter probes also allows monitoring the expression of more than one reporter gene in the same living animal [811].

III-B.2. Categories of Reporter Imaging Systems

Current reporter gene imaging tools are essentially based on the recognition of a specific reporter system expressed at the cellular level by means of three main categories including optical imaging (bioluminescent and fluorescent proteins), nuclear imaging (positron emission tomography (PET) and single photon emission computed tomography (SPECT)) and magnetic resonance imaging (MRI) (Figure 17).

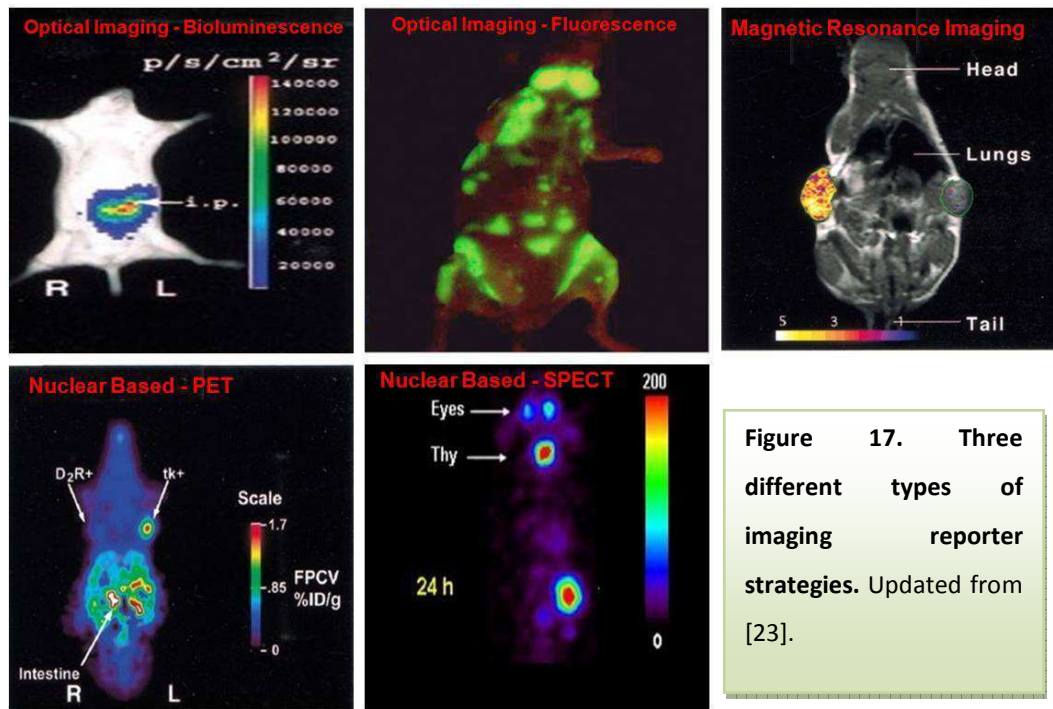


Figure 17. Three different types of imaging reporter strategies. Updated from [23].

a) Optical Imaging: Bioluminescence and Fluorescence

Bioluminescence is the production and emission of light by a living organism as the result of an exo-ergonic reaction during which chemical energy is converted to light energy. Bioluminescence is generated by an enzyme-catalyzed chemiluminescence reaction, wherein the substrate pigment is oxidized by the luciferase enzyme. Adenosine triphosphate (ATP) is involved in most instances. Luciferases represent a large family of proteins expressed in nature by a wide variety of living organisms, including bacteria, marine invertebrates, fish, insects, fungi and worms, but not mammals. Therefore, optical imaging with luciferases in higher animals has the advantage of low background [812]. The detection and measurement of bioluminescence is operated by means of image sensors, named charged coupled device (CCD) cameras, consisting of an integrated circuit containing an array of linked, or coupled, light-sensitive capacitors. This newer generation devices are the cooled CCD cameras, where reduced thermal noise lowers background noise, resulting in a significantly increased in signal-to-noise ratio [813]. Bioluminescence acquisition is usually performed in a dark room supplied by Isoflurane gas anesthesia and connected to a computer for data analysis. The whole system is named “Xenogen” (Figure18).

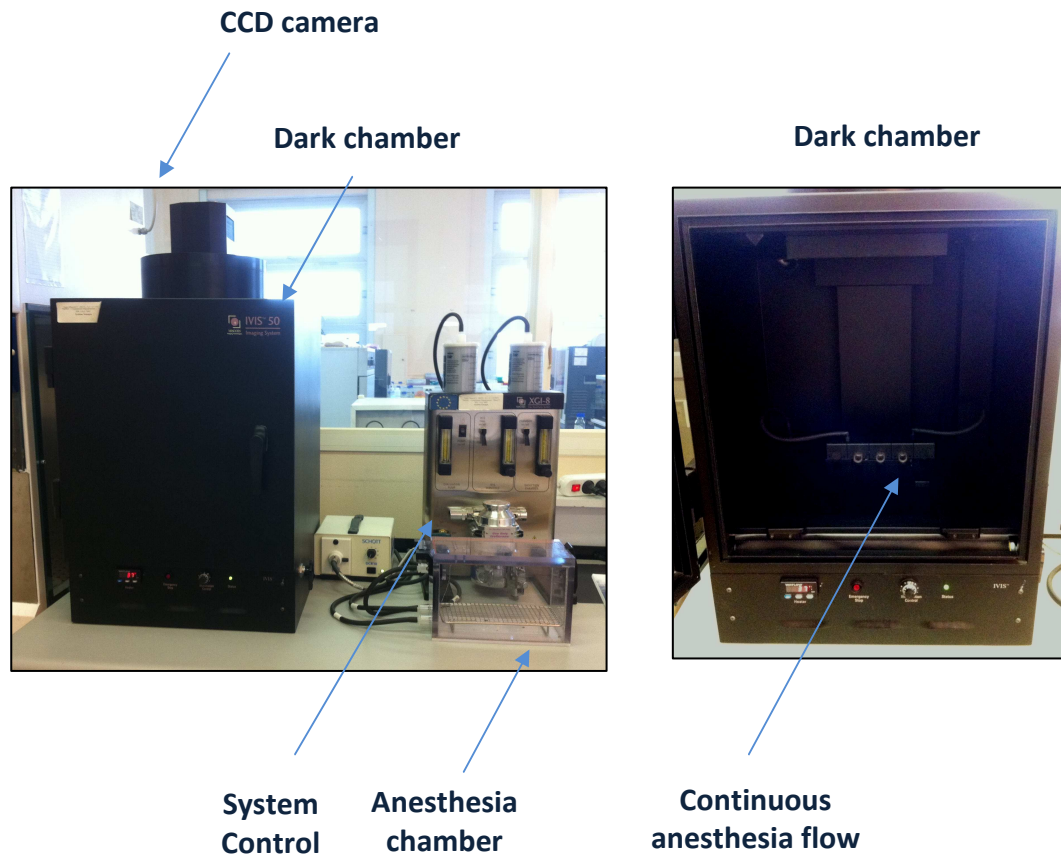


Figure 18. Xenogen IVIS for bioluminescence detection. Xenogen's IVIS 50 imaging system includes a highly sensitive CCD camera-cryogenically cooled to -105°C , a dark imaging chamber to minimize incident light, and software to quantify the results. The system is connected to a gas anesthesia unit that allows continuous administration of Isoflurane gas for anesthetizing animals during imaging (Plateforme d'Imagerie Cellulaire et Tissulaire, Lille).

One of the first luciferases molecular biologists used is firefly *Photinus pyralis* (FLuc) which can oxidize the natural substrate, luciferin, in the presence of oxygen and ATP as a source of energy (Figure 19) [814]. Several unique features make this enzyme particularly suited for whole-body imaging: its emission spectrum peaks at 560 nm and includes a significant fraction of light in the near infrared region that can penetrate biological tissues; second, luciferin, the natural substrate, diffuses well in mammalian tissues [815], even through the blood-brain, blood-testis or placental barriers [816]; moreover, luciferin possesses low toxicity and poor antigenicity (Figure 20).

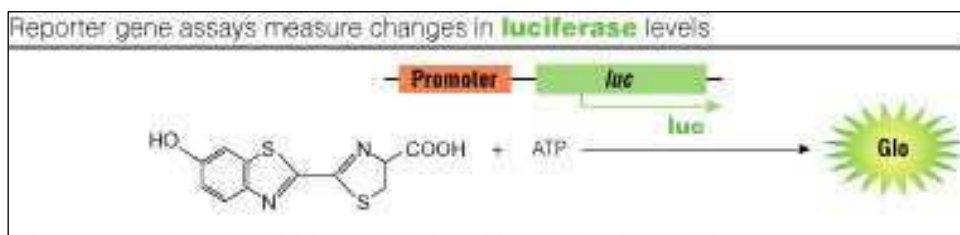


Figure 19. Luciferin oxidation by *Firefly* luciferase. Luciferase catalyzes the oxidation of F-luciferin in the presence of ATP, leading to light (photon) emission, called luminescence. (www.promege.com)

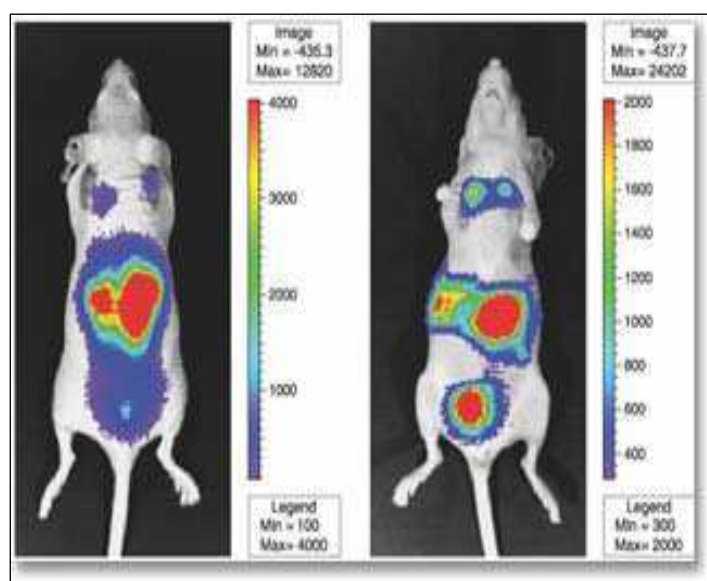


Figure 20. Examples of *in vivo* bioluminescence imaging. The bioluminescence is induced by injecting cancerous cells transfected with the *Firefly* luciferin. This image shows metastatic lesions from both the ventral and dorsal view. The color of bioluminescence reflecting the intensity of the luciferase reaction is evaluated according to the scale (right) using specific software (www.spie.org)

Another luciferase commonly used for expression in mammalian living systems is the *Renilla* luciferase (RLuc), which is purified from a bioluminescent soft coral (*Renilla reniformis*). Studies on this enzyme indicate that RLuc appears to differ from FLuc structurally and in emission range and substrate requirement [817]: The oxidation ATP-independent and Na^{2+} -requiring of the substrate coelenterazine, generates blue-green bioluminescence with a spectrum peak at 480 nm [818]. RLuc, being a small enzyme of about 36 kDa, appears to be highly appropriate for application as a bioluminescent tag conjugated to other proteins like antibodies or growth factors [819]. This enzyme is rapidly inactivated in murine serum, where its half-life is <1 h. The blue emission range, however, limits its use in living animals. Moreover, more accurate evaluation of the toxicity and biodistribution of the coelenterazine substrate, needs to be carried out [820].

A less known luciferase is the bioluminescent enzyme derived from the marine copepod *Gaussia princeps*. *Gaussia* luciferase (GLuc) is the smallest luciferase known (19.9 kDa) and it catalyzes the oxidation ATP-independent and Na²⁺-requiring of the substrate coelenterazine in the same way as RLuc, generating light at a peak of 480 nm, with a broad emission spectrum that expands up to 600 nm. In mammalian cell cultures, it generates a high intensity signal stronger than those emitted by RLuc or FLuc, and flash kinetics, with a more rapid decay over time than luciferases from *Renilla* and *firefly*. Despite its blue emission range, the high luminescence of GLuc allows it to efficiently penetrate biological tissues, making it detectable also in inner tissues [821]. As a result, GLuc holds great promise for the future, if the use of coelenterazine analogues or mutations in the amino acid sequence could create an ultrasensitive and red-shifted enzyme ideal for *in vivo* imaging.

Fluorescence is a luminescent process in which the molecular absorption of a photon triggers the emission of another photon with a longer wavelength. The energy difference between the absorbed and the emitted photons ends up as molecular vibrations or heat. Usually, the absorbed photon is in the ultraviolet range and the emitted light is in the visible range, but this depends on the absorbance curve and stroke shift of the particular fluorophore.

Over the past few years, the discovery and development of naturally occurring fluorescent proteins and mutated derivatives has rapidly improved the investigation of a broad spectrum of intracellular processes in living cells. A variety of marine organisms has been the source of over 25 fluorescent proteins and their analogs, providing an ample set of non-invasive biological probes for single, dual, and multispectral fluorescence analysis. The possibility to specifically target fluorescent probes in subcellular compartments, coupled with extremely low or absent toxicity and widespread compatibility with tissues and intact organisms, opens new opportunities for these biological macromolecules in live-cell imaging. Actually, the most common fluorescent protein for real time imaging in cells, the green fluorescent protein (GFP) isolated from the jellyfish *Aequorea Victoria*, has been used in whole-body imaging experimentations, to visualize tumor cells and to monitor cancer growth *in vivo* [822, 823]. In these experiments, despite a GFP emission peak at 510 nm, the

activity of injected cancer cells was followed. Numerous efforts are being made to identify new fluorescent proteins with red-shifted optical qualities for small-animal imaging, given their reduced auto-fluorescence at longer wavelengths. The DsRed fluorescent protein originally isolated from the sea anemone *Discosoma striata* (emission peak 583 nm) is currently the most widely used analog for fluorescence analysis in the 575 to 650 nm region [824]. This protein, however, carries several draw-backs for its use as a fluorescent probe: forced tetrameric structure, slow chromophore maturation and poor solubility. Specific amino acid substitutions in the wild-type form yielded a fluorescent protein in which most of the problems were overcome [825]. With further genetic modifications, a series of novel proteins with new emission colors, from yellow-orange to red-orange were generated [826]. A broader field where fluorescent proteins have been applied *in vivo* is cancer: these probes permit the visualization of tumor cell proliferation, apoptosis, metastasis, angiogenesis and the effect of therapeutics. To this end, tumor cells were stably transfected with the genes encoding the reporter for non-invasive analysis in intact animals.

Over 100 fluorescent organic dyes are currently available but just a fraction displays the essential features listed below. However, the growth in the number of fluorescent compounds in the red portion of the spectrum has led to a novel branch of fluorescence, near-infrared fluorescence imaging (NIRF), which was expressly generated for imaging small living animals. Novel fluorescent organic proteins should possess: **1)** a peak of excitation-emission close to a spectrum range of 600-900 nm; **2)** a large stroke shift (difference between the maximum level in the excitation and emission spectra), critical for effectively obtaining high sensitivity of fluorescence imaging with low noise background; **3)** a high quantum yield representing a quantitative measure of fluorescence emission efficiency, expressed as the ratio between the number of photons emitted and those absorbed, typically expressed as a value between 0 and 1; **4)** high chemical and photostability; **5)** low toxicity and good biocompatibility, with predictable biodistribution.

A major advance in modern fluorophore technology is exemplified by the Alexa Fluor dyes, which are sulfonated rhodamine derivatives with high quantum yields, enhanced photostability, pH insensitivity, and a high degree of water solubility. These fluorophores are commercially available in a broad range of fluorescence excitation and emission

wavelength maxima; in particular, some are specifically designed for near-infrared fluorescence analysis. Utilizing an Alexa Fluor dye emitting in the far-red region, a caspase-activable red fluorescent peptide was recently generated to image apoptosis *in vivo*, thanks to its small size that allows easy membrane-permeability [827]. New members of the cyanine dye family (as long-wavelengths Cy5 derivatives) were synthesized to shift their optical properties towards the red region of the spectrum. Compared with traditional dyes, they show enhanced water solubility, photostability, and higher quantum yields; moreover, most cyanine dyes are more environmentally stable than their traditional counterparts, rendering their fluorescence emission intensity less sensitive to pH and organic mounting media. Cyanine dyes could be used to label specific antibodies or binding proteins to facilitate the study of molecular interactions in living animals. For instance, this approach was used to follow the growth of an intracranial tumor *in vivo* through semiquantitative imaging analysis of a specific integrin expression [828]. This method may provide a solution to assess the efficacy of brain tumors antibody treatment in preclinical studies.

b) Nuclear-based imaging

An alternative to optical imaging is positron emission tomography (PET) and single photon emission tomography (SPET) technology, by which reporter expression is localized with reporter probes, i.e. radiotracers that bind specifically to the reporter itself. So far, the best reporters developed for PET-based imaging of small animals are the dopamine D2 receptor [829, 830] and the viral thymidine kinase [23, 831]. Efficient radiotracers are available for both approaches [830, 832-836]. Many other radiotracers have been developed to study cell proliferation, metabolism, apoptosis and other processes as well [837-841]. By combining the radionuclide approach with computed tomography techniques 3D images can be generated with a resolution approaching 1mm³ (using dedicated animal PET scanners) [842], a degree of resolution not yet achievable with optical imaging (Table 1). Conversely, the sensitivity of animal PET remains lower than that of optical imaging. Furthermore, the need of a cyclotron to produce isotopes and the difficulties in developing novel radiotracers still pose major limitations to the use of this technology. The temporal resolution of optical imaging is actually far superior to that of radionuclide techniques and allows better assessment of rapidly cycling processes.

c) Magnetic resonance imaging

Although a very interesting imaging technique, to date Magnetic Resonance Imaging (MRI) and more specifically functional MRI (fMRI) has been limited to neurosciences in small animals such as rodents. However, fMRI of small animal models will contribute to this knowledge as a supplement to the existing vast amount of *in vitro* and invasively-obtained data that make up the present molecular background knowledge of neuronal communication. Although fMRI appears to be a major tool for longitudinal studies of brain diseases and functional reorganization and plasticity, these methods, however, do not allow to study directly neuronal electrical activity [843]. fMRI offers a set of different variables to measure functional brain activity including measuring either blood oxygenation level dependent (BOLD) changes or changes in cerebral blood flow (CBF) or cerebral blood volume (CBV). The most widely used variable is based on the BOLD effect, being a rather complex combination of the physiological changes in blood oxygenation, CBF and CBV. The use of intravascular tracers, in particular iron oxide nanoparticles, provides access to CBV changes upon stimulation, but makes this technique slightly invasive. These three fMRI variables all reflect haemodynamic changes in response to the stimulus. Their temporal response patterns, however, differ, with CBV being the slowest to reach a new steady-state after the change between the resting and activated state of the brain. It should be noted that, results may depend on the choice of variable, as these measurement variables reflect different physiological aspects. Interestingly, fMRI can be also combined with other modalities (e.g. invasive electrophysiology, pharmacochemical manipulations, etc) to supplement existing data and to provide a more comprehensive picture and will lead to better answers to the scientific questions [844].

Two general types of reporter systems are applicable to MRI-based molecular imaging. One imaging system relies on molecular probes that are sensitive to the activity of β -galactosidase, the product of the classic marker gene *LacZ*. The synthesis of a molecular probe in which gadolinium is protected by a carbohydrate “cap” that is β -galactosidase-cleavable would result in a probe with variable water access. However, the ability of these probes and MRI to detect gene expression in living subjects is very limited. Unfortunately, present enzyme-cleavable probes do not freely cross cell membranes (thus requiring direct

intracellular injection), and the kinetics of cleavage are quite slow [845]. An alternative to this conditional reporter probe is to couple MRI probes to targeting moieties. *Weissleder et al.* have highlighted the use of the transferrin receptor as a potential intracellular transporter of iron oxide nanoparticles [846]. The transferrin receptor is found in most cells and is part of the iron regulatory system. The receptor binds to transferrin, an iron-carrying protein, and transports it into the cell. Normal expression of the receptor is under feedback regulation to prevent excessive cellular iron uptake. However, cells can be engineered to overexpress transferrin and, therefore, accumulate monocrystalline iron oxide nanoparticles intracellularly. This increased iron content results in differential MRI signal from these cells. Additional studies are required to assess this system further, including measurement of the effect of overexpressing the receptor on normal cellular function, and how well normal systems tolerate increased levels of freely circulating and intracellular iron.

III-B.3. Optical imaging as current methodology of choice

The major shortcoming to the application of nuclear imaging such as PET and SPECT in preclinical studies is that most imaging technologies now available were originally developed for clinical use and adapt poorly to the necessities of a preclinical research laboratory. For instance, in nuclear imaging techniques such as PET and SPECT, their limited resolution may sometimes be problematic for applications in small animals, and the throughput capacity inadequate for screening or pharmacological analysis of drug candidates. But, the major obstacle to pre-clinical use of PET and SPECT is that they require specific, low half-life radioisotopes. MRI and functional MRI are certainly more suitable for the study of small animals, because of their ability to provide finely detailed anatomical/functional information on drug action even in organs as complex as the brain. However, their output capacity is very low and, in addition, MRI is burdened by the high operative costs of instrumentation and dedicated personnel.

Optical technologies, such as bioluminescence and fluorescence, also have shortcomings since these techniques were originally developed by cell biologists and later adapted to the study of molecular events in living organisms. Peculiar to animal imaging is that the fluorescent or luminescent signal has to propagate through several tissue layers.

The obstacles along this journey arise from two physical effects: absorption by chromophores and scattering by interaction with cellular components. Absorption differs in the each tissue type owing to molecules such hemoglobin in the blood, melanin in the skin and hair, or water. These chromophores have a low absorption coefficient in the near infrared region of the spectrum (from 650 to 900 nm), so they absorb mainly in the blue and green region (up to 600 nm approximately). Consequently, the red portion (near infrared and mid infrared) of the emitted light is transmitted through the animal body and can be collected by detectors located outside the animal's body (Figure 21). Scattering of photons, in contrast, is less subject to the specificity of the tissue crossed by the signal. Scattering is linked to photon interplay with cellular components, especially membranes. Experimental evidence shows that near infrared and mid-infrared emitted light, although scattered, is preferentially transmitted through the animal tissues and that signaling from inner organs can be detected outside the body of a small animal, such as the experimental mouse [847].

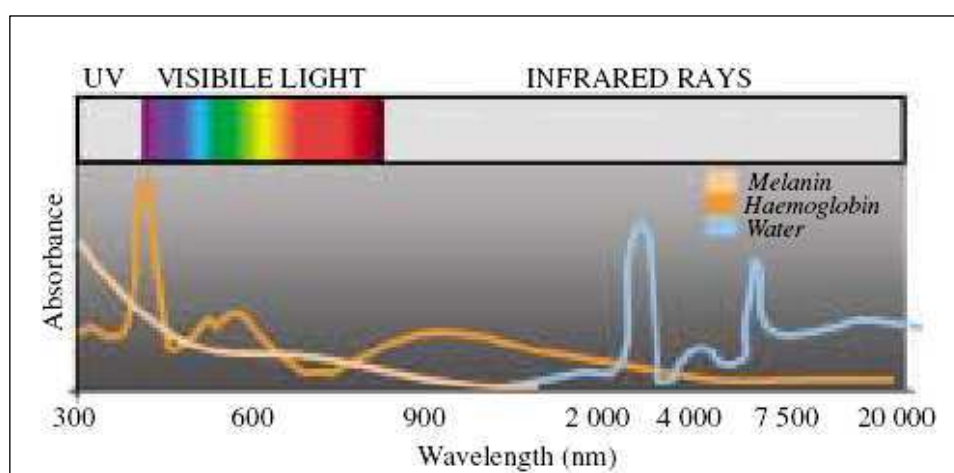


Figure 21. Graph reporting the absorbing spectra of biological tissue components melanin, hemoglobin and water. The absorbance of these chromophores is minimal in the near infrared region of the spectrum (650-900 nm) [6].

Furthermore, the major limitation to the application of fluorescence *in vivo* imaging is that fluorochrome emission requires prior photon absorption: if the exciting source is located outside the animal's body, it will be severely quenched by tissue absorption and scattering, greatly limiting the application of fluorescence probes to *in vivo* imaging. The fluorescent system approach may exhibit some qualitative advantages over bioluminescent analysis, in such as the possibility to obtain dual-color images or substrate-independence.

Table 5 summarizes the advantage and disadvantages of optical imaging versus nuclear-based imaging. Briefly, optical imaging provides a feasible quantification of the emitted signal without endogenous background, high sensitivity and rapidity, low cost and less labor. Currently, Bioluminescence is the method of choice for investigation in the field of drug discovery, owing to its efficiency, rapidity, dynamicity and low cost.

Table 5. Radionuclide and optic imaging: pros and cons [19]

	Optical imaging	Radionuclide imaging
Radiation	Visible light or near infrared	γ-rays at high or low energy
Spatial resolution	About the depth of the object observed (3–5 mm)	1–2 mm
Depth	1 cm–variable	No limit
Temporal resolution	Seconds to minutes	10 s to minutes
Sensitivity	10 ⁻¹⁵ to 10 ⁻¹⁷ mol/L bioluminescence 10 ⁻⁹ to 10 ⁻¹² mol/L fluorescence	10 ⁻¹⁰ to 10 ⁻¹² mol/L
Type of probe	Fluorescent or enzymatic activity leading to bioluminescence	Radiolabeled
Amount of probe needed	Micrograms to milligrams	Nanograms
Quantitative analysis	Relative quantification	Absolute quantification
Cost	Low	High
Advantages	High sensitivity, rapidity, low-cost, low labor consuming	Simultaneously imaging with multiple probes, high sensitivity, quantitative, three-dimensional images
Disadvantages	2D images, depth-dependent intensity of signals (tissue absorption and scattering), poorly quantitative	High background, need of a cyclotron, high cost

III-C. Applications of Reporter Mouse Technology

The most significant revolution in drug discovery has been a shift from a disease-based approach — that is, screening drugs in an assay to look for a disease phenotype — to an inductive research model in which the molecular pathways involved in a disease are examined to identify novel drug targets. However, most of the drugs developed using post-genomic research strategies do not provide the desired therapeutic effect owing to unexpected biological interactions or, worse still, fail in the late stages of drug development as a result of unforeseen toxicity. Clearly, one crucial addition to the drug discovery toolbox would be the development of novel and more predictive models of drug effect and toxicity. In particular, reporter animals could make a significant contribution to drug discovery programs by facilitating target identification and improving the efficacy and reliability of all phases of preclinical drug development (Table 6).

Table 6. Applications of reporter animals.

Applications of reporter animals

- Characterization of the distribution and the pharmacodynamic profile of drugs in entire living organisms after acute or repeated administration of drugs.
- Analysis drugs metabolism; those active on transcription factors by using reporter animals in which liver metabolism has been humanized by substituting the genes that encode drug-metabolizing enzymes with their human form.
- Analysis of gender- and age-dependent effects on physiological conditions.
- Identification of novel drug targets by the *in vivo* examination of selected metabolic pathways.
- Verification of the activity of investigational compounds on selective pathological processes (such as tumor growth, angiogenesis, inflammation and diabetes).
- Facilitation of toxicological analysis by providing the opportunity to evaluate for each given investigational drug all the organs affected by the drug activity; gender-specific effects of the drug; and sites of drug accumulation and eventually its release following stress or fasting.
- Evaluation of drug effect in a disease model

III-C.1. Studying Healthy and Pathophysiological Conditions

By providing novel insights into mammalian physiology, reporter animals could add significant value to knowledge obtained from molecular approaches or systems biology. The recent studies carried out with a mouse reporter (estrogen receptor element-Luciferase mice, ERE-Luc mice) on the systemic activities of oestradiol provide the first example of this — the reporter animal provided completely new information on the physiological activity of estrogen receptors by demonstrating that in non-reproductive organs of fertile mice estrogen receptors are transcriptionally activated by factors other than oestradiol [815]. This is particularly relevant for understanding the susceptibility of postmenopausal women to diseases of the cardiovascular, nervous and skeletal systems, and for the design of innovative hormone-replacement therapies.

Reporter mice developed to visualize the activity of promoters responsive to inflammatory agents (NF- κ B-luciferase mice) could be of significant value in unraveling the pathogenesis of human disorders involving inappropriate regulation of NF- κ B, including cancer, neurodegeneration, asthma, atherogenesis, inflammatory bowel disease and rheumatoid arthritis [848, 849]. Other models, such as activator protein 1 (AP1)-Luc mice, can be used to monitor the effects of carcinogens such as tetradecanoylphorbol acetate (TPA) and could provide relevant clues about the process of tumor initiation and promotion [850]. It would therefore be possible to generate 'libraries' of animals engineered to visualize key reactions of the major biochemical processes. Furthermore, such animals reporting on the pathway of interest could be bred into models of the given disease to investigate the molecular events leading to the onset and progression of the pathology. For instance, reporter mice for peroxisome proliferator activated receptors; peroxisome proliferator response element-luciferase (PPRE-luc mice) could be bred into mice that develop diabetes [851] or colorectal carcinoma [852] to study the relevance of PPAR activity in the aetio-pathogenesis of these diseases. Animals such as these, or others carrying the appropriate reporter in models of diseases, would complement programs of target discovery based on genomics and proteomics and could be used to examine the sequence of events leading to the changes observed in the pathological versus the healthy sample. A further advantage of reporter animals, with respect to other transgenic and knockout

models, is that the expression of the transgene does not perturb the normal physiology of the animals and therefore does not induce compensatory metabolic effects. Furthermore, the reporter transgene allows the detection of the activity of one single metabolic pathway at a time, which can simplify analysis of the experimental results.

III-C.2. From Basic Science to Pharmacology

The increased demand of safety tests that should be carried out before approval of drugs has inflated costs; but, more importantly, has limited the flexibility in the testing procedures [853, 854]. Toxicity tests are carried out in absolute compliance with government regulations with little attempt to specifically adapt the study to the molecule in development. This limits the possibilities of understanding the full range of pharmacological actions. The consequence is that, as stated in the beginning of this chapter, the number of drugs withdrawn from the US market for safety reasons has grown considerably: of all the drugs withdrawn from the market since 1982, almost 70% were pulled in the past decade [854]!!!

a) Pharmacodynamics and specificity

Currently, preclinical testing is carried out following well-defined phases; without doubt, the introduction of reporter mice could significantly change the investigational approach and significantly improve the predictability of these studies. One application of reporter mice in drug development is for the study of pharmacodynamics and organ-specificity of drug action. The activity of the compound in organs other than the intended target can be readily assessed for all routes of administration and for different pharmaceutical preparations by simple analysis of the reporter activity. The minimal dose required to produce a therapeutic effect can also be calculated directly by verifying the state of activation of the target in the organ subject to therapy. If reporter animals can be generated that are suitable for *in vivo* imaging, this could open up the possibility of studying the effect of the drug on reporter-gene expression on a daily basis and without sacrificing the mice. This is particularly relevant for chronic treatments, for which it is important to determine that the dosage and administration schedule leads to the best therapeutic effect

with minimal side effects. For instance, for drugs that act on membrane receptors, reporter systems could be used to verify whether continuous exposure to specific ligands induces receptor downregulation or desensitization. In this case, discontinuous administration of the compound, allowing for receptor replenishment, could increase the efficacy of the treatment and limit its toxicity. Using reporter mice and *in vivo* imaging, gender- and age-specific studies of the efficacy of drug treatment would be possible with limited costs.

Furthermore, the pharmacodynamic profile of the molecules with the best range of action could be further evaluated after repeated administrations in which the scheduling of the drug could be appropriately set to obtain the maximal therapeutic effect at the minimal dosage. The analysis of the extent of metabolite excretion would enable prediction of the drug-metabolism pathway, which could help in the prediction of any potential adverse interactions.

b) Toxicity assessment

Another application of reporter mice is as an alternative to acute toxicity tests. Several objections have been made regarding the use of toxicity tests as they are carried out at present [855] and proposals have been made to limit their use to specific classes of drugs, such as, for example, anti-neoplastic therapeutics [856]. The introduction of reporter animal models would simplify the definition of the full range of organs affected by drug action; this would certainly facilitate the prediction of side effects. The analysis of reporter activity over time would reveal hypersensitive tissues or potential sites of accumulation of drugs administered for a prolonged period of time. For drugs that accumulate in fat tissue, it would become feasible to measure the effects of the drug when lipolysis occurs — for example, after strenuous exercise or fasting. Dietary or environmental interferences could also be revealed, as well as negative interactions with other drugs that are often co-administered. In the last case, it would be very useful to use animals that have been genetically altered to mimic drug metabolism in humans [857]. Furthermore, reporter animals can aid the determination of drug dosage, particularly for anticancer agents, to obtain the maximal effect on the tumor while limiting undesired systemic effects.

With appropriately designed reporters that can image particular metabolic pathways in embryos, the embryonic toxicity of single and repeated administration of investigational drugs could be analyzed during pregnancy and later on during lactation. Furthermore, using *in vivo* imaging methodologies, the effects of the drug could be analyzed in embryos, mature and ageing mice of both genders using a very limited number of animals.

c) Preclinical drug development rule: ADMET

One potential major impact of the use of reporter mice is that the five steps of preclinical drug development — Absorption, Distribution, Metabolism, Excretion and Toxicology (ADMET) — could be increasingly integrated, thereby leading to significant savings in time and the use of animals. For example, screening and pharmacodynamics profiling could be integrated: the battery of compounds to be investigated would be prescreened in a limited number of cell lines for the accurate measurement of their potency and affinity for the target. The compounds with the best profile of action could then be screened for their acute effects in reporter animals. Dose–response studies would clearly illustrate the response profile in different organs. The use of small animals would allow continuation of the study with limited quantities of compound without scaling up the synthesis procedures. These *in vivo* studies would vastly improve the prediction of investigational compound efficacy compared with current methods that use lines of immortalized human cells and in which *in vivo* drug potency is predicted on the basis of a series of complex algorithms designed to take into consideration all of the factors that might influence the response to the treatment [858]. The use of reporter mice would immediately provide the full picture of absorption, distribution, potency and range of actions *in vivo*.

The use of reporter mice could therefore greatly improve current ADMET methodology, overcoming most of the present limitations and allowing a much more predictive analysis of the dosage and profile of action of the drug before it is studied in humans.

III-D. Peroxisome Proliferator-Response Element-Luciferase Mice – PPRE-luc Mice

III-D.1. Principle of PPRE-luc mice

In view of the significant relevance of reporter mouse technology in studying physiological targets through molecular imaging, we developed a model of reporter mice for the peroxisome proliferator-activated receptors (PPARs). These mice (PPRE-luc) were generated by genomic integration of a transgene flanked by insulators and carrying a reporter gene, the *Firefly luciferase (Photinus pyralis)* gene, under the control of PPAR responsive regulatory element (Figure 22). PPRE-luc mice would allow for the ubiquitous and quantitative measurement of the transcriptional activity of PPARs.

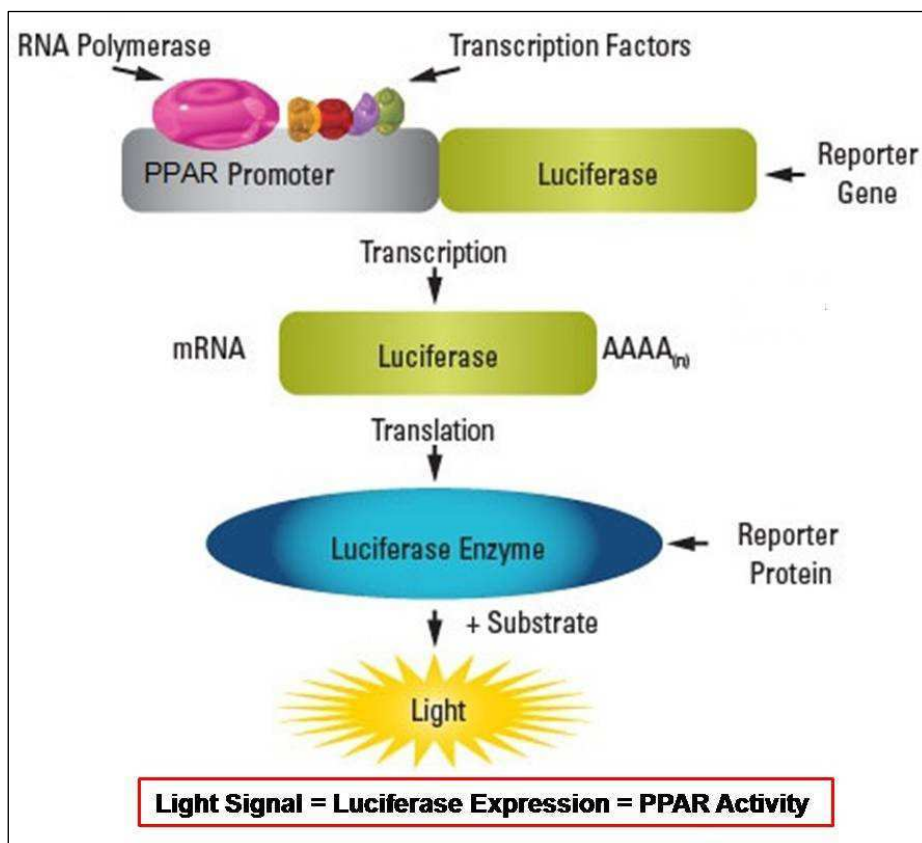


Figure 22. PPRE-Luc mice model. The PPRE-luc mice carry the *luciferase* gene expression under the control of a PPAR-inducible promoter in all organs. Upon PPAR transcriptional activation, the *luciferase* gene is transcribed and translated and the luciferase protein is synthesized. The administered substrate luciferin will be degraded by the luciferase leading to bioluminescence emission. The light intensity reflects the level of luciferase expression and thus the transcriptional activation of PPARs. Updated from (www.piercenet.com).

III-D.2. Construction of PPRE-luc mice

As stated above, PPRE-luc mice were generated by genomic integration of a transgene flanked by insulators and carrying the *Firefly luciferase*, as reporter gene, under the control of PPAR responsive regulatory element or PPRE. The exogenous DNA contains 3 binding sites of the PPAR/RXR heterodimer (PPRE) upstream from the luciferase gene. Generation of PPRE-luc was performed using classical techniques of microinjection of fertilized eggs. Cloning strategy was based on the pdLuc farnesoid X receptor (FXR) plasmid [859]. FXR sequence was replaced by a sequence containing 3 peroxisome proliferator response elements (PPRE). This fragment was ligated into the *Sna*BI and *Not*I sites (Figure 23). To remove the backbone of the transgenic vector, the plasmid was linearized using *Cla*I plus *As*eI and purified after gel electrophoresis. Transgenic mouse lines were obtained after pronuclear DNA injection according to established procedures [860]. Screening for founders and routine genotyping was performed by PCR on genomic DNA isolated from the tail. In mice carrying the transgene, PCRs yield 417 bp and 281 bp products. The transgene was kept in a hemizygous state by crossing founders and their offspring with C57BL/6J mice. For the characterization of founders, mice were treated for 12 h with Rosiglitazone (PPAR γ agonist, 100 mg/kg), L165041 (PPAR β/δ agonist, 100 mg/kg) and WY14643 (PPAR α agonist, 45 mg/kg).

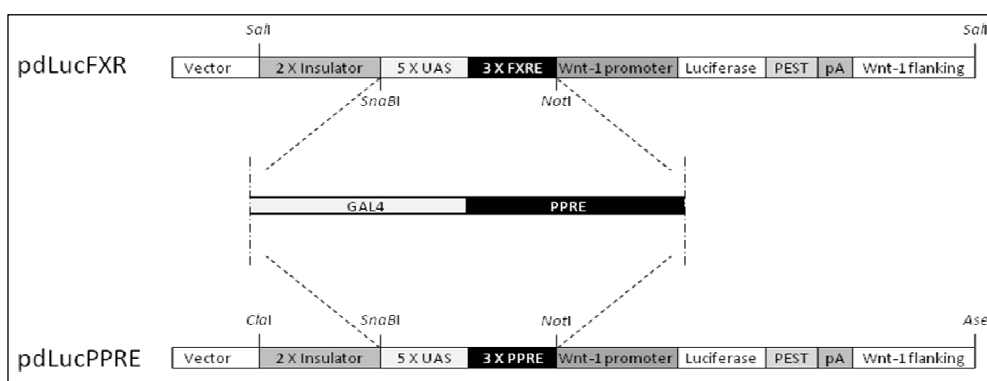


Figure 23. Schematic representation of the cloning strategy performed to obtain the construct used to get transgenic mice. Insulator correspond to chicken beta-globin insulator. UAS, FXRE and PPRE denote the GAL4, FXR and PPAR response elements, respectively. PEST encodes for a proline, glutamic acid, serine and threonine rich region used to destabilize luciferase. pA indicates the position of polyadenylation site. *Sall* are restriction sites used to linearized the transgenic vector. *Sna*BI and *Not*I are used to replace FXRE by PPRE.

III-D.3. Considerations for the Choice of Reporter Gene

Reporter genes such as those encoding β -galactosidase or GFP could not be selected because it is well known that these proteins accumulate in mammalian cells and would not have enabled a dynamic view of PPARs activity to be obtained. The fact that firefly (*Photinus pyralis*) luciferase has a very short turnover rate and does not accumulate in target cells, supporting the selection of the luciferase gene as the reporter in the generation of the PPRE-luc mouse. The advantages of using this gene include the fact that: (i) the gene encodes an insect enzyme that is not present in mammals, and thus the signal to noise ratio was believed to be optimal; (ii) luciferase is easily quantified in tissue extracts by a highly sensitive assay; (iii) antibodies for the immunohistochemical localization of cells expressing the insect enzyme are commercially available; and (iv) optical imaging can be applied to detect the enzyme activity *in vivo*.

III-D.4. PPRE-luc Mice: A tool to Gain New Insights into PPARs

To date, very few studies have addressed the PPRE-luc mice in terms of model characterization and as tool for studying the regulation of the transcriptional activity of PPARs [9, 11, 861].

a) Gender-specific differences in PPARs activity

In a first study, *Ciana et al.* have shown by *in vivo* imaging and that in the liver of female PPRE-luc mice, the PPAR reporter transgene is more than one order of magnitude less expressed, implying that the PPARs signaling in females liver is much less activated than in males. Furthermore, diet or hormonal manipulations did not modulate this low activation [861]. It is in fact well known that PPAR α is the mediator of the hepatic, gender-specific response to fibrates [862]; this receptor subtype is also important for gender-specific lipid and glucose metabolism [863], fat storage [864], and responses to food assumption [865] or inflammatory stimuli [866]. Moreover, it has also been demonstrated that the liver content of PPAR α mRNA and protein is significantly lower in females than in males, and that testosterone is able to elevate female PPAR α to male levels. This body of evidence revealed

that levels of PPAR α expression are directly responsible for the above reported sex differences [867]. However, the authors showed that hepatic PPAR activity in females, remains significantly lower than in males, despite any hormonal or dietary manipulation. They suggested that such impairment implies that factors other than receptor content or hormonal influences would contribute to the limited activity of PPAR α , such as epigenetic events. They have concluded that female liver cells might have impaired transcriptional activity through DR1 elements resulting in this phenotype. It is worth emphasizing that only the use of the PPRE-luc reporter mouse could provide a direct demonstration of impaired PPAR signaling in female liver. Overall, the molecular characterization of this gender-specific transcriptional blockage is of relevance considering the key role of PPAR signaling in the liver physiology.

b) Pharmacological profiling of PPARs ligands

The use of PPRE-luc mice was instrumental to obtain the global pharmacological profiling for the activity of PPAR ligands, not underlined by previous traditional, cell-based studies. The authors showed by *in vivo* imaging differential activation of PPAR α and PPAR γ by their well-known corresponding ligands WY14643 and rosiglitazone, respectively, in different organs such as brain, liver, heart, and testis (Figure 24). The specific PPARs activation was confirmed by the administration of specific antagonists and by the measurement of luciferase activity in protein extracts of corresponding tissues [9]. A complete picture of the different ligands effects on the transcriptional activation of PPARs was illustrated by performing time and dose responses of the modulators, hence specifying the optimal timing and concentrations for further use *in vivo*. The authors were also able to characterize the effect of novel ligands on PPARs activation in various organs [9]. This study was the first to provide evidence of the power of PPRE-luc mice when applied to selective modulation of PPARs activity. For the first time, the introduction of a surrogate marker offers the possibility to directly titrate the action of a drug on its target in space and time, avoiding extrapolations based on drug distribution parameters.

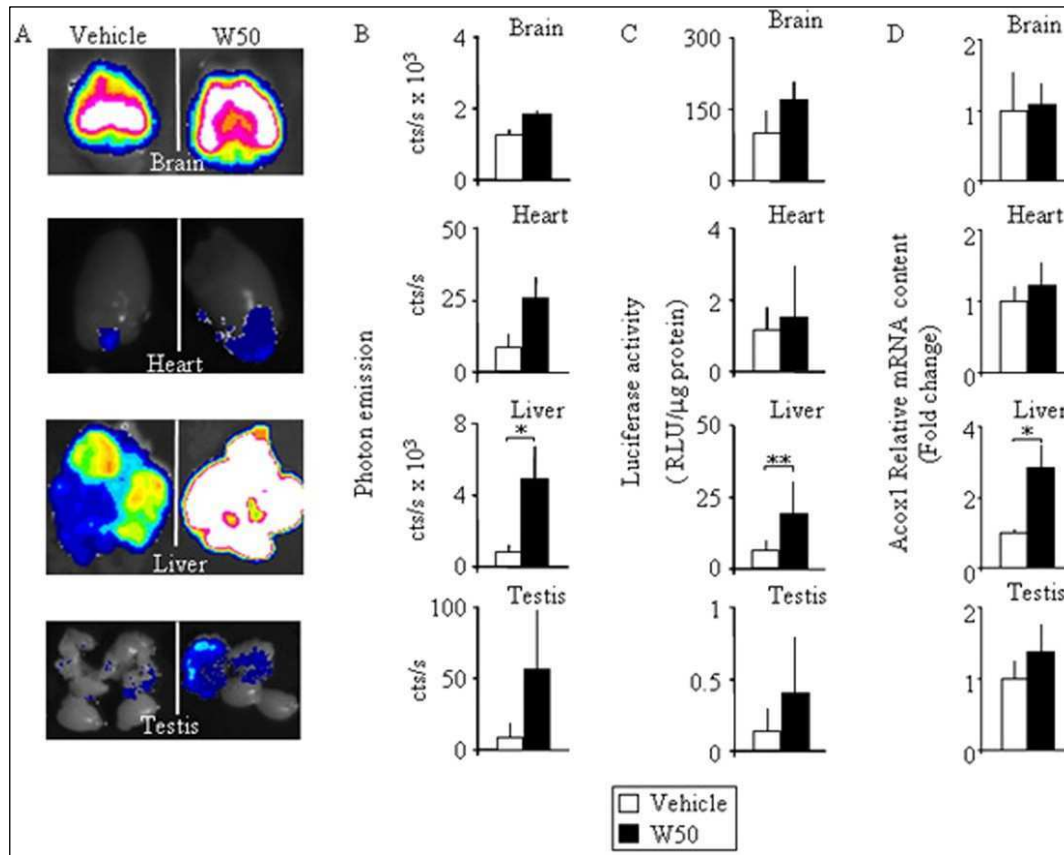


Figure 24. Ex vivo evaluation of the luciferase activity. PPRE-Luc male mice were treated subcutaneously with 50 mg/kg WY14643 (W50) or vehicle and euthanized after 6 h. Organs were rapidly dissected for imaging (A) and photon counting (B) and then frozen for subsequent luciferase analysis by enzymatic assay (C). Luciferase activity was measured by enzymatic assay on protein extracts and expressed as RLU per microgram of proteins. D, the expression of the endogenous PPAR target gene, Acox1, was determined by semi-quantitative real-time polymerase chain reaction (PCR) carried on total mRNA extracted from the indicated tissues [9]. Bars represent mean ± S.E.M. of five mice. *, $P < 0.05$; **, $P < 0.005$ compared with vehicle treatment. P values were calculated by t test. The experiment was repeated three times [9].

In a more recent study, *Nebbioso et al.* have shown that a novel selective inhibitor (MC1568) of class II-histone deacetylase (HDAC) interferes with the PPAR γ -mediated cell differentiation. They have shown that MC1568 blocks the differentiation of 3T3-L1 pre-adipocytes induced by PPAR γ agonists' troglitazone or rosiglitazone by inhibiting the induction of PPAR γ expression. They have demonstrated by *in vivo* imaging that MC1568 impairs PPAR γ -mediated adipogenesis mostly in the heart and adipose tissues (Figure 25)

[11]. The observation that HDAC class II inhibition decreases thiazolidinediones activation of PPAR γ in cell culture as well as *in vivo* suggests that HDAC class II inhibition could potentially be used in combination with PPAR γ agonists to modulate PPAR γ activation in a tissue-specific manner. This would require that these inhibitors preferentially interfere with the deleterious side effects rather than the many beneficial effects of thiazolidinediones.

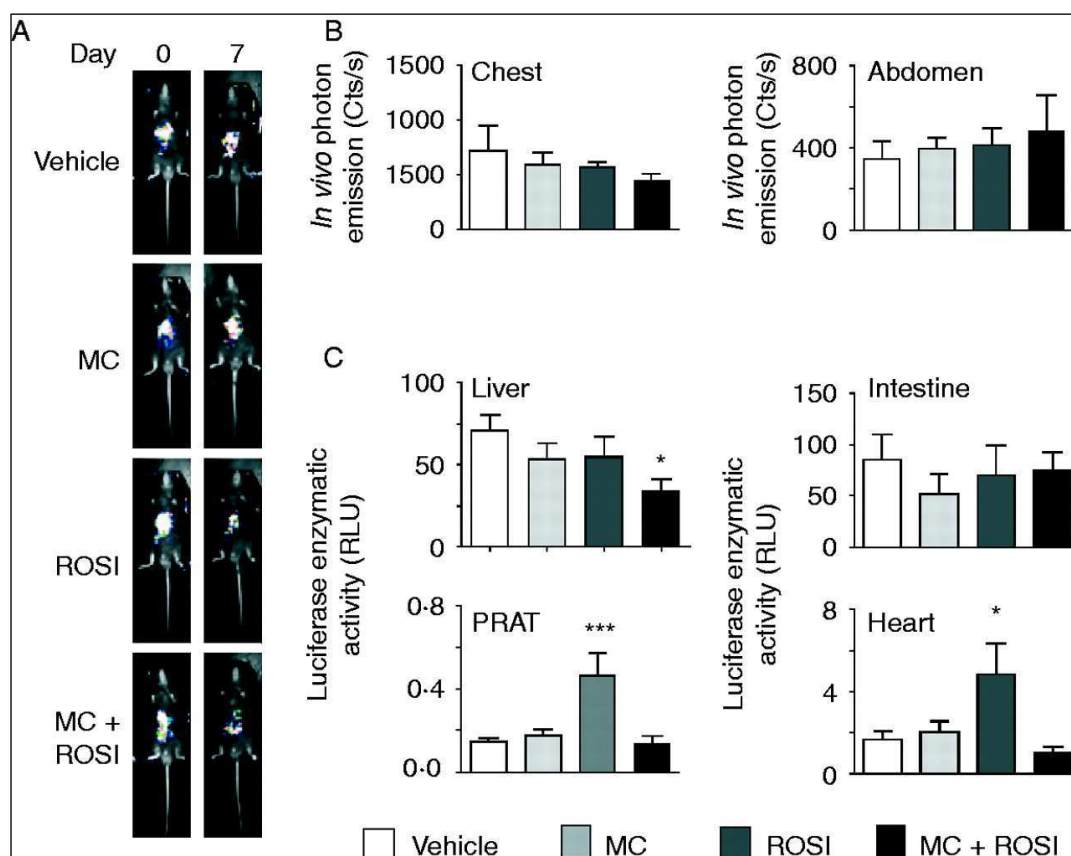


Figure 25. MC1568 antagonizes rosiglitazone-dependent induction of luciferase expression in adipose tissue and heart of the PPRE-Luc mouse. PPRE-Luc mice were treated by gavage daily with 5 mg/kg rosiglitazone (ROSI), 50 mg/kg MC1568 (MC), or vehicle (water solution of 0.5% carboxymethylcellulose); when co-administered, MC1568 was given 30 min before rosiglitazone for 7 days. Photon emission in chest and abdomen was measured at 1700h at day 0 and at the end of the treatment (day 7). **(A)** Pictures of photon emission measured *in vivo* of a single, representative, individual at day 0 and 7. **(B)** Photon emission measured *in vivo* after 7 days treatment; bars represent the mean \pm S.E.M. **(C)** Luciferase contents from liver, intestine, perirenal adipose tissue (PRAT), and heart. Relative luciferase units (RLU, photon counts/ μ g of proteins) were determined by enzymatic assay as described in Materials and methods. Bars represent the mean RLU \pm S.E.M. * P <0.05 and *** P <0.001 versus vehicle-treated group [11].

(C) AIM OF THE WORK

The biological significance of PPARs in skin homeostasis could be dissected at several levels. PPARs play a physiological role in skin maturation, proliferation and differentiation and an important regulatory role in several skin disorders. Experimental studies have shown that PPAR signaling pathways may represent interesting therapeutic targets for a broad variety of inflammatory skin diseases and skin malignancies. Therefore, the generation of specific and valid animal models that would contribute into the characterization and development of new PPAR ligands as therapeutics in skin diseases is necessary.

It has been obvious from the bibliographical data presented above that reporter mouse technology would represent a valuable tool for endless scientific objectives. Reporter mice would allow the characterization of special phenotypes of mice models and the discovery of unrevealed yet, molecular or cellular targets. Besides, they would serve a valuable tool for the pharmacological profiling of novel molecules with specific molecular target. Recent studies have contributed to the elucidation of the biological significance of PPRE-luc mice; however, still too much to be explored. PPRE-luc mice would greatly help, through *in vivo* imaging, in examining the biological role of PPARs and their ligands in specific organs and also in selecting novel molecules with PPARs modulating properties. In this work, we have focused on the transcriptional activation of PPARs in the skin. Our first aim was to establish the necessary parameters for the *in vivo* evaluation through optical imaging of basal PPARs activation in the skin, and only in the skin, without the need to extrapolate based on luciferin distribution. The second goal of this study is to present PPRE-luc mice as credible tool for the *in vivo* evaluation of selective modulation of PPARs activation in the skin through topical application of specific PPARs ligands and subsequent *in vivo* imaging. Overall, this work aimed at establishing the optimal conditions for further assessment of novel PPARs-targeting molecules with potential therapeutic benefit.

(D) PERSONAL WORK

Exp Dermatol – Accepted

In Vivo Imaging Reveals Selective PPAR Activity in the Skin of Peroxisome Proliferator-Activated Receptor Responsive Element-Luciferase Reporter Mice

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Running head: Evaluation of skin PPAR activity by in vivo imaging

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors playing relevant roles in mammalian physiology. Three receptor subtypes have been characterized and named PPAR α , - γ , and - δ (PPAR β). To regulate gene transcription, PPARs heterodimerize with the retinoid-X-receptor and bind to PPAR responsive elements (PPREs) located in the promoter of target genes (1, 2). PPARs and corresponding ligands have been shown in skin to regulate important cellular functions, including cell proliferation and differentiation, as well as inflammatory responses (3-5). It has been shown that the expression of both PPAR α and PPAR γ was decreased in inflammatory skin disorders, including hyperproliferative psoriatic epidermis and atopic dermatitis (6-8). Conversely studies have suggested that thiazolidinediones, PPAR γ ligands, may be beneficial for moderate chronic plaque psoriasis by suppressing proliferation and inducing differentiation of keratinocytes (6, 9). Moreover, recent findings indicated that PPAR-signaling pathways induced apoptosis and terminal differentiation in various human malignant tumors (10-12). Therefore, an increasing body of evidence indicated that PPAR signaling pathways may represent interesting therapeutic targets for skin disorders, including inflammatory diseases and skin malignancies (13).

Thus, major efforts should be made to generate valid models for the characterization and development of new PPAR ligands as therapeutics in skin diseases. A boost to the drug discovery process may come from the combined application of imaging technologies and animal engineering. This is represented by developing reporter animals in which selected molecular events can be visualized in real time in living animals (14, 15). To this aim, we have recently developed a reporter mouse for the ubiquitous and quantitative measurement of the transcriptional activity of PPARs. These mice (PPRE-Luc) were generated by genomic integration of a transgene flanked by insulators and carrying a reporter gene, the *Firefly luciferase* gene, under the control of PPAR responsive regulatory element. The present study highlights the usefulness of PPRE-Luc mice to investigate PPARs activity *in vivo* and demonstrates that PPRE-Luc reporter mouse is a non-invasive powerful tool for selective screening of novel molecules with pharmacological properties.

Evaluation of skin PPAR activity by in vivo imaging

MATERIALS AND METHODS

Chemicals

Pioglitazone, WY14643, and GW6471 were obtained from Sigma-Aldrich (France). GW9662 was purchased from Cayman Chemical, (Interchim, France). D-Luciferin Na salt was obtained from Euromedex (France).

Experimental Animals and Pharmacological Manipulations

Experiments performed in this study were conducted according to governmental guidelines for animal experimentation. Eight weeks male PPRE-Luc mice were shaved on the back one day before the experiment to minimize trimming effect. The effect of PPAR agonists on photons emission was evaluated after topical application of 50 μ l of pioglitazone or WY14643. PPAR α and γ antagonists GW6471 and GW9662 were as well topically applied at 10mM, 10 min before agonist or vehicle treatment. Control mice were treated with the vehicle (acetone).

Bioluminescence Reporter Imaging

Bioluminescence imaging was assessed using a charge-coupled device camera IVIS Imaging system (IVIS Imaging System 50 Series, Caliper Life Sciences, France) consisting of an EMCCD camera equipped with a 50-mm, f/0.95 lens. Mice were anesthetized using Isoflurane gas anesthesia (2%) and 50 μ l of D-luciferin 40mM was topically applied on mice skin 10 min before bioluminescence acquisition. Mice were placed in the light-tight chamber and a grayscale photo of the animals was first taken with dimmed light. Photon emission was then integrated over a period of 5 min. Images were processed using Living Image[®]3.2 Software (Caliper Life Sciences, France). Luminescence measurements are expressed as the integration of the average brightness/pixel unit expressed as photon counts per second (p/s).

Luciferase Enzymatic Assay

Skin from euthanized mice was dissected and protein extracts were prepared by homogenization in Glo Lysis Buffer (Promega, France). Homogenates were centrifuged twice at 1500 rpm for 10 min, supernatants containing luciferase were collected, and protein concentrations were determined by Bradford assay (Sigma-Aldrich, France). Luciferase enzymatic activity was measured by a commercial kit (Bright-Glo[™] Luciferase Assay System, Promega, France). The light intensity was measured using a microplate luminometer (Mithras Berthold, France) over a 10-s time period and expressed as relative light units per milligram of protein (RLU/mg).

Evaluation of skin PPAR activity by in vivo imaging

Statistical Analysis

Statistical analyses were performed using Wilcoxon Test for two independent samples (SPSS Inc., Chicago, USA). Differences were considered statistically significant if $p < 0.05$.

The construction of the transgenic mice is detailed in the supplementary materials and methods

Evaluation of skin PPAR activity by *in vivo* imaging

RESULTS

We first defined the optimal luciferin conditions for topical application on mice skin. Different luciferin concentrations were applied topically (50 μ l) on mice skin and photons emission was measured 10-12 min afterward. Skin bioluminescence augmented gradually as luciferin concentration increased (supp figure 1A). At 40mM the maximal signal was detected and maintained at 60 and 80mM. We have also examined the kinetics of photons emission following luciferin application on mice skin at 40mM and 60mM (supp figure 1B and data not shown). Photons emission was quantified by a series of 2-min acquisitions in each animal from 0 to 40 minutes. Our results revealed that the highest signal was obtained 10 minutes after luciferin application and maintained for at least 30 minutes afterward. Thus, the optimal measurement of local PPAR activity in the skin was obtained by evaluating photon emission 10min after topical application of luciferin at 40mM. These conditions have been therefore applied throughout the study.

The primary objective of this study was to demonstrate that PPRE-Luc mice represent a valuable model for the evaluation of PPAR activity in the skin. Therefore, two concentrations of 10 and 100mM of pioglitazone, PPAR γ ligand, were topically applied on mice skin and *in vivo* luciferase activity was quantified at 1, 2, 4, 6, 8, and 24 hours afterward. As shown in figure 1A, 100mM of pioglitazone induced a stronger signal than the 10mM dose. However, both concentrations displayed the same profile of photon emission with a maximal bioluminescence detected 6 hours after PPAR γ agonist application. In order to determine the optimal dose of pioglitazone, we have tested a larger range of agonist concentrations at 6 hours post treatment. A comparable increase in photon emission was detected at 10, 50 and 75mM of pioglitazone, and a significantly higher photon flux was observed at 100 and 200mM reflecting a biphasic response (Figure 1B). Since these results revealed two distinct peaks with two different concentration ranges (10-75mM and 100-200mM), the 50 and 100mM doses were chosen to further investigate the specificity of the signals. To this aim, PPRE-Luc mice were treated with pioglitazone in the presence of specific PPAR γ and/or PPAR α antagonists, GW9662 and GW6471, respectively (Figure 1C and 1D). The photon flux induced by pioglitazone 50mM was significantly inhibited by GW9662 (86% decrease, $p < 0.005$) and barely affected by GW6471. Co-treatment with GW9662 and GW6471 did not further decrease luciferase activity compared to GW9662 alone. When using 100mM of pioglitazone the response profile to PPAR antagonists was quite different. The bioluminescence induced by pioglitazone was significantly decreased by the addition of either GW9662 or GW6471 (73% decrease and 55% decrease respectively; $p < 0.005$). Moreover a strong additive effect was obtained in the presence of both inhibitors (95% decrease, $p < 0.001$ compared to GW9662 or GW6471 alone).

Evaluation of skin PPAR activity by *in vivo* imaging

We extended the evaluation of PPAR skin activation by testing the effect of WY14643, PPAR α agonist, on skin bioluminescence of PPRE-Luc mice. Again, the profile of WY14643 effect on PPRE-Luc mice skin revealed two characteristic plateau of photon emission between 10 to 50mM and 200 to 300mM (Figure 2A). Similarly to pioglitazone, significant increase in bioluminescence was detected at 2 hours post-treatment reaching the maximum activation at 6 hours and returning to basal levels at 24 hours (Figure 2B). The increase in bioluminescence following treatment with 10mM WY14643 was significantly inhibited by GW6471 (80% decrease, $p<0.005$) but not by GW9662 (Figure 2C). The addition of GW9662 to GW6471 did not modify the inhibitory effect of this latter (80% decrease, $p<0.005$). However, the photon emission induced by 200mM of WY14643 was significantly suppressed by either GW6471 or GW9662 (54%decrease and 42% decrease respectively; $p<0.005$) and a greater inhibition was detected in the presence of both antagonists (86% decrease, $p<0.001$ compared to GW9662 or GW6471 alone) (Figure 2D).

To confirm our *in vivo* data of selective modulation of PPAR activity and to eliminate potential irrelevant signals from deeper tissues such as subcutaneous fat, *in vitro* luciferase activity was assayed in protein extracts from PPRE-Luc mice skin. Figure3 A-D showed that at the lowest concentrations of pioglitazone and WY14643, luciferase activity was solely inhibited by corresponding antagonists GW9662 and GW6471 respectively (Figure 3A and 3C). But at higher concentrations, the effects of pioglitazone and WY14643 were reduced by GW9662 and also GW6471. Further inhibition was obtained by the simultaneous application of both PPAR antagonists (Figure 3B and 3D). These results correlate to those obtained in living animals, indicating that luciferase activity measured following topical application of PPARs modulators reflects skin specific PPAR activity.

DISCUSSION

The development of novel drugs is a lengthy process requiring years of preclinical research. Thus, novel methodologies that shorten the time for pharmacokinetic and toxicological studies in animals are needed (15-17). The present study provides a novel usage of PPRE-Luc reporter mouse model for identification of new drug candidates targeting PPARs in the skin.

To date, few studies have considered PPRE-Luc mice for the assessment of transcriptional activity of PPARs. Described protocol allowed for broad luciferin distribution into the animal body and organ-specific evaluation of PPAR activity was based on projected localization of the target organ and in some cases *ex vivo* measurement of luciferase activity (18-20). Here, we showed, for the first time, that specific evaluation of skin PPAR activity in PPRE-Luc mice is feasible by topical application of 40mM luciferin allowing direct diffusion into skin layers within 10 minutes. Using this protocol, we were able to eliminate irrelevant signals from internal organs, with the exception for limited signal from subcutaneous adipose tissue to which luciferin could diffuse. Nevertheless, comparison of luciferase activity profiles between living animals and dissected skin samples indicated a negligible contribution of this fat depot.

The foremost aim of this model is testing pharmacological properties of new molecules and formulations. Given the growing interest in the role of PPARs in skin homeostasis, we made a novel usage of PPRE-Luc mice as a new tool dedicated to evaluate PPAR modulators onto skin of living animals. The advantages of PPRE-Luc mice, or reporter mice in general, over the methods currently in use for preclinical drug development can be summarized as follows: (i) it is a non-invasive way allowing a quantitative measurement and direct visualization of the process in a specific target organ or tissue; (ii) The possibility to carry out longitudinal studies in single individuals during repeated drug treatment to unravel sites of drug accumulation and activity, or the dynamics of the target response to the treatment; (iii) the possibility to perform time course studies with limited use of experimental animals; (iv) The great advantage of using the same animal for consecutive analysis is that each animal acts as its own statistical control for previous and subsequent examinations.

Using PPAR ligands, we tried to modulate and measure PPAR activity by evaluating *in vivo* bioluminescence and *in vitro* luciferase activity. Following treatment with pioglitazone and WY14643, PPAR activation was maximal at 6 hours and returned to basal levels at 24 hours. At low to moderate concentrations, pioglitazone and WY14643 selectively activated PPAR γ and PPAR α respectively since photons emission was specifically inhibited by corresponding antagonists GW9662 and GW6471. However, at excessive concentrations, the signal emitted by either ligand was significantly reduced

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by both GW9662 and GW6471 and further decreased when both inhibitors were added. These observations revealed decreased PPAR ligand specificity at high concentrations and potential overlapping transactivation of PPAR α and PPAR γ . *Welch, J.S. et al.* have previously demonstrated an overlapping transactivation and transrepression functions of PPAR γ and PPAR β/δ . They have shown that in PPAR γ -deficient macrophages, high concentrations of rosiglitazone induced regulation of several genes by activating PPAR β/δ (21). In this study, we showed that the quantification of selective activation and inhibition of PPAR subtypes is achievable in skin of living PPRE-Luc mice. Our model simplifies significantly the analysis of drug activity targeting PPARs, providing the possibility to directly titer the effect of a drug on its target in space and time, avoiding extrapolations based on drug distribution parameters.

The model described here could be further improved by crossing PPRE-Luc mice with mice models knockout for a specific PPAR isoform in the skin (22). These models would complement in vitro pharmacological evaluation of PPAR ligands by in vivo assessment of isoforms specificity. Furthermore, PPRE-Luc mice could be bred into skin disease mouse models, an approach that will allow in the same animal to test therapeutic utility and signaling mechanisms of new chemical entities (14, 23).

This study provides evidence of the powerfulness of the PPRE-Luc mouse model for the characterization and development of PPARs ligands targeting skin diseases. Offering the possibility of carrying quantitative longitudinal and time course studies, this non-invasive technique shows superiority over the conventional cell-based tests in the prediction of ligand potency and efficacy.

REFERENCES

1. Clark, R.B. The role of PPARs in inflammation and immunity. *J Leukoc Biol*: 2002: 71: 388-400.
2. Daynes, R.A. and D.C. Jones. Emerging roles of PPARs in inflammation and immunity. *Nat Rev Immunol*: 2002: 2: 748-59.
3. Friedmann, P.S., H.L. Cooper, and E. Healy. Peroxisome proliferator-activated receptors and their relevance to dermatology. *Acta Derm Venereol*: 2005: 85: 194-202.
4. Kuenzli, S. and J.H. Saurat. Peroxisome proliferator-activated receptors in cutaneous biology. *Br J Dermatol*: 2003: 149: 229-36.
5. Sertznig, P. and J. Reichrath. Peroxisome proliferator-activated receptors (PPARs) in dermatology: Challenge and promise. *Dermatoendocrinol*: 2011: 3: 130-5.
6. Ellis, C.N., J. Varani, G.J. Fisher, et al. Troglitazone improves psoriasis and normalizes models of proliferative skin disease: ligands for peroxisome proliferator-activated receptor-gamma inhibit keratinocyte proliferation. *Arch Dermatol*: 2000: 136: 609-16.
7. Plager, D.A., A.A. Leontovich, S.A. Henke, et al. Early cutaneous gene transcription changes in adult atopic dermatitis and potential clinical implications. *Exp Dermatol*: 2007: 16: 28-36.
8. Rivier, M., I. Safonova, P. Lebrun, C.E. Griffiths, G. Ailhaud, and S. Michel. Differential expression of peroxisome proliferator-activated receptor subtypes during the differentiation of human keratinocytes. *J Invest Dermatol*: 1998: 111: 1116-21.
9. Robertshaw, H. and P.S. Friedmann. Pioglitazone: a promising therapy for psoriasis. *Br J Dermatol*: 2005: 152: 189-91.
10. Sertznig, P., M. Seifert, W. Tilgen, and J. Reichrath. Present concepts and future outlook: function of peroxisome proliferator-activated receptors (PPARs) for pathogenesis, progression, and therapy of cancer. *J Cell Physiol*: 2007: 212: 1-12.
11. Hanley, K., Y. Jiang, S.S. He, et al. Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPARalpha. *J Invest Dermatol*: 1998: 110: 368-75.
12. Mossner, R., P. Meyer, F. Jankowski, et al. Variations in the peroxisome proliferator-activated receptor-gamma gene and melanoma risk. *Cancer Lett*: 2007: 246: 218-23.
13. Kopelovich, L., J.R. Fay, R.I. Glazer, and J.A. Crowell. Peroxisome proliferator-activated receptor modulators as potential chemopreventive agents. *Mol Cancer Ther*: 2002: 1: 357-63.
14. Maggi, A. and P. Ciana. Reporter mice and drug discovery and development. *Nat Rev Drug Discov*: 2005: 4: 249-55.
15. Lucignani, G., L. Ottobrini, C. Martelli, M. Rescigno, and M. Clerici. Molecular imaging of cell-mediated cancer immunotherapy. *Trends Biotechnol*: 2006: 24: 410-8.
16. Ramachandran, B., A. Stell, L. Maravigna, A. Maggi, and P. Ciana. Novel insights on imaging sex-hormone-dependent tumorigenesis in vivo. *Endocr Relat Cancer*: 2011: 18: R41-51.
17. Ottobrini, L., P. Ciana, A. Biserni, G. Lucignani, and A. Maggi. Molecular imaging: a new way to study molecular processes in vivo. *Mol Cell Endocrinol*: 2006: 246: 69-75.
18. Biserni, A., F. Giannessi, A.F. Sciarroni, F.M. Milazzo, A. Maggi, and P. Ciana. In vivo imaging reveals selective peroxisome proliferator activated receptor modulator activity of the synthetic ligand 3-(1-(4-chlorobenzyl)-3-t-butylthio-5-isopropylindol-2-yl)-2,2-dimethylpropanoic acid (MK-886). *Mol Pharmacol*: 2008: 73: 1434-43.
19. Ciana, P., A. Biserni, L. Tatangelo, et al. A novel peroxisome proliferator-activated receptor responsive element-luciferase reporter mouse reveals gender specificity of peroxisome proliferator-activated receptor activity in liver. *Mol Endocrinol*: 2007: 21: 388-400.
20. Nebbioso, A., C. Dell'Aversana, A. Bugge, et al. HDACs class II-selective inhibition alters nuclear receptor-dependent differentiation. *J Mol Endocrinol*: 2010: 45: 219-28.
21. Welch, J.S., M. Ricote, T.E. Akiyama, F.J. Gonzalez, and C.K. Glass. PPARgamma and PPARdelta negatively regulate specific subsets of lipopolysaccharide and IFN-gamma target genes in macrophages. *Proc Natl Acad Sci U S A*: 2003: 100: 6712-7.

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22. Sahu, R.P., S.C. DaSilva, B. Rashid, et al. Mice lacking epidermal PPARgamma exhibit a marked augmentation in photocarcinogenesis associated with increased UVB-induced apoptosis, inflammation and barrier dysfunction. *Int J Cancer*: 2012: 131: E1055-66.
23. Maggi, A. and G. Rando. Reporter mice for the study of intracellular receptor activity. *Methods Mol Biol*: 2009: 590: 307-16.

FIGURES

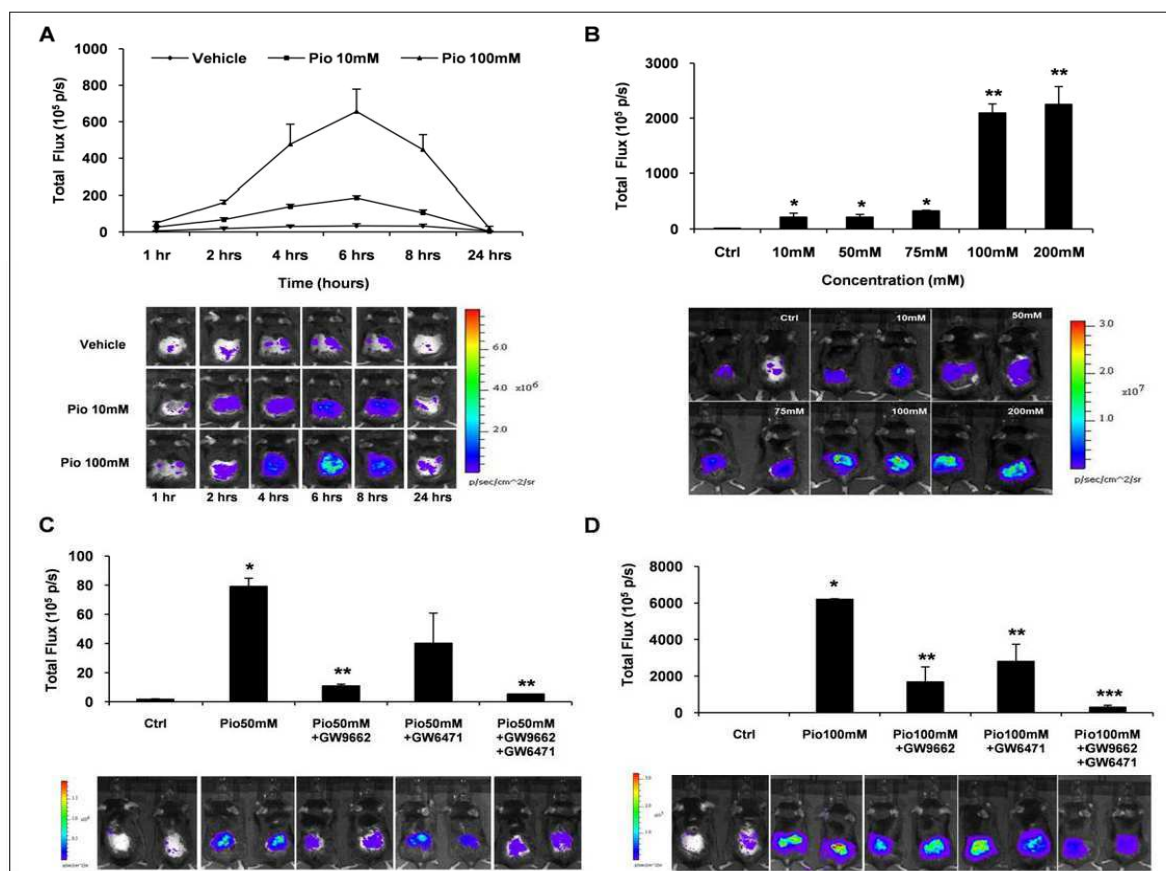


FIGURE 1: Modulation of bioluminescence emission from skin of PPRE-Luc mice after pioglitazone treatment. **A**, Optical imaging (1 representative animal) and photon counting (p/s = photons per second) of luciferase activity of PPRE-Luc mice topically treated with pioglitazone at 10mM, 100mM or vehicle (acetone). Photon counting was performed at 1, 2, 4, 6, 8, and 24 hours. **B**, Optical imaging (2 representative animals) and photon counting of luciferase activity of PPRE-Luc mice topically treated topically for 6 h with vehicle (acetone) or pioglitazone at 10mM, 50mM, 75mM, 100mM, or 200mM. * $P < 0.05$ versus control, ** $P < 0.05$ versus 75mM. **C and D**, Optical imaging (1 representative animal) and photon counting of luciferase activity in PPRE-Luc mice topically treated for 6 h with vehicle (acetone), pioglitazone at 50mM (**C**) or pioglitazone at 100mM (**D**), with or without GW9662 (10mM) and/or GW6471 (10mM). * $P < 0.05$ versus control, ** $P < 0.05$ versus agonist, *** $P < 0.05$ versus agonist + antagonist.

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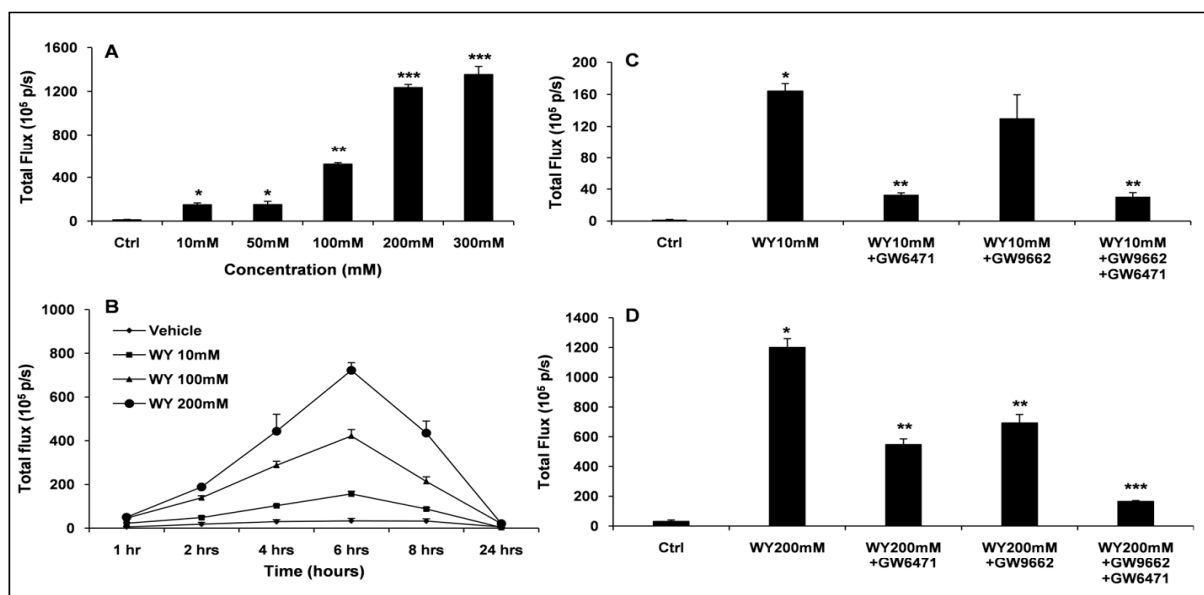


FIGURE 2: Modulation of bioluminescence emission from skin of PPRE-Luc mice after WY14643 treatment. **A**, Photon counting (p/s = photons per second) of luciferase activity of PPRE-Luc mice topically treated topically for 6 h with vehicle (acetone) or WY14643 at 10mM, 50mM, 100mM, 200mM or 300mM. * $P < 0.05$ versus control. ** $P < 0.05$ versus 50mM, *** $P < 0.05$ versus 100mM. **B**, Photon counting of luciferase activity of PPRE-Luc mice topically treated with WY14643 at 10mM, 100mM, 200mM or vehicle (acetone). Photon counting was performed at 1, 2, 4, 6, 8, and 24 hours. **C and D**, photon counting of luciferase activity of PPRE-Luc mice topically treated for 6 h with vehicle (acetone), WY14643 at 10mM (**C**) or WY14643 at 200mM (**D**), with or without GW9662 (10mM) and/or GW6471 (10mM). # $P < 0.05$ versus control, ** $P < 0.05$ versus agonist, *** $P < 0.05$ versus agonist + antagonist.

Evaluation of skin PPAR activity by in vivo imaging

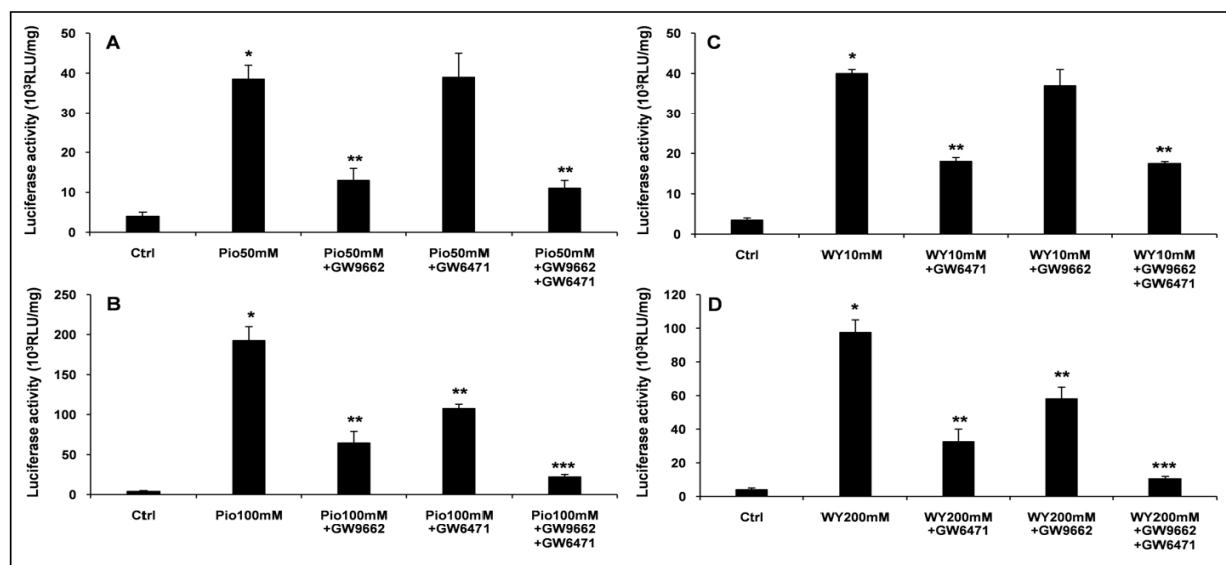


FIGURE 3: Luciferase enzymatic activity in skin extracts of PPRE-Luc mice after treatment with pioglitazone or WY14643. A-D, mice were topically treated for 6 hours with pioglitazone at 50 and 100mM (A and B), or WY14643 at 10 and 200mM (C and D) with or without GW9662 and/or GW6471. Skin proteins were extracted and luciferase activity was measured and expressed as relative luciferase units (RLU) per milligrams of protein. * $P < 0.05$ versus control, ** $P < 0.05$ versus agonist, * $P < 0.05$ versus agonist + antagonist.**

SUPPLEMENTARY DATA

MATERIALS AND METHODS

Transgenic Mice

To study PPAR activation *in vivo* under different conditions, we developed a model of transgenic mice expressing the luciferase gene after PPAR activation. The exogenous DNA contains 3 binding sites of the PPAR/RXR heterodimer (PPRE) upstream from the luciferase gene. Generation of these transgenic mice (PPRE-*Luc*) was performed in collaboration with Professors Pierre Chambon and Johan Auwerx at the Mouse Clinic Institute (Illkirch, France) using classical techniques of microinjection of fertilized eggs. Briefly, cloning strategy was based on the pdLuc FXR plasmid (received from Prof. Johan Auwerx). FXR sequence was replaced by a sequence containing 3 peroxisome proliferator response elements (PPRE). This fragment was ligated into the SnaBI and NotI sites (Figure S2). To remove the backbone of the transgenic vector, the plasmid was linearized using *ClaI* plus *AseI* and purified after gel electrophoresis. Transgenic mouse lines were obtained after pronuclear DNA injection according to established procedures. Screening for founders and routine genotyping was performed by PCR on genomic DNA isolated from the tail using primers in table S1. In mice carrying the transgene, PCRs yield 417 bp and 281 bp products. The transgene was kept in a hemizygous state by crossing founders and their offspring with C57BL/6J mice. For the characterization of founders, mice were treated for 12 h with Rosiglitazone (PPAR γ agonist, 100 mg/kg), L165041 (PPAR β/δ agonist, 100 mg/kg) and WY14643 (PPAR α agonist, 45 mg/kg).

Evaluation of skin PPAR activity by in vivo imaging

SUPPLEMENTARY FIGURES

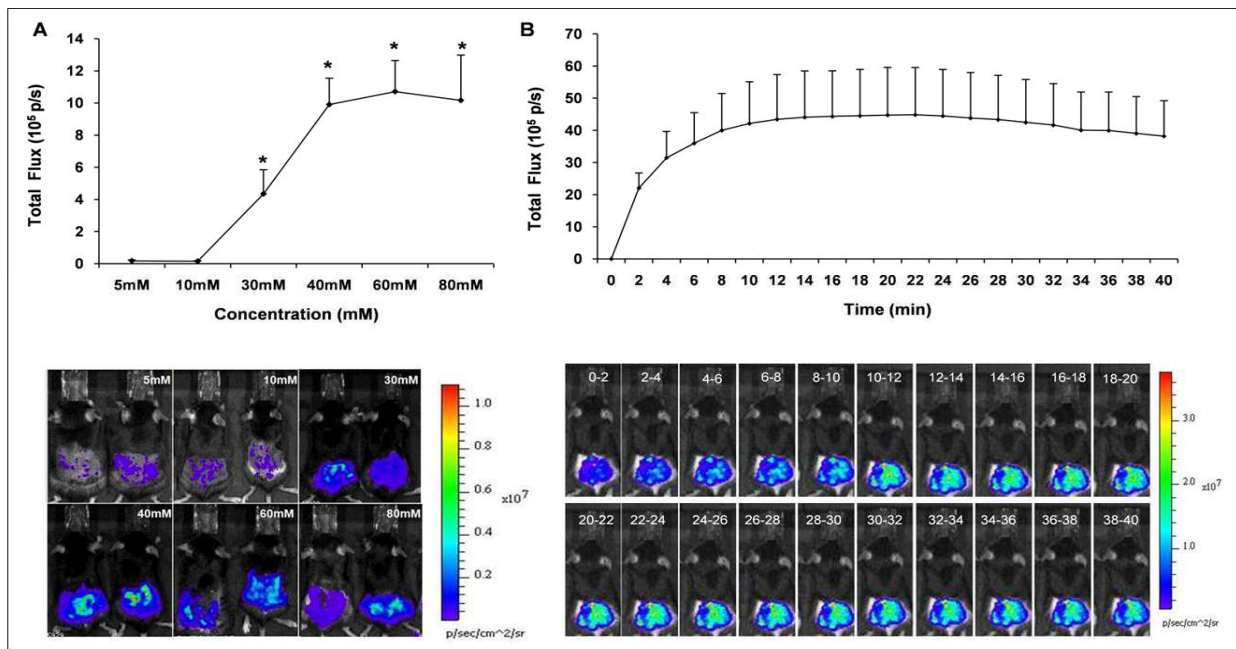


FIGURE S1: Concentration and time study for D-Luciferin application on skin. A, Optical imaging and photon counting (p/s = photons per second) of the luciferase activity of male PPRE-Luc mice, 10 min after topical application of 5, 10, 20, 40, 60, and 80mM of luciferin. Photon emission was quantified in skin by a series of 5-min CCD camera acquisitions in each animal. Data represent the mean \pm S.E.M. of acquisitions made in groups of 5 to 10 animals and images show acquisition of 2 representative animals; * $p < 0.05$. B, Optical imaging and photon counting (p/s = photons per second) of the luciferase activity of male PPRE-Luc mice following application of 40mM of luciferin. Photon emission was quantified in skin by a series of 2-min CCD camera acquisitions in each animal from 0 to 40 minutes following luciferin application. Data represent the mean \pm S.E.M. of acquisitions made in groups of 5 to 10 animals and images show acquisition of 1 representative animal.

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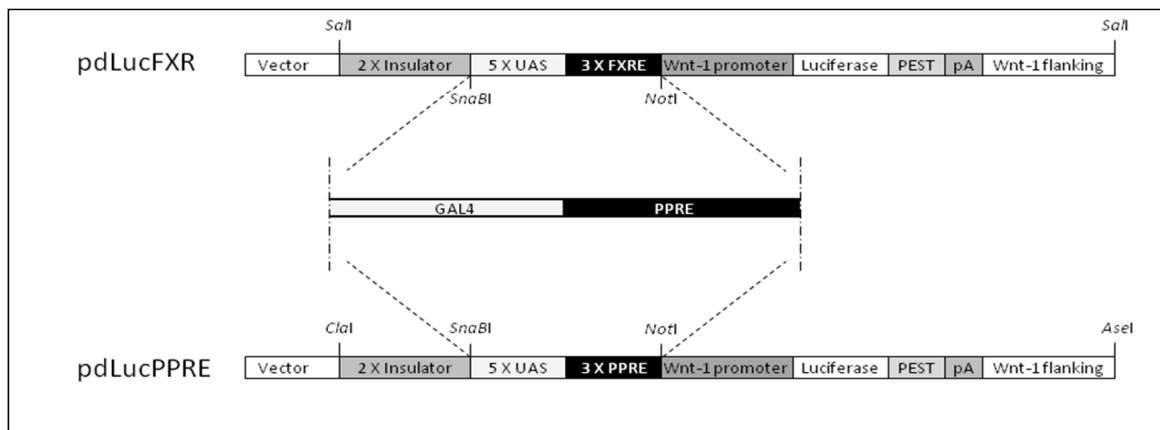


FIGURE S2: Schematic representation of the cloning strategy performed to obtain the construct used to get transgenic mice. Insulator correspond to chicken beta-globin insulator. UAS, FXRE and PPRE denote the GAL4, FXR and PPAR response elements, respectively. PEST encodes for a proline, glutamic acid, serine and threonine rich region used to destabilize luciferase. pA indicates the position of polyadenylation site. *SalI* are restriction sites used to linearized the transgenic vector. *SnaBI* and *NotI* are used to replace FXRE by PPRE.

(E) CONCLUSION AND PERSPECTIVES

Since their initial characterization more than two decades ago, the PPARs have been shown essential for the normal physiological function of most mammalian cell types, tissues and organ systems. Consequently, it is not surprising that many disease states have now been linked to abnormalities in the function of the PPAR-regulated transcription factors. In particular, PPARs are involved in diverse biological activities in the epidermis, such as keratinocyte proliferation and differentiation, epidermal barrier maturation and recovery, sebocyte activity, and melanocyte differentiation. In addition, their overall anti-inflammatory functions are well characterized [868]. Therefore, PPARs represent a major research target for the understanding and treatment of many skin diseases, particularly those involving inflammation and epidermal hyperproliferation, such as benign epidermal tumors, papillomas, and psoriasis. Due to involvement in the control of sebocyte differentiation, the modulation of PPAR activity in order to interfere with sebum production may also be an interesting development for the treatment of acne vulgaris, characterized by excess sebum production. Thus, targeting PPARs signaling pathways may represent interesting therapeutic ways for various dermatologic diseases.

However, the development of novel drugs is a lengthy process requiring years of preclinical research and many steps indispensable to ensure that the molecule of interest can be administered to humans with a minimal risk of toxic effects. Therefore, pharmaceutical companies are eager to apply novel methodologies that shorten the time required for pharmacodynamic, pharmacokinetic and toxicological studies to be carried out *in vitro* and in animal systems. Despite their success, conventional microscopy methods suffer significant limitations when used in biological experimentation. They usually require chemical fixation of removed tissues, involve the observation of biological samples under non-physiological conditions, can generally not resolve the dynamics of cellular processes, and most importantly, it has been very difficult to generate quantitative data using conventional microscopy. The impact of these relative drawbacks of microscopy may be lessened to some extent by the use of noninvasive molecular imaging techniques in intact living subjects. Currently, quantitative analysis of molecular events in living organisms is done with the combined application of imaging and genetic engineering technologies,

leading to the generation of reporter mice where molecular events can be visualized in real time *in vivo*. Functional, and efficient, results of *in vivo* imaging assays are potentially attainable quicker and less labor-intensively than with conventional *in vitro* or cell culture study of biological materials.

In this work, we have established all necessary parameters that would define PPRE-luc as a valid model for the *in vivo* evaluation of basal and induced transcriptional activation of PPARs in the skin. Overall, this work might serve as basis for further studies aiming at the pharmacological profiling of novel molecules targeting PPARs. Interestingly, nowadays, a large number of pharmacological PPAR ligands (thiazolidinediones, fibrates) are already registered and clinically used for other diseases. Knowing that the route from identification to registration of drugs is long and expensive, drugs already registered for the treatment of one disease and eventually effective for others are of high interest, and might reveal interesting potentials when investigated using the PPRE-luc mice model.

A very important question needs to be addressed when applying the reporter mouse technology in molecular imaging: it is mainly about the sensitivity of the reporter system. Effectively, available data in the literature showed that the transcriptional regulation of PPARs is modulated by a multitude of factors which vary spatio-temporally. The metabolic state of the animal, the circadian rhythm, the presence of any kind of stress, and many various stimuli, would affect PPARs activity, thus limiting the possibilities to obtain a clear picture of the exact state of the activation of PPARs. Thus, several preventive measures were carried out in order to maximally limit inter-individual variability. First, even though this study addressed PPARs activation in the skin, a non-metabolic organ; the feeding of mice was limited to the night period, an approach that has significantly contributed to the stability of basal PPARs activity in the skin. Second, all animals used in this study were from the same sex and approximately the same age. Third, skin mice were always shaved one day before experimentation in order to limit the effect of shaving and skin irritation on PPARs activation. Fourth, unlike other studies, we have avoided the use of olive oil as a vehicle for PPARs ligands, knowing that it is rich in unsaturated fatty acids which act as natural PPARs activators. Fifth, the examination of PPRE-luc mice using *in vivo* optical imaging prevented animal sacrifice; thus each animal would act as its own control for previous or subsequent

experiments leading to reduction of experimental uncertainties arising from physiological deviation. The use of these standardized procedures has decreased the intra-group variability from 50% to about 15%. Once the relatively “stable” physiological picture of PPARs activity in the skin has been defined, the effects of PPARs ligands on its transcriptional activation can be identified.

Compared with classical preclinical investigation models, PPRE-luc mouse model has a much broader potential because it can: (a) reveal in which organs or tissues a given compound might exert a certain effect on PPAR, independently of previously acquired knowledge of the target tissue; (b) assess the effects of and changes in response after repeated administration of a given PPAR modulator; (c) perform time course and dose response studies on the same animal; (d) test the effect of PPARs-targeting molecule using different routes of administration; (e) evaluate the minimal drug concentration needed to stimulate PPARs independently of the drug’s plasma levels; (f) help combining pharmacological and toxicological studies to verify potential sites of drug accumulation during chronic administration. In fact, imaging technologies provide a global view of drug actions, permitting its effects to be observed also in non target tissues, thus facilitating the prediction of potential adverse effects in organs other than the defined target; (g) verify whether repeated administration of a PPARs activator causes tachyphylaxis (rapidly decreasing response to a drug after administration of a few doses) phenomena during response (h) limit the number of animals used; (i) limit or completely abolish the pain of the animals used since it is as a non-invasive technique. Nevertheless, a possible drawback for the use of the PPRE-luc reporter mouse is the limited possibility to discriminate which subtype of PPARs is actually contributing to the luciferase expression; however, this problem may be overcome by breeding the model with subtype-specific knockout models or using selective antagonists as we have shown. In conclusion, the availability of reporter mice would greatly improve the predictability of substance efficiency and safety, and could produce more critical and accurate information much earlier in drug development process, thus reducing late-stage withdrawal risk.

One should not forget that the utility of PPRE-luc mice extends largely beyond the skin as target-organ. Considering that most tissues in mammals express PPARs and that each

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synthetic chemical may have an unpredictable tissue-specific action on PPARs, the PPRE-Luc mouse model represents at present the most appropriate model to obtain a realistic, systemic view of the effects of endogenous or exogenous potential PPARs ligands. In view of these observations, the model here described, particularly if crossed with other mice models in which the ablation of a specific PPAR subtype has been operated, will be of major interest for the study of disorders where PPARs are implicated and for the identification of novel synthetic ligands for therapeutic use. Moreover, PPRE-luc mice could be bred into diabetes or colorectal carcinoma model mice to investigate the relevance of PPAR activity and its pharmacological control in the etiopathogenesis of these disorders. Also, with the application of PPRE-luc mouse model, abnormal and healthy organisms can be compared to identify phenotype alterations elicited by a disease in PPARs might play a major regulatory role.

In the preclinical research, reporter systems combining imaging technologies and animal engineering would give a new impetus to the biomedical research facilitating pharmacological profiling of drugs; open thereby new avenues for the development of innovative drugs with by limited side effects.

SYNOPSIS

- The *in vitro* expression of GLP-2R has been detected only in human intestinal myofibroblasts, mouse enteroendocrine cells, and the rat enteric nervous system.
- The *in vivo* expression of GLP-2R transcript extends to several organs, notably, bladder, central nervous system, mesenteric adipose tissue, spleen and liver.
- The expression of GLP-2R in the gastrointestinal tract follows an increasing gradient toward the colon and rectum
- The intestinal expression of GLP-2R is decreased in mice models of colitis and in IBD patients.
 - Future studies should aim at examining the full range of potential GLP-2 actions.
 - The high colonic GLP-2R expression could reflect important role for GLP-2 in the colon under healthy and pathophysiological states.
 - The decreased GLP-2R expression under inflammatory conditions might redirect the therapeutic use of GLP-2 in inflammatory bowel disease.

- The anti-proliferative and pro-apoptotic properties on tumor-derived intestinal epithelial cell lines of 5-ASA exhibits are significantly inhibited by GW9662 (PPAR γ antagonist)
- The tumor limiting effect of 5-ASA on mass volume and weight is suppressed by co-treatment of GW9662 in a model of Xenograft tumor in SCID mice
- The anti-neoplastic effect of 5-ASA on aberrant crypt formation in a mouse model of colon carcinogenesis is abolished by co-administration of GW9662.
 - The well-recognized anti-neoplastic effect of 5-ASA are mediated at least partially via PPAR γ .
 - This work provide rational for further designing more effective and safe anti-neoplastic PPAR γ ligands with topical effects

- The *in vivo* evaluation of basal PPAR transcriptional activation in the skin of PPRE-luc mice is achievable by topically applying 40mM of luciferin followed by optical imaging 10 minutes afterwards.
- The *in vivo* selective modulation of PPARs subtypes in the skin of PPRE-luc mice can be measured through optical imaging after topical application of specific PPARs ligands.
 - PPRE-luc mice represent a valuable reporter mouse model for the *in vivo* pharmacological profiling of drugs targeting PPARs in the skin

REFERENCE LIST

1. Terzic, J., et al., *Inflammation and colon cancer*. Gastroenterology, 2010. **138**(6): p. 2101-2114 e5.
2. Straus, D.S. and C.K. Glass, *Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms*. Trends Immunol, 2007. **28**(12): p. 551-8.
3. Iacucci, M., S. de Silva, and S. Ghosh, *Mesalazine in inflammatory bowel disease: a trendy topic once again?* Can J Gastroenterol, 2010. **24**(2): p. 127-33.
4. Chourasia, M.K. and S.K. Jain, *Pharmaceutical approaches to colon targeted drug delivery systems*. J Pharm Pharm Sci, 2003. **6**(1): p. 33-66.
5. Drucker, D.J., et al., *Induction of intestinal epithelial proliferation by glucagon-like peptide 2*. Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(15): p. 7911-6.
6. Stell, A., et al., *Multimodality imaging: novel pharmacological applications of reporter systems*. Q J Nucl Med Mol Imaging, 2007. **51**(2): p. 127-38.
7. Michalik, L. and W. Wahli, *Peroxisome proliferator-activated receptors (PPARs) in skin health, repair and disease*. Biochim Biophys Acta, 2007. **1771**(8): p. 991-8.
8. Montagner, A., et al., *New insights into the role of PPARs*. Prostaglandins Leukot Essent Fatty Acids, 2011. **85**(5): p. 235-43.
9. Biserni, A., et al., *In vivo imaging reveals selective peroxisome proliferator activated receptor modulator activity of the synthetic ligand 3-(1-(4-chlorobenzyl)-3-t-butylthio-5-isopropylindol-2-yl)-2,2-dimethylpropanoic acid (MK-886)*. Mol Pharmacol, 2008. **73**(5): p. 1434-43.
10. Yessoufou, A. and W. Wahli, *Multifaceted roles of peroxisome proliferator-activated receptors (PPARs) at the cellular and whole organism levels*. Swiss Med Wkly, 2010. **140**: p. w13071.
11. Nebbioso, A., et al., *HDACs class II-selective inhibition alters nuclear receptor-dependent differentiation*. J Mol Endocrinol, 2010. **45**(4): p. 219-28.
12. Phillips, S., *Acute Colitis and Ulcerative Colitis*. Br Med J, 1907. **1**(2423): p. 1355-60.
13. Tanaka, T., *Development of an inflammation-associated colorectal cancer model and its application for research on carcinogenesis and chemoprevention*. Int J Inflam, 2012. **2012**: p. 658786.
14. Han, S.W. and J. Roman, *Anticancer actions of PPARgamma ligands: Current state and future perspectives in human lung cancer*. World J Biol Chem, 2010. **1**(3): p. 31-40.

15. Krishnan, A., S.A. Nair, and M.R. Pillai, *Biology of PPAR gamma in cancer: a critical review on existing lacunae*. *Curr Mol Med*, 2007. **7**(6): p. 532-40.
16. Jahn, K.A., Y. Su, and F. Braet, *Multifaceted nature of membrane microdomains in colorectal cancer*. *World J Gastroenterol*, 2011. **17**(6): p. 681-90.
17. Moraes, L.A., L. Piqueras, and D. Bishop-Bailey, *Peroxisome proliferator-activated receptors and inflammation*. *Pharmacol Ther*, 2006. **110**(3): p. 371-85.
18. Drucker, D.J., *Biologic actions and therapeutic potential of the proglucagon-derived peptides*. *Nat Clin Pract Endocrinol Metab*, 2005. **1**(1): p. 22-31.
19. Ottobriani, L., et al., *Molecular imaging: a new way to study molecular processes in vivo*. *Mol Cell Endocrinol*, 2006. **246**(1-2): p. 69-75.
20. Tee, C.T., K. Wallis, and S.M. Gabe, *Emerging treatment options for short bowel syndrome: potential role of teduglutide*. *Clin Exp Gastroenterol*, 2011. **4**: p. 189-96.
21. Ullman, T.A. and S.H. Itzkowitz, *Intestinal inflammation and cancer*. *Gastroenterology*, 2011. **140**(6): p. 1807-16.
22. Peters, J.M., Y.M. Shah, and F.J. Gonzalez, *The role of peroxisome proliferator-activated receptors in carcinogenesis and chemoprevention*. *Nat Rev Cancer*, 2012. **12**(3): p. 181-95.
23. Massoud, T.F. and S.S. Gambhir, *Molecular imaging in living subjects: seeing fundamental biological processes in a new light*. *Genes Dev*, 2003. **17**(5): p. 545-80.
24. Yazbeck, R., G.S. Howarth, and C.A. Abbott, *Growth factor based therapies and intestinal disease: is glucagon-like peptide-2 the new way forward?* *Cytokine Growth Factor Rev*, 2009. **20**(2): p. 175-84.
25. Sertznig, P., et al., *Peroxisome proliferator-activated receptors (PPARs) and the human skin: importance of PPARs in skin physiology and dermatologic diseases*. *Am J Clin Dermatol*, 2008. **9**(1): p. 15-31.
26. Round, J.L. and S.K. Mazmanian, *The gut microbiota shapes intestinal immune responses during health and disease*. *Nat Rev Immunol*, 2009. **9**(5): p. 313-23.
27. Drucker, D.J., et al., *Human [Gly2]GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis*. *Am J Physiol*, 1999. **276**(1 Pt 1): p. G79-91.
28. Baumgart, D.C. and S.R. Carding, *Inflammatory bowel disease: cause and immunobiology*. *Lancet*, 2007. **369**(9573): p. 1627-40.
29. Sherwood, N.M., S.L. Krueckl, and J.E. McRory, *The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily*. *Endocr Rev*, 2000. **21**(6): p. 619-70.
30. Holst, J.J., *Glucagon and glucagon-like peptides 1 and 2*. *Results Probl Cell Differ*, 2010. **50**: p. 121-35.

31. Xiao, Q., et al., *Secretion of the intestinotropic hormone glucagon-like peptide 2 is differentially regulated by nutrients in humans*. *Gastroenterology*, 1999. **117**(1): p. 99-105.
32. Brubaker, P.L., *The glucagon-like peptides: pleiotropic regulators of nutrient homeostasis*. *Ann N Y Acad Sci*, 2006. **1070**: p. 10-26.
33. Burrin, D.G., B. Stoll, and X. Guan, *Glucagon-like peptide 2 function in domestic animals*. *Domest Anim Endocrinol*, 2003. **24**(2): p. 103-22.
34. Tavares, W., D.J. Drucker, and P.L. Brubaker, *Enzymatic- and renal-dependent catabolism of the intestinotropic hormone glucagon-like peptide-2 in rats*. *Am J Physiol Endocrinol Metab*, 2000. **278**(1): p. E134-9.
35. Ruiz-Grande, C., et al., *Renal catabolism of human glucagon-like peptides 1 and 2*. *Can J Physiol Pharmacol*, 1990. **68**(12): p. 1568-73.
36. Jeppesen, P.B., et al., *Teduglutide (ALX-0600), a dipeptidyl peptidase IV resistant glucagon-like peptide 2 analogue, improves intestinal function in short bowel syndrome patients*. *Gut*, 2005. **54**(9): p. 1224-31.
37. Wallis, K., J.R. Walters, and A. Forbes, *Review article: glucagon-like peptide 2--current applications and future directions*. *Aliment Pharmacol Ther*, 2007. **25**(4): p. 365-72.
38. Jeppesen, P.B., et al., *Short bowel patients treated for two years with glucagon-like Peptide 2: effects on intestinal morphology and absorption, renal function, bone and body composition, and muscle function*. *Gastroenterol Res Pract*, 2009. **2009**: p. 616054.
39. Wojdemann, M., et al., *Inhibition of sham feeding-stimulated human gastric acid secretion by glucagon-like peptide-2*. *J Clin Endocrinol Metab*, 1999. **84**(7): p. 2513-7.
40. Nagell, C.F., et al., *Glucagon-like peptide-2 inhibits antral emptying in man, but is not as potent as glucagon-like peptide-1*. *Scand J Gastroenterol*, 2004. **39**(4): p. 353-8.
41. Cheeseman, C.I., *Upregulation of SGLT-1 transport activity in rat jejunum induced by GLP-2 infusion in vivo*. *Am J Physiol*, 1997. **273**(6 Pt 2): p. R1965-71.
42. Ramsanahie, A., et al., *Effect of GLP-2 on mucosal morphology and SGLT1 expression in tissue-engineered neointestine*. *Am J Physiol Gastrointest Liver Physiol*, 2003. **285**(6): p. G1345-52.
43. Guan, X., et al., *GLP-2 receptor localizes to enteric neurons and endocrine cells expressing vasoactive peptides and mediates increased blood flow*. *Gastroenterology*, 2006. **130**(1): p. 150-64.
44. Guan, X., et al., *GLP-2-mediated up-regulation of intestinal blood flow and glucose uptake is nitric oxide-dependent in TPN-fed piglets 1*. *Gastroenterology*, 2003. **125**(1): p. 136-47.
45. Burrin, D.G., et al., *Glucagon-like peptide 2 dose-dependently activates intestinal cell survival and proliferation in neonatal piglets*. *Endocrinology*, 2005. **146**(1): p. 22-32.

46. Tsai, C.H., M. Hill, and D.J. Drucker, *Biological determinants of intestinotrophic properties of GLP-2 in vivo*. Am J Physiol, 1997. **272**(3 Pt 1): p. G662-8.
47. Kouris, G.J., et al., *The effect of glucagon-like peptide 2 on intestinal permeability and bacterial translocation in acute necrotizing pancreatitis*. Am J Surg, 2001. **181**(6): p. 571-5.
48. Burrin, D.G., et al., *GLP-2 stimulates intestinal growth in premature TPN-fed pigs by suppressing proteolysis and apoptosis*. Am J Physiol Gastrointest Liver Physiol, 2000. **279**(6): p. G1249-56.
49. Yusta, B., R.P. Boushey, and D.J. Drucker, *The glucagon-like peptide-2 receptor mediates direct inhibition of cellular apoptosis via a cAMP-dependent protein kinase-independent pathway*. J Biol Chem, 2000. **275**(45): p. 35345-52.
50. Brubaker, P.L., et al., *Intestinal function in mice with small bowel growth induced by glucagon-like peptide-2*. Am J Physiol, 1997. **272**(6 Pt 1): p. E1050-8.
51. Kato, Y., D. Yu, and M.Z. Schwartz, *Glucagonlike peptide-2 enhances small intestinal absorptive function and mucosal mass in vivo*. J Pediatr Surg, 1999. **34**(1): p. 18-20; discussion 20-1.
52. Shin, E.D., et al., *Mucosal adaptation to enteral nutrients is dependent on the physiologic actions of glucagon-like peptide-2 in mice*. Gastroenterology, 2005. **128**(5): p. 1340-53.
53. Dube, P.E., et al., *The essential role of insulin-like growth factor-1 in the intestinal tropic effects of glucagon-like peptide-2 in mice*. Gastroenterology, 2006. **131**(2): p. 589-605.
54. Anini, Y., et al., *Role of phosphatidylinositol-3 kinase-gamma in the actions of glucagon-like peptide-2 on the murine small intestine*. Am J Physiol Endocrinol Metab, 2007. **292**(6): p. E1599-606.
55. Sigalet, D.L., et al., *Enteric neural pathways mediate the anti-inflammatory actions of glucagon-like peptide 2*. American journal of physiology. Gastrointestinal and liver physiology, 2007. **293**(1): p. G211-21.
56. Bremholm, L., et al., *Glucagon-like peptide-2 increases mesenteric blood flow in humans*. Scand J Gastroenterol, 2009. **44**(3): p. 314-9.
57. Hsieh, J., et al., *Glucagon-like peptide-2 increases intestinal lipid absorption and chylomicron production via CD36*. Gastroenterology, 2009. **137**(3): p. 997-1005, 1005 e1-4.
58. Cani, P.D., et al., *Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability*. Gut, 2009. **58**(8): p. 1091-103.
59. Hadjiyanni, I., K.K. Li, and D.J. Drucker, *Glucagon-like peptide-2 reduces intestinal permeability but does not modify the onset of type 1 diabetes in the nonobese diabetic mouse*. Endocrinology, 2009. **150**(2): p. 592-9.

60. Booth, C., et al., *Teduglutide ([Gly2]GLP-2) protects small intestinal stem cells from radiation damage*. *Cell Prolif*, 2004. **37**(6): p. 385-400.
61. Bjerknes, M. and H. Cheng, *Modulation of specific intestinal epithelial progenitors by enteric neurons*. *Proc Natl Acad Sci U S A*, 2001. **98**(22): p. 12497-502.
62. Dube, P.E., K.J. Rowland, and P.L. Brubaker, *Glucagon-like peptide-2 activates beta-catenin signaling in the mouse intestinal crypt: role of insulin-like growth factor-I*. *Endocrinology*, 2008. **149**(1): p. 291-301.
63. Vrang, N. and P.J. Larsen, *Preproglucagon derived peptides GLP-1, GLP-2 and oxyntomodulin in the CNS: role of peripherally secreted and centrally produced peptides*. *Prog Neurobiol*, 2010. **92**(3): p. 442-62.
64. Lovshin, J.A., et al., *Extrahypothalamic expression of the glucagon-like peptide-2 receptor is coupled to reduction of glutamate-induced cell death in cultured hippocampal cells*. *Endocrinology*, 2004. **145**(7): p. 3495-506.
65. Velazquez, E., E. Blazquez, and J.M. Ruiz-Albusac, *Synergistic effect of glucagon-like peptide 2 (GLP-2) and of key growth factors on the proliferation of cultured rat astrocytes. Evidence for reciprocal upregulation of the mRNAs for GLP-2 and IGF-I receptors*. *Mol Neurobiol*, 2009. **40**(2): p. 183-93.
66. Velazquez, E., J.M. Ruiz-Albusac, and E. Blazquez, *Glucagon-like peptide-2 stimulates the proliferation of cultured rat astrocytes*. *European journal of biochemistry / FEBS*, 2003. **270**(14): p. 3001-9.
67. Voss, U., et al., *Glucagon-like peptides 1 and 2 and vasoactive intestinal peptide are neuroprotective on cultured and mast cell co-cultured rat myenteric neurons*. *BMC Gastroenterol*, 2012. **12**: p. 30.
68. Iwai, T., et al., *Antidepressant-like effects of glucagon-like peptide-2 in mice occur via monoamine pathways*. *Behav Brain Res*, 2009. **204**(1): p. 235-40.
69. Jeppesen, P.B., et al., *Randomised placebo-controlled trial of teduglutide in reducing parenteral nutrition and/or intravenous fluid requirements in patients with short bowel syndrome*. *Gut*, 2011. **60**(7): p. 902-14.
70. Jeppesen, P.B., et al., *Short bowel patients treated for two years with glucagon-like peptide 2 (GLP-2): compliance, safety, and effects on quality of life*. *Gastroenterol Res Pract*, 2009. **2009**: p. 425759.
71. Buchman, A.L., et al., *Teduglutide, a novel mucosally active analog of glucagon-like peptide-2 (GLP-2) for the treatment of moderate to severe Crohn's disease*. *Inflammatory bowel diseases*, 2010. **16**(6): p. 962-73.

72. Thulesen, J., et al., *The truncated metabolite GLP-2 (3-33) interacts with the GLP-2 receptor as a partial agonist*. Regul Pept, 2002. **103**(1): p. 9-15.
73. Bahrami, J., et al., *Glucagon-like peptide-2 receptor modulates islet adaptation to metabolic stress in the ob/ob mouse*. Gastroenterology, 2010. **139**(3): p. 857-68.
74. Dahly, E.M., et al., *Role of luminal nutrients and endogenous GLP-2 in intestinal adaptation to mid-small bowel resection*. Am J Physiol Gastrointest Liver Physiol, 2003. **284**(4): p. G670-82.
75. Martin, G.R., L.E. Wallace, and D.L. Sigalet, *Glucagon-like peptide-2 induces intestinal adaptation in parenterally fed rats with short bowel syndrome*. Am J Physiol Gastrointest Liver Physiol, 2004. **286**(6): p. G964-72.
76. Bahrami, J., B. Yusta, and D.J. Drucker, *ErbB activity links the glucagon-like peptide-2 receptor to refeeding-induced adaptation in the murine small bowel*. Gastroenterology, 2010. **138**(7): p. 2447-56.
77. Nelson, D.W., et al., *Insulin-like growth factor I and glucagon-like peptide-2 responses to fasting followed by controlled or ad libitum refeeding in rats*. Am J Physiol Regul Integr Comp Physiol, 2008. **294**(4): p. R1175-84.
78. Hartmann, B., et al., *Immunoneutralization of endogenous glucagon-like peptide-2 reduces adaptive intestinal growth in diabetic rats*. Regul Pept, 2002. **105**(3): p. 173-9.
79. Munroe, D.G., et al., *Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2*. Proc Natl Acad Sci U S A, 1999. **96**(4): p. 1569-73.
80. Lovshin, J., et al., *Glucagon-like peptide (GLP)-2 action in the murine central nervous system is enhanced by elimination of GLP-1 receptor signaling*. The Journal of biological chemistry, 2001. **276**(24): p. 21489-99.
81. Nelson, D.W., et al., *Localization and activation of glucagon-like peptide-2 receptors on vagal afferents in the rat*. Endocrinology, 2007. **148**(5): p. 1954-62.
82. Yusta, B., et al., *Enteroendocrine localization of GLP-2 receptor expression in humans and rodents*. Gastroenterology, 2000. **119**(3): p. 744-55.
83. de Heer, J., et al., *The alpha cell expresses glucagon-like peptide-2 receptors and glucagon-like peptide-2 stimulates glucagon secretion from the rat pancreas*. Diabetologia, 2007. **50**(10): p. 2135-42.
84. Orskov, C., et al., *GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors*. Regulatory peptides, 2005. **124**(1-3): p. 105-12.
85. Yusta, B., et al., *Identification of glucagon-like peptide-2 (GLP-2)-activated signaling pathways in baby hamster kidney fibroblasts expressing the rat GLP-2 receptor*. J Biol Chem, 1999. **274**(43): p. 30459-67.

86. Koehler, J.A., B. Yusta, and D.J. Drucker, *The HeLa cell glucagon-like peptide-2 receptor is coupled to regulation of apoptosis and ERK1/2 activation through divergent signaling pathways*. Mol Endocrinol, 2005. **19**(2): p. 459-73.
87. Yusta, B., J. Estall, and D.J. Drucker, *Glucagon-like peptide-2 receptor activation engages bad and glycogen synthase kinase-3 in a protein kinase A-dependent manner and prevents apoptosis following inhibition of phosphatidylinositol 3-kinase*. J Biol Chem, 2002. **277**(28): p. 24896-906.
88. Estall, J.L., et al., *The glucagon-like peptide-2 receptor C terminus modulates beta-arrestin-2 association but is dispensable for ligand-induced desensitization, endocytosis, and G-protein-dependent effector activation*. J Biol Chem, 2005. **280**(23): p. 22124-34.
89. Estall, J.L., B. Yusta, and D.J. Drucker, *Lipid raft-dependent glucagon-like peptide-2 receptor trafficking occurs independently of agonist-induced desensitization*. Mol Biol Cell, 2004. **15**(8): p. 3673-87.
90. Sams, A., et al., *Naturally occurring glucagon-like peptide-2 (GLP-2) receptors in human intestinal cell lines*. European journal of pharmacology, 2006. **532**(1-2): p. 18-23.
91. Hoosein, N.M. and R.S. Gurd, *Human glucagon-like peptides 1 and 2 activate rat brain adenylate cyclase*. FEBS Lett, 1984. **178**(1): p. 83-6.
92. Walsh, N.A., et al., *Glucagon-like peptide-2 receptor activation in the rat intestinal mucosa*. Endocrinology, 2003. **144**(10): p. 4385-92.
93. Desbois-Mouthon, C., et al., *Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3beta inhibition and Ras activation*. Oncogene, 2001. **20**(2): p. 252-9.
94. Muncan, V., et al., *Rapid loss of intestinal crypts upon conditional deletion of the Wnt/Tcf-4 target gene c-Myc*. Mol Cell Biol, 2006. **26**(22): p. 8418-26.
95. Pinto, D., et al., *Canonical Wnt signals are essential for homeostasis of the intestinal epithelium*. Genes Dev, 2003. **17**(14): p. 1709-13.
96. Bennett, W.R., et al., *Structural-proliferative units and organ growth: effects of insulin-like growth factor 2 on the growth of colon and skin*. Development, 2003. **130**(6): p. 1079-88.
97. Powell, D.W., et al., *Myofibroblasts. II. Intestinal subepithelial myofibroblasts*. The American journal of physiology, 1999. **277**(2 Pt 1): p. C183-201.
98. Iwakiri, D. and D.K. Podolsky, *Keratinocyte growth factor promotes goblet cell differentiation through regulation of goblet cell silencer inhibitor*. Gastroenterology, 2001. **120**(6): p. 1372-80.

99. Washizawa, N., et al., *Comparative effects of glucagon-like peptide-2 (GLP-2), growth hormone (GH), and keratinocyte growth factor (KGF) on markers of gut adaptation after massive small bowel resection in rats*. JPEN J Parenter Enteral Nutr, 2004. **28**(6): p. 399-409.
100. Helmrich, M.A., C.R. Erwin, and B.W. Warner, *A defective EGF-receptor in waved-2 mice attenuates intestinal adaptation*. J Surg Res, 1997. **69**(1): p. 76-80.
101. Yusta, B., et al., *ErbB signaling is required for the proliferative actions of GLP-2 in the murine gut*. Gastroenterology, 2009. **137**(3): p. 986-96.
102. Hare, K.J., et al., *The intestinotrophic peptide, glp-2, counteracts intestinal atrophy in mice induced by the epidermal growth factor receptor inhibitor, gefitinib*. Clin Cancer Res, 2007. **13**(17): p. 5170-5.
103. MacDonald, T.T., *Epithelial proliferation in response to gastrointestinal inflammation*. Ann N Y Acad Sci, 1992. **664**: p. 202-9.
104. Burrin, D.G., et al., *GLP-2 rapidly activates divergent intracellular signaling pathways involved in intestinal cell survival and proliferation in neonatal piglets*. Am J Physiol Endocrinol Metab, 2007. **292**(1): p. E281-91.
105. O'Brien, D.P., et al., *Intestinal adaptation: structure, function, and regulation*. Semin Pediatr Surg, 2001. **10**(2): p. 56-64.
106. McMellen, M.E., et al., *Growth factors: possible roles for clinical management of the short bowel syndrome*. Semin Pediatr Surg, 2010. **19**(1): p. 35-43.
107. Jeppesen, P.B., *Glucagon-like peptide-2: update of the recent clinical trials*. Gastroenterology, 2006. **130**(2 Suppl 1): p. S127-31.
108. Marier, J.F., et al., *Population pharmacokinetics of teduglutide following repeated subcutaneous administrations in healthy participants and in patients with short bowel syndrome and Crohn's disease*. J Clin Pharmacol, 2010. **50**(1): p. 36-49.
109. Mardini, H.E. and W.J. de Villiers, *Teduglutide in intestinal adaptation and repair: light at the end of the tunnel*. Expert Opin Investig Drugs, 2008. **17**(6): p. 945-51.
110. Sigalet, D.L., et al., *GLP-2 levels in infants with intestinal dysfunction*. Pediatr Res, 2004. **56**(3): p. 371-6.
111. Sangild, P.T., et al., *Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs*. Gastroenterology, 2006. **130**(6): p. 1776-92.
112. Mouksassi, M.S., et al., *Clinical trial simulations in pediatric patients using realistic covariates: application to teduglutide, a glucagon-like peptide-2 analog in neonates and infants with short-bowel syndrome*. Clin Pharmacol Ther, 2009. **86**(6): p. 667-71.

113. Taqi, E., et al., *The influence of nutrients, biliary-pancreatic secretions, and systemic trophic hormones on intestinal adaptation in a Roux-en-Y bypass model*. J Pediatr Surg, 2010. **45**(5): p. 987-95.
114. Kaji, T., et al., *Temporal changes in the intestinal growth promoting effects of glucagon-like peptide 2 following intestinal resection*. J Surg Res, 2009. **152**(2): p. 271-80.
115. Alavi, K., et al., *Treatment of inflammatory bowel disease in a rodent model with the intestinal growth factor glucagon-like peptide-2*. J Pediatr Surg, 2000. **35**(6): p. 847-51.
116. Arthur, G.L., et al., *Glucagonlike peptide-2 analogue: a possible new approach in the management of inflammatory bowel disease*. J Pediatr Surg, 2004. **39**(3): p. 448-52; discussion 448-52.
117. Lomax, A.E., et al., *Persistent alterations to enteric neural signaling in the guinea pig colon following the resolution of colitis*. Am J Physiol Gastrointest Liver Physiol, 2007. **292**(2): p. G482-91.
118. O'Hara, J.R., et al., *Ileitis alters neuronal and enteroendocrine signalling in guinea pig distal colon*. Gut, 2007. **56**(2): p. 186-94.
119. Ivory, C.P., et al., *Interleukin-10-independent anti-inflammatory actions of glucagon-like peptide 2*. American journal of physiology. Gastrointestinal and liver physiology, 2008. **295**(6): p. G1202-10.
120. L'Heureux, M.C. and P.L. Brubaker, *Glucagon-like peptide-2 and common therapeutics in a murine model of ulcerative colitis*. J Pharmacol Exp Ther, 2003. **306**(1): p. 347-54.
121. Geier, M.S., et al., *Development and resolution of experimental colitis in mice with targeted deletion of dipeptidyl peptidase IV*. J Cell Physiol, 2005. **204**(2): p. 687-92.
122. Yazbeck, R., et al., *Inhibiting dipeptidyl peptidase activity partially ameliorates colitis in mice*. Front Biosci, 2008. **13**: p. 6850-8.
123. Xiao, Q., et al., *Circulating levels of glucagon-like peptide-2 in human subjects with inflammatory bowel disease*. Am J Physiol Regul Integr Comp Physiol, 2000. **278**(4): p. R1057-63.
124. Schmidt, P.T., et al., *Tissue levels and post-prandial secretion of the intestinal growth factor, glucagon-like peptide-2, in controls and inflammatory bowel disease: comparison with peptide YY*. Eur J Gastroenterol Hepatol, 2005. **17**(2): p. 207-12.
125. Schnabel, C.A., M. Wintle, and O. Kolterman, *Metabolic effects of the incretin mimetic exenatide in the treatment of type 2 diabetes*. Vasc Health Risk Manag, 2006. **2**(1): p. 69-77.
126. Ahren, B., et al., *Improved meal-related beta-cell function and insulin sensitivity by the dipeptidyl peptidase-IV inhibitor vildagliptin in metformin-treated patients with type 2 diabetes over 1 year*. Diabetes Care, 2005. **28**(8): p. 1936-40.

127. Orskov, C., J.J. Holst, and O.V. Nielsen, *Effect of truncated glucagon-like peptide-1 [proglucagon-(78-107) amide] on endocrine secretion from pig pancreas, antrum, and nonantral stomach*. *Endocrinology*, 1988. **123**(4): p. 2009-13.
128. Meier, J.J., et al., *Glucagon-like peptide 2 stimulates glucagon secretion, enhances lipid absorption, and inhibits gastric acid secretion in humans*. *Gastroenterology*, 2006. **130**(1): p. 44-54.
129. Christensen, M., et al., *Glucagon-like peptide-2, but not glucose-dependent insulinotropic polypeptide, stimulates glucagon release in patients with type 1 diabetes*. *Regul Pept*, 2010. **163**(1-3): p. 96-101.
130. Cameron, H.L. and M.H. Perdue, *Stress impairs murine intestinal barrier function: improvement by glucagon-like peptide-2*. *J Pharmacol Exp Ther*, 2005. **314**(1): p. 214-20.
131. Watson, A.J. and C.A. Duckworth, *Gut microbiota control gut permeability through GLP-2*. *Gastroenterology*, 2010. **138**(2): p. 779-81.
132. Boeckstaens, G.E. and W.J. de Jonge, *Neuroimmune mechanisms in postoperative ileus*. *Gut*, 2009. **58**(9): p. 1300-11.
133. Augestad, K.M. and C.P. Delaney, *Postoperative ileus: impact of pharmacological treatment, laparoscopic surgery and enhanced recovery pathways*. *World J Gastroenterol*, 2010. **16**(17): p. 2067-74.
134. Fukuda, H., et al., *The selective mu opioid receptor antagonist, alvimopan, improves delayed GI transit of postoperative ileus in rats*. *Brain Res*, 2006. **1102**(1): p. 63-70.
135. Shibata, C., et al., *Effect of glucagon, glicentin, glucagon-like peptide-1 and -2 on interdigestive gastroduodenal motility in dogs with a vagally denervated gastric pouch*. *Scand J Gastroenterol*, 2001. **36**(10): p. 1049-55.
136. Moore, B.A., et al., *GLP-2 receptor agonism ameliorates inflammation and gastrointestinal stasis in murine postoperative ileus*. *J Pharmacol Exp Ther*, 2010. **333**(2): p. 574-83.
137. Turler, A., et al., *Endogenous endotoxin participates in causing a panenteric inflammatory ileus after colonic surgery*. *Ann Surg*, 2007. **245**(5): p. 734-44.
138. Sonis, S.T., et al., *Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients*. *Cancer*, 2004. **100**(9 Suppl): p. 1995-2025.
139. Hill, G.R. and J.L. Ferrara, *The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation*. *Blood*, 2000. **95**(9): p. 2754-9.
140. Yazbeck, R., C.A. Abbott, and G.S. Howarth, *The use of GLP-2 and related growth factors in intestinal diseases*. *Curr Opin Investig Drugs*, 2010. **11**(4): p. 440-6.

141. Moon, Y., H. Yang, and Y.B. Kim, *Up-regulation of early growth response gene 1 (EGR-1) via ERK1/2 signals attenuates sulindac sulfide-mediated cytotoxicity in the human intestinal epithelial cells*. *Toxicol Appl Pharmacol*, 2007. **223**(2): p. 155-63.
142. Enestvedt, C.K., et al., *Clinical review: Healing in gastrointestinal anastomoses, part II*. *Microsurgery*, 2006. **26**(3): p. 137-43.
143. Wheeless, C.R., Jr., et al., *The effect of growth hormone on the bursting strength of ileal anastomotic segments in radiation-injured rat bowel*. *Gynecol Oncol*, 1998. **70**(1): p. 121-2.
144. Zacharakis, E., et al., *Contribution of insulin-like growth factor I to the healing of colonic anastomoses in rats*. *J Invest Surg*, 2007. **20**(1): p. 9-14.
145. Redstone, H.A., et al., *The effect of glucagon-like Peptide-2 receptor agonists on colonic anastomotic wound healing*. *Gastroenterol Res Pract*, 2010. **2010**.
146. Koehler, J.A., et al., *Glucagon-like peptide-2 does not modify the growth or survival of murine or human intestinal tumor cells*. *Cancer Res*, 2008. **68**(19): p. 7897-904.
147. Iakoubov, R., et al., *Carcinogenic effects of exogenous and endogenous glucagon-like peptide-2 in azoxymethane-treated mice*. *Endocrinology*, 2009. **150**(9): p. 4033-43.
148. Thulesen, J., et al., *Glucagon-like peptide 2 (GLP-2) accelerates the growth of colonic neoplasms in mice*. *Gut*, 2004. **53**(8): p. 1145-50.
149. Markowitz, S.D. and M.M. Bertagnolli, *Molecular origins of cancer: Molecular basis of colorectal cancer*. *N Engl J Med*, 2009. **361**(25): p. 2449-60.
150. Dube, P.E. and P.L. Brubaker, *Frontiers in glucagon-like peptide-2: multiple actions, multiple mediators*. *American journal of physiology. Endocrinology and metabolism*, 2007. **293**(2): p. E460-5.
151. Henriksen, D.B., et al., *Reduction of nocturnal rise in bone resorption by subcutaneous GLP-2*. *Bone*, 2004. **34**(1): p. 140-7.
152. Cao, X., et al., *Aberrant regulation of human intestinal proglucagon gene expression in the NCI-H716 cell line*. *Endocrinology*, 2003. **144**(5): p. 2025-33.
153. Nian, M., D.J. Drucker, and D. Irwin, *Divergent regulation of human and rat proglucagon gene promoters in vivo*. *Am J Physiol*, 1999. **277**(4 Pt 1): p. G829-37.
154. Powers, A.C., et al., *Sodium butyrate increases glucagon and insulin gene expression by recruiting immunocytochemically negative cells to produce hormone*. *Diabetes*, 1988. **37**(10): p. 1405-10.
155. Hanauer, S.B., *Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities*. *Inflamm Bowel Dis*, 2006. **12 Suppl 1**: p. S3-9.
156. Ardizzone, S. and G. Bianchi Porro, *Inflammatory bowel disease: new insights into pathogenesis and treatment*. *J Intern Med*, 2002. **252**(6): p. 475-96.

157. Cho, J.H., *Inflammatory bowel disease: genetic and epidemiologic considerations*. World J Gastroenterol, 2008. **14**(3): p. 338-47.
158. Xie, J. and S.H. Itzkowitz, *Cancer in inflammatory bowel disease*. World J Gastroenterol, 2008. **14**(3): p. 378-89.
159. Hendrickson, B.A., R. Gokhale, and J.H. Cho, *Clinical aspects and pathophysiology of inflammatory bowel disease*. Clin Microbiol Rev, 2002. **15**(1): p. 79-94.
160. Shih, D.Q. and S.R. Targan, *Immunopathogenesis of inflammatory bowel disease*. World J Gastroenterol, 2008. **14**(3): p. 390-400.
161. Kozuch, P.L. and S.B. Hanauer, *Treatment of inflammatory bowel disease: a review of medical therapy*. World J Gastroenterol, 2008. **14**(3): p. 354-77.
162. Ardizzone, S. and G. Bianchi Porro, *Biologic therapy for inflammatory bowel disease*. Drugs, 2005. **65**(16): p. 2253-86.
163. Anonymous, *Wilhelm Fabry (1560-1624)--the Other Fabricius*. JAMA, 1964. **190**: p. 933.
164. Crohn, B.B., L. Ginzburg, and G.D. Oppenheimer, *Landmark article Oct 15, 1932. Regional ileitis. A pathological and clinical entity. By Burril B. Crohn, Leon Ginzburg, and Gordon D. Oppenheimer*. JAMA, 1984. **251**(1): p. 73-9.
165. Loftus, E.V., Jr., *Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences*. Gastroenterology, 2004. **126**(6): p. 1504-17.
166. Nguyen, G.C., et al., *Inflammatory bowel disease characteristics among African Americans, Hispanics, and non-Hispanic Whites: characterization of a large North American cohort*. Am J Gastroenterol, 2006. **101**(5): p. 1012-23.
167. Kurata, J.H., et al., *Crohn's disease among ethnic groups in a large health maintenance organization*. Gastroenterology, 1992. **102**(6): p. 1940-8.
168. Hampe, J., et al., *Anticipation in inflammatory bowel disease: a phenomenon caused by an accumulation of confounders*. Am J Med Genet, 2000. **92**(3): p. 178-83.
169. Niv, Y., G. Abuksis, and G.M. Fraser, *Epidemiology of ulcerative colitis in Israel: a survey of Israeli kibbutz settlements*. Am J Gastroenterol, 2000. **95**(3): p. 693-8.
170. Leong, R.W., J.Y. Lau, and J.J. Sung, *The epidemiology and phenotype of Crohn's disease in the Chinese population*. Inflamm Bowel Dis, 2004. **10**(5): p. 646-51.
171. Cho, J.H. and C.T. Weaver, *The genetics of inflammatory bowel disease*. Gastroenterology, 2007. **133**(4): p. 1327-39.
172. Russell, R.K. and J. Satsangi, *IBD: a family affair*. Best Pract Res Clin Gastroenterol, 2004. **18**(3): p. 525-39.
173. Cosnes, J., *Tobacco and IBD: relevance in the understanding of disease mechanisms and clinical practice*. Best Pract Res Clin Gastroenterol, 2004. **18**(3): p. 481-96.

174. D'Haens, G.R., et al., *Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum*. *Gastroenterology*, 1998. **114**(2): p. 262-7.
175. Duchmann, R., et al., *Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD)*. *Clin Exp Immunol*, 1995. **102**(3): p. 448-55.
176. Duchmann, R., E. Marker-Hermann, and K.H. Meyer zum Buschenfelde, *Bacteria-specific T-cell clones are selective in their reactivity towards different enterobacteria or H. pylori and increased in inflammatory bowel disease*. *Scand J Immunol*, 1996. **44**(1): p. 71-9.
177. Barrett, J.C., et al., *Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease*. *Nat Genet*, 2008. **40**(8): p. 955-62.
178. Duerr, R.H., et al., *A genome-wide association study identifies IL23R as an inflammatory bowel disease gene*. *Science*, 2006. **314**(5804): p. 1461-3.
179. Abraham, C. and J.H. Cho, *Functional consequences of NOD2 (CARD15) mutations*. *Inflamm Bowel Dis*, 2006. **12**(7): p. 641-50.
180. Economou, M., et al., *Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis*. *Am J Gastroenterol*, 2004. **99**(12): p. 2393-404.
181. Hedl, M., et al., *Chronic stimulation of Nod2 mediates tolerance to bacterial products*. *Proc Natl Acad Sci U S A*, 2007. **104**(49): p. 19440-5.
182. Lesage, S., et al., *CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease*. *Am J Hum Genet*, 2002. **70**(4): p. 845-57.
183. Hampe, J., et al., *A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1*. *Nat Genet*, 2007. **39**(2): p. 207-11.
184. Homer, C.R., et al., *ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis*. *Gastroenterology*, 2010. **139**(5): p. 1630-41, 1641 e1-2.
185. Travassos, L.H., et al., *Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry*. *Nat Immunol*, 2010. **11**(1): p. 55-62.
186. Ahmad, T., et al., *The molecular classification of the clinical manifestations of Crohn's disease*. *Gastroenterology*, 2002. **122**(4): p. 854-66.
187. Cuthbert, A.P., et al., *The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease*. *Gastroenterology*, 2002. **122**(4): p. 867-74.
188. Cleyneen, I., et al., *Molecular reclassification of Crohn's disease by cluster analysis of genetic variants*. *PLoS One*, 2010. **5**(9): p. e12952.
189. Moum, B., et al., *Seasonal variations in the onset of ulcerative colitis*. *Gut*, 1996. **38**(3): p. 376-8.

190. Sonnenberg, A., S.J. Jacobsen, and I.H. Wasserman, *Periodicity of hospital admissions for inflammatory bowel disease*. Am J Gastroenterol, 1994. **89**(6): p. 847-51.
191. Lewis, J.D., et al., *Seasonal variation in flares of inflammatory bowel disease*. Gastroenterology, 2004. **126**(3): p. 665-73.
192. Soncini, M., et al., *Seasonal patterns of hospital treatment for inflammatory bowel disease in Italy*. Digestion, 2006. **73**(1): p. 1-8.
193. Schack-Nielsen, L. and K.F. Michaelsen, *Breast feeding and future health*. Curr Opin Clin Nutr Metab Care, 2006. **9**(3): p. 289-96.
194. Klement, E., et al., *Breastfeeding and risk of inflammatory bowel disease: a systematic review with meta-analysis*. Am J Clin Nutr, 2004. **80**(5): p. 1342-52.
195. Kane, S. and N. Lemieux, *The role of breastfeeding in postpartum disease activity in women with inflammatory bowel disease*. Am J Gastroenterol, 2005. **100**(1): p. 102-5.
196. Riordan, A.M., C.H. Ruxton, and J.O. Hunter, *A review of associations between Crohn's disease and consumption of sugars*. Eur J Clin Nutr, 1998. **52**(4): p. 229-38.
197. Geerling, B.J., et al., *Diet as a risk factor for the development of ulcerative colitis*. Am J Gastroenterol, 2000. **95**(4): p. 1008-13.
198. Weylandt, K.H. and J.X. Kang, *Rethinking lipid mediators*. Lancet, 2005. **366**(9486): p. 618-20.
199. Desai, H.G. and P.A. Gupte, *Increasing incidence of Crohn's disease in India: is it related to improved sanitation?* Indian J Gastroenterol, 2005. **24**(1): p. 23-4.
200. Gent, A.E., et al., *Inflammatory bowel disease and domestic hygiene in infancy*. Lancet, 1994. **343**(8900): p. 766-7.
201. Hampe, J., et al., *Association of inflammatory bowel disease with indicators for childhood antigen and infection exposure*. Int J Colorectal Dis, 2003. **18**(5): p. 413-7.
202. McCormick, P. and D. Manning, *Chronic inflammatory bowel disease and the 'over-clean' environment: rarity in the Irish 'traveller' community*. Ir Med J, 2001. **94**(7): p. 203-4.
203. Ingram, J.R., et al., *A randomized trial of nicotine enemas for active ulcerative colitis*. Clin Gastroenterol Hepatol, 2005. **3**(11): p. 1107-14.
204. McGrath, J., J.W. McDonald, and J.K. Macdonald, *Transdermal nicotine for induction of remission in ulcerative colitis*. Cochrane Database Syst Rev, 2004(4): p. CD004722.
205. Koutroubakis, I.E., I.G. Vlachonikolis, and E.A. Kouroumalis, *Role of appendicitis and appendectomy in the pathogenesis of ulcerative colitis: a critical review*. Inflamm Bowel Dis, 2002. **8**(4): p. 277-86.
206. Radford-Smith, G.L., et al., *Protective role of appendectomy on onset and severity of ulcerative colitis and Crohn's disease*. Gut, 2002. **51**(6): p. 808-13.

207. Andersson, R.E., et al., *Appendectomy is followed by increased risk of Crohn's disease*. *Gastroenterology*, 2003. **124**(1): p. 40-6.
208. Cosnes, J., et al., *Prior appendectomy and the phenotype and course of Crohn's disease*. *World J Gastroenterol*, 2006. **12**(8): p. 1235-42.
209. Darfeuille-Michaud, A., et al., *High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease*. *Gastroenterology*, 2004. **127**(2): p. 412-21.
210. Garcia Rodriguez, L.A., A. Ruigomez, and J. Panes, *Acute gastroenteritis is followed by an increased risk of inflammatory bowel disease*. *Gastroenterology*, 2006. **130**(6): p. 1588-94.
211. Swidsinski, A., et al., *Mucosal flora in inflammatory bowel disease*. *Gastroenterology*, 2002. **122**(1): p. 44-54.
212. Godet, P.G., G.R. May, and L.R. Sutherland, *Meta-analysis of the role of oral contraceptive agents in inflammatory bowel disease*. *Gut*, 1995. **37**(5): p. 668-73.
213. Neuman, M.G. and R.M. Nanau, *Inflammatory bowel disease: role of diet, microbiota, life style*. *Transl Res*, 2012. **160**(1): p. 29-44.
214. Mawdsley, J.E. and D.S. Rampton, *Psychological stress in IBD: new insights into pathogenic and therapeutic implications*. *Gut*, 2005. **54**(10): p. 1481-91.
215. Abraham, C. and J.H. Cho, *Inflammatory bowel disease*. *N Engl J Med*, 2009. **361**(21): p. 2066-78.
216. Strober, W., I.J. Fuss, and R.S. Blumberg, *The immunology of mucosal models of inflammation*. *Annu Rev Immunol*, 2002. **20**: p. 495-549.
217. Fuss, I.J., et al., *Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5*. *J Immunol*, 1996. **157**(3): p. 1261-70.
218. Kobayashi, T., et al., *IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease*. *Gut*, 2008. **57**(12): p. 1682-9.
219. Reimund, J.M., et al., *Mucosal inflammatory cytokine production by intestinal biopsies in patients with ulcerative colitis and Crohn's disease*. *J Clin Immunol*, 1996. **16**(3): p. 144-50.
220. Barnes, M.J. and F. Powrie, *Regulatory T cells reinforce intestinal homeostasis*. *Immunity*, 2009. **31**(3): p. 401-11.
221. Maul, J., et al., *Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease*. *Gastroenterology*, 2005. **128**(7): p. 1868-78.
222. Saruta, M., et al., *Characterization of FOXP3+CD4+ regulatory T cells in Crohn's disease*. *Clin Immunol*, 2007. **125**(3): p. 281-90.

223. Duerkop, B.A., S. Vaishnava, and L.V. Hooper, *Immune responses to the microbiota at the intestinal mucosal surface*. *Immunity*, 2009. **31**(3): p. 368-76.
224. Abrams, G.D., H. Bauer, and H. Sprinz, *Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germ-free and conventional mice*. *Lab Invest*, 1963. **12**: p. 355-64.
225. Bry, L., et al., *A model of host-microbial interactions in an open mammalian ecosystem*. *Science*, 1996. **273**(5280): p. 1380-3.
226. Videla, S., et al., *Role of intestinal microflora in chronic inflammation and ulceration of the rat colon*. *Gut*, 1994. **35**(8): p. 1090-7.
227. Sellon, R.K., et al., *Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice*. *Infect Immun*, 1998. **66**(11): p. 5224-31.
228. Taurog, J.D., et al., *The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats*. *J Exp Med*, 1994. **180**(6): p. 2359-64.
229. Cahill, R.J., et al., *Inflammatory bowel disease: an immunity-mediated condition triggered by bacterial infection with *Helicobacter hepaticus**. *Infect Immun*, 1997. **65**(8): p. 3126-31.
230. Kullberg, M.C., et al., *Induction of colitis by a CD4+ T cell clone specific for a bacterial epitope*. *Proc Natl Acad Sci U S A*, 2003. **100**(26): p. 15830-5.
231. Frank, D.N., et al., *Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases*. *Proc Natl Acad Sci U S A*, 2007. **104**(34): p. 13780-5.
232. Sokol, H., et al., *Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients*. *Proc Natl Acad Sci U S A*, 2008. **105**(43): p. 16731-6.
233. Zaneveld, J., et al., *Host-bacterial coevolution and the search for new drug targets*. *Curr Opin Chem Biol*, 2008. **12**(1): p. 109-14.
234. Pineton de Chambrun, G., et al., *Pathogenic agents in inflammatory bowel diseases*. *Curr Opin Gastroenterol*, 2008. **24**(4): p. 440-7.
235. Coombes, J.L. and F. Powrie, *Dendritic cells in intestinal immune regulation*. *Nat Rev Immunol*, 2008. **8**(6): p. 435-46.
236. Elphick, D.A. and Y.R. Mahida, *Paneth cells: their role in innate immunity and inflammatory disease*. *Gut*, 2005. **54**(12): p. 1802-9.
237. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses*. *Nat Immunol*, 2004. **5**(10): p. 987-95.

238. Strober, W., et al., *Signalling pathways and molecular interactions of NOD1 and NOD2*. Nat Rev Immunol, 2006. **6**(1): p. 9-20.
239. Netea, M.G., et al., *NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease*. Eur J Immunol, 2004. **34**(7): p. 2052-9.
240. Watanabe, T., et al., *NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses*. Nat Immunol, 2004. **5**(8): p. 800-8.
241. Cooney, R., et al., *NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation*. Nat Med, 2010. **16**(1): p. 90-7.
242. Artis, D., *Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut*. Nat Rev Immunol, 2008. **8**(6): p. 411-20.
243. Lampen, A., et al., *Metabolism of vitamin A and its active metabolite all-trans-retinoic acid in small intestinal enterocytes*. J Pharmacol Exp Ther, 2000. **295**(3): p. 979-85.
244. Maynard, C.L. and C.T. Weaver, *Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation*. Immunol Rev, 2008. **226**: p. 219-33.
245. Rimoldi, M., et al., *Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells*. Nat Immunol, 2005. **6**(5): p. 507-14.
246. Saurer, L., K.C. McCullough, and A. Summerfield, *In vitro induction of mucosa-type dendritic cells by all-trans retinoic acid*. J Immunol, 2007. **179**(6): p. 3504-14.
247. Zaph, C., et al., *Commensal-dependent expression of IL-25 regulates the IL-23-IL-17 axis in the intestine*. J Exp Med, 2008. **205**(10): p. 2191-8.
248. Smith, P.D., et al., *Intestinal macrophages lack CD14 and CD89 and consequently are down-regulated for LPS- and IgA-mediated activities*. J Immunol, 2001. **167**(5): p. 2651-6.
249. Smythies, L.E., et al., *Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity*. J Clin Invest, 2005. **115**(1): p. 66-75.
250. Chen, W., et al., *Conversion of peripheral CD4⁺CD25⁻ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF-beta induction of transcription factor Foxp3*. J Exp Med, 2003. **198**(12): p. 1875-86.
251. Perruche, S., et al., *CD3-specific antibody-induced immune tolerance involves transforming growth factor-beta from phagocytes digesting apoptotic T cells*. Nat Med, 2008. **14**(5): p. 528-35.
252. Boirivant, M., et al., *Stimulated human lamina propria T cells manifest enhanced Fas-mediated apoptosis*. J Clin Invest, 1996. **98**(11): p. 2616-22.
253. Qiao, L., et al., *Activation and signaling status of human lamina propria T lymphocytes*. Gastroenterology, 1991. **101**(6): p. 1529-36.

254. Mowat, A.M., *Anatomical basis of tolerance and immunity to intestinal antigens*. Nat Rev Immunol, 2003. **3**(4): p. 331-41.
255. Zhu, J. and W.E. Paul, *Heterogeneity and plasticity of T helper cells*. Cell Res, 2010. **20**(1): p. 4-12.
256. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages*. Nat Immunol, 2005. **6**(11): p. 1123-32.
257. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17*. Nat Immunol, 2005. **6**(11): p. 1133-41.
258. Manel, N., D. Unutmaz, and D.R. Littman, *The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat*. Nat Immunol, 2008. **9**(6): p. 641-9.
259. Valmori, D., et al., *Human RORgammat+ TH17 cells preferentially differentiate from naive FOXP3+Treg in the presence of lineage-specific polarizing factors*. Proc Natl Acad Sci U S A, 2010. **107**(45): p. 19402-7.
260. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells*. Immunity, 2006. **24**(2): p. 179-89.
261. Volpe, E., et al., *A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses*. Nat Immunol, 2008. **9**(6): p. 650-7.
262. Crome, S.Q., A.Y. Wang, and M.K. Levings, *Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease*. Clin Exp Immunol, 2010. **159**(2): p. 109-19.
263. Coombes, J.L., et al., *A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism*. J Exp Med, 2007. **204**(8): p. 1757-64.
264. Feuerer, M., et al., *Foxp3+ regulatory T cells: differentiation, specification, subphenotypes*. Nat Immunol, 2009. **10**(7): p. 689-95.
265. Ito, T., et al., *Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery*. Immunity, 2008. **28**(6): p. 870-80.
266. Williams, L.M. and A.Y. Rudensky, *Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3*. Nat Immunol, 2007. **8**(3): p. 277-84.
267. Wing, K., Z. Fehervari, and S. Sakaguchi, *Emerging possibilities in the development and function of regulatory T cells*. Int Immunol, 2006. **18**(7): p. 991-1000.

268. Xu, L., et al., *Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta*. J Immunol, 2007. **178**(11): p. 6725-9.
269. Murai, M., et al., *Regulatory T-cell stability and plasticity in mucosal and systemic immune systems*. Mucosal Immunol, 2010. **3**(5): p. 443-9.
270. Ahern, P.P., et al., *Interleukin-23 drives intestinal inflammation through direct activity on T cells*. Immunity, 2010. **33**(2): p. 279-88.
271. Izcue, A., et al., *Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis*. Immunity, 2008. **28**(4): p. 559-70.
272. Johansson-Lindbom, B., et al., *Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant*. J Exp Med, 2003. **198**(6): p. 963-9.
273. Mora, J.R., et al., *Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells*. Nature, 2003. **424**(6944): p. 88-93.
274. Bouma, G. and W. Strober, *The immunological and genetic basis of inflammatory bowel disease*. Nat Rev Immunol, 2003. **3**(7): p. 521-33.
275. Fuss, I.J., et al., *Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis*. J Clin Invest, 2004. **113**(10): p. 1490-7.
276. Boirivant, M., et al., *Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis*. Gastroenterology, 1999. **116**(3): p. 557-65.
277. Ina, K., et al., *Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance*. J Immunol, 1999. **163**(2): p. 1081-90.
278. Fujino, S., et al., *Increased expression of interleukin 17 in inflammatory bowel disease*. Gut, 2003. **52**(1): p. 65-70.
279. Rovedatti, L., et al., *Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease*. Gut, 2009. **58**(12): p. 1629-36.
280. Noguchi, D., et al., *Blocking of IL-6 signaling pathway prevents CD4+ T cell-mediated colitis in a T(h)17-independent manner*. Int Immunol, 2007. **19**(12): p. 1431-40.
281. Boirivant, M., et al., *Inhibition of Smad7 with a specific antisense oligonucleotide facilitates TGF-beta1-mediated suppression of colitis*. Gastroenterology, 2006. **131**(6): p. 1786-98.
282. Monteleone, G., et al., *Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease*. J Clin Invest, 2001. **108**(4): p. 601-9.
283. Marks, D.J. and A.W. Segal, *Innate immunity in inflammatory bowel disease: a disease hypothesis*. J Pathol, 2008. **214**(2): p. 260-6.

284. Segal, A.W. and G. Loewi, *Neutrophil dysfunction in Crohn's disease*. Lancet, 1976. **2**(7979): p. 219-21.
285. Smith, A.M., et al., *Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease*. J Exp Med, 2009. **206**(9): p. 1883-97.
286. Soderholm, J.D., et al., *Augmented increase in tight junction permeability by luminal stimuli in the non-inflamed ileum of Crohn's disease*. Gut, 2002. **50**(3): p. 307-13.
287. Cario, E. and D.K. Podolsky, *Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease*. Infect Immun, 2000. **68**(12): p. 7010-7.
288. Rhee, S.H., et al., *Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation*. Proc Natl Acad Sci U S A, 2005. **102**(38): p. 13610-5.
289. Berrebi, D., et al., *Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon*. Gut, 2003. **52**(6): p. 840-6.
290. Hart, A.L., et al., *Characteristics of intestinal dendritic cells in inflammatory bowel diseases*. Gastroenterology, 2005. **129**(1): p. 50-65.
291. Ashcroft, A.J., et al., *Colonic dendritic cells, intestinal inflammation, and T cell-mediated bone destruction are modulated by recombinant osteoprotegerin*. Immunity, 2003. **19**(6): p. 849-61.
292. Cruickshank, S.M., et al., *Colonic epithelial cell mediated suppression of CD4 T cell activation*. Gut, 2004. **53**(5): p. 678-84.
293. Nakazawa, A., et al., *The expression and function of costimulatory molecules B7H and B7-H1 on colonic epithelial cells*. Gastroenterology, 2004. **126**(5): p. 1347-57.
294. Van den Brande, J.M., et al., *Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease*. Gastroenterology, 2003. **124**(7): p. 1774-85.
295. Demaude, J., et al., *Phenotypic changes in colonocytes following acute stress or activation of mast cells in mice: implications for delayed epithelial barrier dysfunction*. Gut, 2006. **55**(5): p. 655-61.
296. Furlan, R., et al., *Sympathetic overactivity in active ulcerative colitis: effects of clonidine*. Am J Physiol Regul Integr Comp Physiol, 2006. **290**(1): p. R224-32.
297. Hanauer, S.B. and F. Baert, *Medical therapy of inflammatory bowel disease*. Med Clin North Am, 1994. **78**(6): p. 1413-26.
298. Pearson, D.C., et al., *Azathioprine and 6-mercaptopurine in Crohn disease. A meta-analysis*. Ann Intern Med, 1995. **123**(2): p. 132-42.

299. Aberra, F.N., et al., *Corticosteroids and immunomodulators: postoperative infectious complication risk in inflammatory bowel disease patients*. *Gastroenterology*, 2003. **125**(2): p. 320-7.
300. Lowry, P.W., et al., *Leucopenia resulting from a drug interaction between azathioprine or 6-mercaptopurine and mesalamine, sulphasalazine, or balsalazide*. *Gut*, 2001. **49**(5): p. 656-64.
301. Haber, C.J., et al., *Nature and course of pancreatitis caused by 6-mercaptopurine in the treatment of inflammatory bowel disease*. *Gastroenterology*, 1986. **91**(4): p. 982-6.
302. Feagan, B.G., et al., *A comparison of methotrexate with placebo for the maintenance of remission in Crohn's disease*. *North American Crohn's Study Group Investigators*. *N Engl J Med*, 2000. **342**(22): p. 1627-32.
303. Pithadia, A.B. and S. Jain, *Treatment of inflammatory bowel disease (IBD)*. *Pharmacol Rep*, 2011. **63**(3): p. 629-42.
304. Lichtiger, S., et al., *Cyclosporine in severe ulcerative colitis refractory to steroid therapy*. *N Engl J Med*, 1994. **330**(26): p. 1841-5.
305. Sandborn, W.J., *Cyclosporine therapy for inflammatory bowel disease: definitive answers and remaining questions*. *Gastroenterology*, 1995. **109**(3): p. 1001-3.
306. De Iudibus, S., et al., *Molecular mechanism of glucocorticoid resistance in inflammatory bowel disease*. *World J Gastroenterol*, 2011. **17**(9): p. 1095-108.
307. Katz, J.A., *Treatment of inflammatory bowel disease with corticosteroids*. *Gastroenterol Clin North Am*, 2004. **33**(2): p. 171-89, vii.
308. Farrell, R.J. and D. Kelleher, *Glucocorticoid resistance in inflammatory bowel disease*. *J Endocrinol*, 2003. **178**(3): p. 339-46.
309. Munkholm, P., et al., *Frequency of glucocorticoid resistance and dependency in Crohn's disease*. *Gut*, 1994. **35**(3): p. 360-2.
310. Faubion, W.A., Jr., et al., *The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study*. *Gastroenterology*, 2001. **121**(2): p. 255-60.
311. Cronin, E., *Prednisolone in the management of patients with Crohn's disease*. *Br J Nurs*, 2010. **19**(21): p. 1333-6.
312. Greenberg, G.R., et al., *Oral budesonide for active Crohn's disease*. *Canadian Inflammatory Bowel Disease Study Group*. *N Engl J Med*, 1994. **331**(13): p. 836-41.
313. Hofer, K.N., *Oral budesonide in the management of Crohn's disease*. *Ann Pharmacother*, 2003. **37**(10): p. 1457-64.
314. Van Deventer, S.J., *Tumour necrosis factor and Crohn's disease*. *Gut*, 1997. **40**(4): p. 443-8.

315. Lichtenstein, G.R., et al., *American Gastroenterological Association Institute technical review on corticosteroids, immunomodulators, and infliximab in inflammatory bowel disease*. *Gastroenterology*, 2006. **130**(3): p. 940-87.
316. Schwartz, D.A., et al., *A comparison of endoscopic ultrasound, magnetic resonance imaging, and exam under anesthesia for evaluation of Crohn's perianal fistulas*. *Gastroenterology*, 2001. **121**(5): p. 1064-72.
317. Rutgeerts, P., S. Vermeire, and G. Van Assche, *Biological therapies for inflammatory bowel diseases*. *Gastroenterology*, 2009. **136**(4): p. 1182-97.
318. Sartor, R.B., *Current concepts of the etiology and pathogenesis of ulcerative colitis and Crohn's disease*. *Gastroenterol Clin North Am*, 1995. **24**(3): p. 475-507.
319. Arnold, G.L., et al., *Preliminary study of ciprofloxacin in active Crohn's disease*. *Inflamm Bowel Dis*, 2002. **8**(1): p. 10-5.
320. Sutherland, L., et al., *Double blind, placebo controlled trial of metronidazole in Crohn's disease*. *Gut*, 1991. **32**(9): p. 1071-5.
321. Engel, M.A. and M.F. Neurath, *New pathophysiological insights and modern treatment of IBD*. *J Gastroenterol*, 2010. **45**(6): p. 571-83.
322. Camma, C., et al., *Mesalamine in the maintenance treatment of Crohn's disease: a meta-analysis adjusted for confounding variables*. *Gastroenterology*, 1997. **113**(5): p. 1465-73.
323. Desreumaux, P. and S. Ghosh, *Review article: mode of action and delivery of 5-aminosalicylic acid - new evidence*. *Aliment Pharmacol Ther*, 2006. **24 Suppl 1**: p. 2-9.
324. McCormack, P.L., D.M. Robinson, and C.M. Perry, *Delayed-release Multi Matrix System (MMX) mesalazine: in ulcerative colitis*. *Drugs*, 2007. **67**(17): p. 2635-42.
325. Rousseaux, C., et al., *Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator-activated receptor-gamma*. *J Exp Med*, 2005. **201**(8): p. 1205-15.
326. Ahnfelt-Ronne, I., et al., *Clinical evidence supporting the radical scavenger mechanism of 5-aminosalicylic acid*. *Gastroenterology*, 1990. **98**(5 Pt 1): p. 1162-9.
327. Egan, L.J., et al., *Inhibition of interleukin-1-stimulated NF-kappaB RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity*. *J Biol Chem*, 1999. **274**(37): p. 26448-53.
328. Kaiser, G.C., F. Yan, and D.B. Polk, *Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor kappaB activation in mouse colonocytes*. *Gastroenterology*, 1999. **116**(3): p. 602-9.
329. Mahida, Y.R., et al., *5-Aminosalicylic acid is a potent inhibitor of interleukin 1 beta production in organ culture of colonic biopsy specimens from patients with inflammatory bowel disease*. *Gut*, 1991. **32**(1): p. 50-4.

330. Stenson, W.F. and E. Lobos, *Sulfasalazine inhibits the synthesis of chemotactic lipids by neutrophils*. J Clin Invest, 1982. **69**(2): p. 494-7.
331. Viennot, S., et al., *Colon cancer in inflammatory bowel disease: recent trends, questions and answers*. Gastroenterol Clin Biol, 2009. **33 Suppl 3**: p. S190-201.
332. Crohn, B.B., *Rectal complications of inflammatory disease of the small and large bowel*. Dis Colon Rectum, 1960. **3**: p. 99-102.
333. Gillen, C.D., et al., *Crohn's disease and colorectal cancer*. Gut, 1994. **35**(5): p. 651-5.
334. Ouaisi, M., et al., *Colorectal cancer complicating inflammatory bowel disease: a comparative study of Crohn's disease vs ulcerative colitis in 34 patients*. Colorectal Dis, 2011. **13**(6): p. 684-8.
335. Eaden, J.A., K.R. Abrams, and J.F. Mayberry, *The risk of colorectal cancer in ulcerative colitis: a meta-analysis*. Gut, 2001. **48**(4): p. 526-35.
336. Bernstein, C.N., et al., *Cancer risk in patients with inflammatory bowel disease: a population-based study*. Cancer, 2001. **91**(4): p. 854-62.
337. Rutter, M.D., et al., *Thirty-year analysis of a colonoscopic surveillance program for neoplasia in ulcerative colitis*. Gastroenterology, 2006. **130**(4): p. 1030-8.
338. Lakatos, L., et al., *Risk factors for ulcerative colitis-associated colorectal cancer in a Hungarian cohort of patients with ulcerative colitis: results of a population-based study*. Inflamm Bowel Dis, 2006. **12**(3): p. 205-11.
339. Ahmadi, A., S. Polyak, and P.V. Draganov, *Colorectal cancer surveillance in inflammatory bowel disease: the search continues*. World J Gastroenterol, 2009. **15**(1): p. 61-6.
340. Lakatos, P.L. and L. Lakatos, *Risk for colorectal cancer in ulcerative colitis: changes, causes and management strategies*. World J Gastroenterol, 2008. **14**(25): p. 3937-47.
341. Canavan, C., K.R. Abrams, and J. Mayberry, *Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease*. Aliment Pharmacol Ther, 2006. **23**(8): p. 1097-104.
342. Loftus, E.V., Jr., *Epidemiology and risk factors for colorectal dysplasia and cancer in ulcerative colitis*. Gastroenterol Clin North Am, 2006. **35**(3): p. 517-31.
343. Rutter, M., et al., *Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis*. Gastroenterology, 2004. **126**(2): p. 451-9.
344. Ekobom, A., et al., *Ulcerative colitis and colorectal cancer. A population-based study*. N Engl J Med, 1990. **323**(18): p. 1228-33.
345. Lutgens, M.W., et al., *High frequency of early colorectal cancer in inflammatory bowel disease*. Gut, 2008. **57**(9): p. 1246-51.
346. Rutter, M.D., et al., *Cancer surveillance in longstanding ulcerative colitis: endoscopic appearances help predict cancer risk*. Gut, 2004. **53**(12): p. 1813-6.

347. Askling, J., et al., *Family history as a risk factor for colorectal cancer in inflammatory bowel disease*. *Gastroenterology*, 2001. **120**(6): p. 1356-62.
348. Nuako, K.W., et al., *Familial predisposition for colorectal cancer in chronic ulcerative colitis: a case-control study*. *Gastroenterology*, 1998. **115**(5): p. 1079-83.
349. Heuschen, U.A., et al., *Backwash ileitis is strongly associated with colorectal carcinoma in ulcerative colitis*. *Gastroenterology*, 2001. **120**(4): p. 841-7.
350. Broome, U., et al., *Primary sclerosing cholangitis and ulcerative colitis: evidence for increased neoplastic potential*. *Hepatology*, 1995. **22**(5): p. 1404-8.
351. Jayaram, H., J. Satsangi, and R.W. Chapman, *Increased colorectal neoplasia in chronic ulcerative colitis complicated by primary sclerosing cholangitis: fact or fiction?* *Gut*, 2001. **48**(3): p. 430-4.
352. Loftus, E.V., Jr., et al., *Risk of colorectal neoplasia in patients with primary sclerosing cholangitis and ulcerative colitis following orthotopic liver transplantation*. *Hepatology*, 1998. **27**(3): p. 685-90.
353. Platz, E.A., et al., *Proportion of colon cancer risk that might be preventable in a cohort of middle-aged US men*. *Cancer Causes Control*, 2000. **11**(7): p. 579-88.
354. Kim, Y.S. and J.A. Milner, *Dietary modulation of colon cancer risk*. *J Nutr*, 2007. **137**(11 Suppl): p. 2576S-2579S.
355. Murillo, G. and R.G. Mehta, *Chemoprevention of chemically-induced mammary and colon carcinogenesis by 1alpha-hydroxyvitamin D5*. *J Steroid Biochem Mol Biol*, 2005. **97**(1-2): p. 129-36.
356. Wilson, A.J., et al., *Novel detection and differential utilization of a c-myc transcriptional block in colon cancer chemoprevention*. *Cancer Res*, 2002. **62**(21): p. 6006-10.
357. Biswas, S., et al., *Inhibition of transforming growth factor-beta signaling in human cancer: targeting a tumor suppressor network as a therapeutic strategy*. *Clin Cancer Res*, 2006. **12**(14 Pt 1): p. 4142-6.
358. Tong, X., L. Yin, and C. Giardina, *Butyrate suppresses Cox-2 activation in colon cancer cells through HDAC inhibition*. *Biochem Biophys Res Commun*, 2004. **317**(2): p. 463-71.
359. Jeong, W.S., et al., *Modulatory properties of various natural chemopreventive agents on the activation of NF-kappaB signaling pathway*. *Pharm Res*, 2004. **21**(4): p. 661-70.
360. Sun, A.Y., et al., *Botanical phenolics and brain health*. *Neuromolecular Med*, 2008. **10**(4): p. 259-74.
361. Farraye, F.A., et al., *AGA technical review on the diagnosis and management of colorectal neoplasia in inflammatory bowel disease*. *Gastroenterology*, 2010. **138**(2): p. 746-74, 774 e1-4; quiz e12-3.

362. Rubin, C.E., et al., *DNA aneuploidy in colonic biopsies predicts future development of dysplasia in ulcerative colitis*. *Gastroenterology*, 1992. **103**(5): p. 1611-20.
363. Itzkowitz, S.H., *Molecular biology of dysplasia and cancer in inflammatory bowel disease*. *Gastroenterol Clin North Am*, 2006. **35**(3): p. 553-71.
364. Willenbacher, R.F., et al., *Genomic instability is an early event during the progression pathway of ulcerative-colitis-related neoplasia*. *Am J Pathol*, 1999. **154**(6): p. 1825-30.
365. Aust, D.E., et al., *The APC/beta-catenin pathway in ulcerative colitis-related colorectal carcinomas: a mutational analysis*. *Cancer*, 2002. **94**(5): p. 1421-7.
366. Redston, M.S., et al., *Common occurrence of APC and K-ras gene mutations in the spectrum of colitis-associated neoplasias*. *Gastroenterology*, 1995. **108**(2): p. 383-92.
367. Tarmin, L., et al., *Adenomatous polyposis coli gene mutations in ulcerative colitis-associated dysplasias and cancers versus sporadic colon neoplasms*. *Cancer Res*, 1995. **55**(10): p. 2035-8.
368. Umetani, N., et al., *Genetic alterations in ulcerative colitis-associated neoplasia focusing on APC, K-ras gene and microsatellite instability*. *Jpn J Cancer Res*, 1999. **90**(10): p. 1081-7.
369. Burmer, G.C., et al., *Neoplastic progression in ulcerative colitis: histology, DNA content, and loss of a p53 allele*. *Gastroenterology*, 1992. **103**(5): p. 1602-10.
370. Yin, J., et al., *p53 point mutations in dysplastic and cancerous ulcerative colitis lesions*. *Gastroenterology*, 1993. **104**(6): p. 1633-9.
371. Brentnall, T.A., et al., *Mutations in the p53 gene: an early marker of neoplastic progression in ulcerative colitis*. *Gastroenterology*, 1994. **107**(2): p. 369-78.
372. Hussain, S.P., et al., *Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease*. *Cancer Res*, 2000. **60**(13): p. 3333-7.
373. Issa, J.P., et al., *Accelerated age-related CpG island methylation in ulcerative colitis*. *Cancer Res*, 2001. **61**(9): p. 3573-7.
374. Fleisher, A.S., et al., *Microsatellite instability in inflammatory bowel disease-associated neoplastic lesions is associated with hypermethylation and diminished expression of the DNA mismatch repair gene, hMLH1*. *Cancer Res*, 2000. **60**(17): p. 4864-8.
375. Hsieh, C.J., et al., *Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis*. *Cancer Res*, 1998. **58**(17): p. 3942-5.
376. Sato, F., et al., *Hypermethylation of the p14(ARF) gene in ulcerative colitis-associated colorectal carcinogenesis*. *Cancer Res*, 2002. **62**(4): p. 1148-51.

377. Barker, N., et al., *Crypt stem cells as the cells-of-origin of intestinal cancer*. Nature, 2009. **457**(7229): p. 608-11.
378. Hussain, S.P., L.J. Hofseth, and C.C. Harris, *Radical causes of cancer*. Nat Rev Cancer, 2003. **3**(4): p. 276-85.
379. Hofseth, L.J., et al., *Nitric oxide-induced cellular stress and p53 activation in chronic inflammation*. Proc Natl Acad Sci U S A, 2003. **100**(1): p. 143-8.
380. Kimura, H., et al., *Increased expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in colonic mucosa of patients with active ulcerative colitis*. Gut, 1998. **42**(2): p. 180-7.
381. Rachmilewitz, D., et al., *Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn's disease*. Gut, 1995. **36**(5): p. 718-23.
382. McKenzie, S.J., et al., *Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease*. J Clin Invest, 1996. **98**(1): p. 136-41.
383. D'Inca, R., et al., *Oxidative DNA damage in the mucosa of ulcerative colitis increases with disease duration and dysplasia*. Inflamm Bowel Dis, 2004. **10**(1): p. 23-7.
384. Marnett, L.J., *Oxyradicals and DNA damage*. Carcinogenesis, 2000. **21**(3): p. 361-70.
385. Chang, C.L., et al., *Oxidative stress inactivates the human DNA mismatch repair system*. Am J Physiol Cell Physiol, 2002. **283**(1): p. C148-54.
386. Brentnall, T.A., et al., *Microsatellite instability in nonneoplastic mucosa from patients with chronic ulcerative colitis*. Cancer Res, 1996. **56**(6): p. 1237-40.
387. Noffsinger, A., et al., *Microsatellite instability is uncommon in intestinal mucosa of patients with Crohn's disease*. Dig Dis Sci, 2000. **45**(2): p. 378-84.
388. Atreya, I. and M.F. Neurath, *Immune cells in colorectal cancer: prognostic relevance and therapeutic strategies*. Expert Rev Anticancer Ther, 2008. **8**(4): p. 561-72.
389. Gabrilovich, D.I. and S. Nagaraj, *Myeloid-derived suppressor cells as regulators of the immune system*. Nat Rev Immunol, 2009. **9**(3): p. 162-74.
390. Erdman, S.E., et al., *CD4+CD25+ regulatory lymphocytes induce regression of intestinal tumors in ApcMin/+ mice*. Cancer Res, 2005. **65**(10): p. 3998-4004.
391. Izcue, A., J.L. Coombes, and F. Powrie, *Regulatory lymphocytes and intestinal inflammation*. Annu Rev Immunol, 2009. **27**: p. 313-38.
392. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The three Es of cancer immunoediting*. Annu Rev Immunol, 2004. **22**: p. 329-60.
393. Karin, M., *Nuclear factor-kappaB in cancer development and progression*. Nature, 2006. **441**(7092): p. 431-6.

394. Kojima, M., et al., *Increased nuclear factor-kB activation in human colorectal carcinoma and its correlation with tumor progression*. *Anticancer Res*, 2004. **24**(2B): p. 675-81.
395. Karin, M. and F.R. Greten, *NF-kappaB: linking inflammation and immunity to cancer development and progression*. *Nat Rev Immunol*, 2005. **5**(10): p. 749-59.
396. Naugler, W.E. and M. Karin, *NF-kappaB and cancer-identifying targets and mechanisms*. *Curr Opin Genet Dev*, 2008. **18**(1): p. 19-26.
397. Greten, F.R., et al., *IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer*. *Cell*, 2004. **118**(3): p. 285-96.
398. Delloye-Bourgeois, C., et al., *Netrin-1 acts as a survival factor for aggressive neuroblastoma*. *J Exp Med*, 2009. **206**(4): p. 833-47.
399. Paradisi, A., et al., *Netrin-1 up-regulation in inflammatory bowel diseases is required for colorectal cancer progression*. *Proc Natl Acad Sci U S A*, 2009. **106**(40): p. 17146-51.
400. Wang, C.Y., M.W. Mayo, and A.S. Baldwin, Jr., *TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB*. *Science*, 1996. **274**(5288): p. 784-7.
401. Chung, D.C., *The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis*. *Gastroenterology*, 2000. **119**(3): p. 854-65.
402. Popivanova, B.K., et al., *Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis*. *J Clin Invest*, 2008. **118**(2): p. 560-70.
403. Rogler, G., et al., *Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa*. *Gastroenterology*, 1998. **115**(2): p. 357-69.
404. Becker, C., et al., *IL-6 signaling promotes tumor growth in colorectal cancer*. *Cell Cycle*, 2005. **4**(2): p. 217-20.
405. Chung, Y.C. and Y.F. Chang, *Serum interleukin-6 levels reflect the disease status of colorectal cancer*. *J Surg Oncol*, 2003. **83**(4): p. 222-6.
406. Becker, C., et al., *TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling*. *Immunity*, 2004. **21**(4): p. 491-501.
407. Grivennikov, S., et al., *IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer*. *Cancer Cell*, 2009. **15**(2): p. 103-13.
408. Li, Y., et al., *Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis*. *Gut*, 2010. **59**(2): p. 227-35.
409. Koehne, C.H. and R.N. Dubois, *COX-2 inhibition and colorectal cancer*. *Semin Oncol*, 2004. **31**(2 Suppl 7): p. 12-21.
410. Sheehan, K.M., et al., *The relationship between cyclooxygenase-2 expression and colorectal cancer*. *JAMA*, 1999. **282**(13): p. 1254-7.

411. Arber, N., et al., *Celecoxib for the prevention of colorectal adenomatous polyps*. N Engl J Med, 2006. **355**(9): p. 885-95.
412. Pugh, S. and G.A. Thomas, *Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E2*. Gut, 1994. **35**(5): p. 675-8.
413. Kawamori, T., et al., *Enhancement of colon carcinogenesis by prostaglandin E2 administration*. Carcinogenesis, 2003. **24**(5): p. 985-90.
414. Castellone, M.D., et al., *Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis*. Science, 2005. **310**(5753): p. 1504-10.
415. Tessner, T.G., et al., *Prostaglandin E2 reduces radiation-induced epithelial apoptosis through a mechanism involving AKT activation and bax translocation*. J Clin Invest, 2004. **114**(11): p. 1676-85.
416. Pozzi, A., et al., *Colon carcinoma cell growth is associated with prostaglandin E2/EP4 receptor-evoked ERK activation*. J Biol Chem, 2004. **279**(28): p. 29797-804.
417. Jones, M.K., et al., *Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing*. Nat Med, 1999. **5**(12): p. 1418-23.
418. Tsujii, M., S. Kawano, and R.N. DuBois, *Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential*. Proc Natl Acad Sci U S A, 1997. **94**(7): p. 3336-40.
419. Khayrullina, T., et al., *In vitro differentiation of dendritic cells in the presence of prostaglandin E2 alters the IL-12/IL-23 balance and promotes differentiation of Th17 cells*. J Immunol, 2008. **181**(1): p. 721-35.
420. Wu, S., et al., *A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses*. Nat Med, 2009. **15**(9): p. 1016-22.
421. Garrett, W.S., et al., *Colitis-associated colorectal cancer driven by T-bet deficiency in dendritic cells*. Cancer Cell, 2009. **16**(3): p. 208-19.
422. Pages, F., et al., *Effector memory T cells, early metastasis, and survival in colorectal cancer*. N Engl J Med, 2005. **353**(25): p. 2654-66.
423. Erdman, S.E. and T. Poutahidis, *Roles for inflammation and regulatory T cells in colon cancer*. Toxicol Pathol, 2010. **38**(1): p. 76-87.
424. Sartor, R.B., *Microbial influences in inflammatory bowel diseases*. Gastroenterology, 2008. **134**(2): p. 577-94.
425. Uronis, J.M., et al., *Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility*. PLoS One, 2009. **4**(6): p. e6026.

426. Gueimonde, M., et al., *Qualitative and quantitative analyses of the bifidobacterial microbiota in the colonic mucosa of patients with colorectal cancer, diverticulitis and inflammatory bowel disease*. World J Gastroenterol, 2007. **13**(29): p. 3985-9.
427. Scanlan, P.D., et al., *Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis*. Environ Microbiol, 2008. **10**(3): p. 789-98.
428. Huycke, M.M., V. Abrams, and D.R. Moore, *Enterococcus faecalis produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA*. Carcinogenesis, 2002. **23**(3): p. 529-36.
429. Itzkowitz, S.H. and X. Yio, *Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation*. Am J Physiol Gastrointest Liver Physiol, 2004. **287**(1): p. G7-17.
430. Moore, K.W., et al., *Interleukin-10 and the interleukin-10 receptor*. Annu Rev Immunol, 2001. **19**: p. 683-765.
431. Berg, D.J., et al., *Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses*. J Clin Invest, 1996. **98**(4): p. 1010-20.
432. O'Mahony, L., et al., *Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice*. Aliment Pharmacol Ther, 2001. **15**(8): p. 1219-25.
433. Balish, E. and T. Warner, *Enterococcus faecalis induces inflammatory bowel disease in interleukin-10 knockout mice*. Am J Pathol, 2002. **160**(6): p. 2253-7.
434. Kado, S., et al., *Intestinal microflora are necessary for development of spontaneous adenocarcinoma of the large intestine in T-cell receptor beta chain and p53 double-knockout mice*. Cancer Res, 2001. **61**(6): p. 2395-8.
435. Chu, F.F., et al., *Bacteria-induced intestinal cancer in mice with disrupted Gpx1 and Gpx2 genes*. Cancer Res, 2004. **64**(3): p. 962-8.
436. Erdman, S.E., et al., *CD4+ CD25+ regulatory T lymphocytes inhibit microbially induced colon cancer in Rag2-deficient mice*. Am J Pathol, 2003. **162**(2): p. 691-702.
437. Erdman, S.E., et al., *CD4(+)CD25(+) regulatory lymphocytes require interleukin 10 to interrupt colon carcinogenesis in mice*. Cancer Res, 2003. **63**(18): p. 6042-50.
438. Rakoff-Nahoum, S. and R. Medzhitov, *Toll-like receptors and cancer*. Nat Rev Cancer, 2009. **9**(1): p. 57-63.
439. Kim, S.W., et al., *Bifidobacterium lactis inhibits NF-kappaB in intestinal epithelial cells and prevents acute colitis and colitis-associated colon cancer in mice*. Inflamm Bowel Dis, 2010. **16**(9): p. 1514-25.

440. Fukata, M., et al., *Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors*. *Gastroenterology*, 2007. **133**(6): p. 1869-81.
441. Itzkowitz, S.H., et al., *Consensus conference: Colorectal cancer screening and surveillance in inflammatory bowel disease*. *Inflamm Bowel Dis*, 2005. **11**(3): p. 314-21.
442. Stange, E.F., et al., *European evidence-based Consensus on the diagnosis and management of ulcerative colitis: Definitions and diagnosis*. *J Crohns Colitis*, 2008. **2**(1): p. 1-23.
443. Choi, P.M., et al., *Colonoscopic surveillance reduces mortality from colorectal cancer in ulcerative colitis*. *Gastroenterology*, 1993. **105**(2): p. 418-24.
444. Karlen, P., et al., *Is colonoscopic surveillance reducing colorectal cancer mortality in ulcerative colitis? A population based case control study*. *Gut*, 1998. **42**(5): p. 711-4.
445. Pinczowski, D., et al., *Risk factors for colorectal cancer in patients with ulcerative colitis: a case-control study*. *Gastroenterology*, 1994. **107**(1): p. 117-20.
446. Connell, W.R., et al., *Factors affecting the outcome of endoscopic surveillance for cancer in ulcerative colitis*. *Gastroenterology*, 1994. **107**(4): p. 934-44.
447. Ahlquist, D.A., et al., *Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel*. *Gastroenterology*, 2000. **119**(5): p. 1219-27.
448. Tagore, K.S., et al., *Sensitivity and specificity of a stool DNA multitarget assay panel for the detection of advanced colorectal neoplasia*. *Clin Colorectal Cancer*, 2003. **3**(1): p. 47-53.
449. Traverso, G., et al., *Detection of APC mutations in fecal DNA from patients with colorectal tumors*. *N Engl J Med*, 2002. **346**(5): p. 311-20.
450. Itzkowitz, S.H. and N. Harpaz, *Diagnosis and management of dysplasia in patients with inflammatory bowel diseases*. *Gastroenterology*, 2004. **126**(6): p. 1634-48.
451. Velayos, F.S., J.P. Terdiman, and J.M. Walsh, *Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies*. *Am J Gastroenterol*, 2005. **100**(6): p. 1345-53.
452. Moody, G.A., et al., *Long-term therapy with sulphasalazine protects against colorectal cancer in ulcerative colitis: a retrospective study of colorectal cancer risk and compliance with treatment in Leicestershire*. *Eur J Gastroenterol Hepatol*, 1996. **8**(12): p. 1179-83.
453. Rubin, D.T., et al., *Aminosalicylate therapy in the prevention of dysplasia and colorectal cancer in ulcerative colitis*. *Clin Gastroenterol Hepatol*, 2006. **4**(11): p. 1346-50.
454. Eaden, J., et al., *Colorectal cancer prevention in ulcerative colitis: a case-control study*. *Aliment Pharmacol Ther*, 2000. **14**(2): p. 145-53.
455. van Staa, T.P., et al., *5-Aminosalicylate use and colorectal cancer risk in inflammatory bowel disease: a large epidemiological study*. *Gut*, 2005. **54**(11): p. 1573-8.

456. Bernstein, C.N., et al., *Does the use of 5-aminosalicylates in inflammatory bowel disease prevent the development of colorectal cancer?* Am J Gastroenterol, 2003. **98**(12): p. 2784-8.
457. Bernstein, C.N., Z. Nugent, and J.F. Blanchard, *5-aminosalicylate is not chemoprophylactic for colorectal cancer in IBD: a population based study.* Am J Gastroenterol, 2011. **106**(4): p. 731-6.
458. Nguyen, G.C., A. Gulamhusein, and C.N. Bernstein, *5-Aminosalicylic Acid Is Not Protective Against Colorectal Cancer in Inflammatory Bowel Disease: A Meta-Analysis of Non-Referral Populations.* Am J Gastroenterol, 2012.
459. Terdiman, J.P., et al., *5-Aminosalicylic acid therapy and the risk of colorectal cancer among patients with inflammatory bowel disease.* Inflamm Bowel Dis, 2007. **13**(4): p. 367-71.
460. Reinacher-Schick, A., et al., *Mesalazine changes apoptosis and proliferation in normal mucosa of patients with sporadic polyps of the large bowel.* Endoscopy, 2000. **32**(3): p. 245-54.
461. Bus, P.J., et al., *Mesalazine-induced apoptosis of colorectal cancer: on the verge of a new chemopreventive era?* Aliment Pharmacol Ther, 1999. **13**(11): p. 1397-402.
462. Clapper, M.L., et al., *5-aminosalicylic acid inhibits colitis-associated colorectal dysplasias in the mouse model of azoxymethane/dextran sulfate sodium-induced colitis.* Inflamm Bowel Dis, 2008. **14**(10): p. 1341-7.
463. Ikeda, I., et al., *5-aminosalicylic acid given in the remission stage of colitis suppresses colitis-associated cancer in a mouse colitis model.* Clin Cancer Res, 2007. **13**(21): p. 6527-31.
464. Reinacher-Schick, A., et al., *Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells.* Carcinogenesis, 2003. **24**(3): p. 443-51.
465. Stolfi, C., et al., *Mesalazine negatively regulates CDC25A protein expression and promotes accumulation of colon cancer cells in S phase.* Carcinogenesis, 2008. **29**(6): p. 1258-66.
466. Shapiro, G.I., *Cyclin-dependent kinase pathways as targets for cancer treatment.* J Clin Oncol, 2006. **24**(11): p. 1770-83.
467. Luciani, M.G., et al., *5-ASA affects cell cycle progression in colorectal cells by reversibly activating a replication checkpoint.* Gastroenterology, 2007. **132**(1): p. 221-35.
468. Baan, B., et al., *5-aminosalicylic acid inhibits cell cycle progression in a phospholipase D dependent manner in colorectal cancer.* Gut, 2011.
469. Koelink, P.J., et al., *5-aminosalicylic acid interferes in the cell cycle of colorectal cancer cells and induces cell death modes.* Inflamm Bowel Dis, 2010. **16**(3): p. 379-89.
470. Lengauer, C., K.W. Kinzler, and B. Vogelstein, *Genetic instabilities in human cancers.* Nature, 1998. **396**(6712): p. 643-9.

471. Ionov, Y., et al., *Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis*. Nature, 1993. **363**(6429): p. 558-61.
472. Campregher, C., et al., *Mesalazine reduces mutations in transforming growth factor beta receptor II and activin type II receptor by improvement of replication fidelity in mononucleotide repeats*. Clin Cancer Res, 2010. **16**(6): p. 1950-6.
473. Gasche, C., et al., *Mesalazine improves replication fidelity in cultured colorectal cells*. Cancer Res, 2005. **65**(10): p. 3993-7.
474. Kastan, M.B. and J. Bartek, *Cell-cycle checkpoints and cancer*. Nature, 2004. **432**(7015): p. 316-23.
475. Bienz, M. and H. Clevers, *Linking colorectal cancer to Wnt signaling*. Cell, 2000. **103**(2): p. 311-20.
476. Bos, C.L., et al., *Protein phosphatase 2A is required for mesalazine-dependent inhibition of Wnt/beta-catenin pathway activity*. Carcinogenesis, 2006. **27**(12): p. 2371-82.
477. Ratcliffe, M.J., K. Itoh, and S.Y. Sokol, *A positive role for the PP2A catalytic subunit in Wnt signal transduction*. J Biol Chem, 2000. **275**(46): p. 35680-3.
478. Jorissen, R.N., et al., *Epidermal growth factor receptor: mechanisms of activation and signalling*. Exp Cell Res, 2003. **284**(1): p. 31-53.
479. Spano, J.P., et al., *Impact of EGFR expression on colorectal cancer patient prognosis and survival*. Ann Oncol, 2005. **16**(1): p. 102-8.
480. Svrcek, M., et al., *Expression of epidermal growth factor receptor (EGFR) is frequent in inflammatory bowel disease (IBD)-associated intestinal cancer*. Virchows Arch, 2007. **450**(2): p. 243-4.
481. Monteleone, G., et al., *Silencing of SH-PTP2 defines a crucial role in the inactivation of epidermal growth factor receptor by 5-aminosalicylic acid in colon cancer cells*. Cell Death Differ, 2006. **13**(2): p. 202-11.
482. Huls, G., J.J. Koornstra, and J.H. Kleibeuker, *Non-steroidal anti-inflammatory drugs and molecular carcinogenesis of colorectal carcinomas*. Lancet, 2003. **362**(9379): p. 230-2.
483. Stolfi, C., et al., *Cyclooxygenase-2-dependent and -independent inhibition of proliferation of colon cancer cells by 5-aminosalicylic acid*. Biochem Pharmacol, 2008. **75**(3): p. 668-76.
484. Joshi, R., et al., *Free radical scavenging reactions of sulfasalazine, 5-aminosalicylic acid and sulfapyridine: mechanistic aspects and antioxidant activity*. Free Radic Res, 2005. **39**(11): p. 1163-72.
485. Rubin, D.T., et al., *Colorectal cancer prevention in inflammatory bowel disease and the role of 5-aminosalicylic acid: a clinical review and update*. Inflamm Bowel Dis, 2008. **14**(2): p. 265-74.

486. Campregher, C., et al., *The position of the amino group on the benzene ring is critical for mesalamine's improvement of replication fidelity*. *Inflamm Bowel Dis*, 2010. **16**(4): p. 576-82.
487. Lopez-Alarcon, C., et al., *Reaction of 5-aminosalicylic acid with peroxy radicals: protection and recovery by ascorbic acid and amino acids*. *Pharm Res*, 2005. **22**(10): p. 1642-8.
488. Lopez-Alarcon, C., H. Speisky, and E. Lissi, *Antioxidant effect of 5-amino salicylic acid on copper-mediated LDL oxidation*. *Biol Res*, 2007. **40**(2): p. 155-62.
489. Ancha, H.R., et al., *Effects of N-acetylcysteine plus mesalamine on prostaglandin synthesis and nitric oxide generation in TNBS-induced colitis in rats*. *Dig Dis Sci*, 2009. **54**(4): p. 758-66.
490. Horvath, K., et al., *The involvement of heme oxygenase-1 activity in the therapeutic actions of 5-aminosalicylic acid in rat colitis*. *Eur J Pharmacol*, 2008. **581**(3): p. 315-23.
491. Mantena, S.K., et al., *In vivo radioprotection by 5-aminosalicylic acid*. *Mutat Res*, 2008. **650**(1): p. 63-79.
492. Kennedy, M., et al., *5-aminosalicylic acid inhibits iNOS transcription in human intestinal epithelial cells*. *Int J Mol Med*, 1999. **4**(4): p. 437-43.
493. Nandi, J., et al., *5-aminosalicylic acid improves indomethacin-induced enteropathy by inhibiting iNOS transcription in rats*. *Dig Dis Sci*, 2008. **53**(1): p. 123-32.
494. Joo, K., et al., *An anti-inflammatory mechanism of taurine conjugated 5-aminosalicylic acid against experimental colitis: taurine chloramine potentiates inhibitory effect of 5-aminosalicylic acid on IL-1beta-mediated NFkappaB activation*. *Eur J Pharmacol*, 2009. **618**(1-3): p. 91-7.
495. Wahl, C., et al., *Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B*. *J Clin Invest*, 1998. **101**(5): p. 1163-74.
496. Swidsinski, A., et al., *Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease*. *J Clin Microbiol*, 2005. **43**(7): p. 3380-9.
497. Darfeuille-Michaud, A., et al., *Presence of adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease*. *Gastroenterology*, 1998. **115**(6): p. 1405-13.
498. Swidsinski, A., et al., *Association between intraepithelial Escherichia coli and colorectal cancer*. *Gastroenterology*, 1998. **115**(2): p. 281-6.
499. Subramanian, S., et al., *Characterization of epithelial IL-8 response to inflammatory bowel disease mucosal E. coli and its inhibition by mesalamine*. *Inflamm Bowel Dis*, 2008. **14**(2): p. 162-75.
500. Kaufman, J., et al., *Effects of mesalamine (5-aminosalicylic acid) on bacterial gene expression*. *Inflamm Bowel Dis*, 2009. **15**(7): p. 985-96.

501. Matthiessen, M.W., et al., *Peroxisome proliferator-activated receptor expression and activation in normal human colonic epithelial cells and tubular adenomas*. Scand J Gastroenterol, 2005. **40**(2): p. 198-205.
502. Osawa, E., et al., *Peroxisome proliferator-activated receptor gamma ligands suppress colon carcinogenesis induced by azoxymethane in mice*. Gastroenterology, 2003. **124**(2): p. 361-7.
503. Tanaka, T., et al., *Ligands for peroxisome proliferator-activated receptors alpha and gamma inhibit chemically induced colitis and formation of aberrant crypt foci in rats*. Cancer Res, 2001. **61**(6): p. 2424-8.
504. Schwab, M., et al., *PPARgamma is involved in mesalazine-mediated induction of apoptosis and inhibition of cell growth in colon cancer cells*. Carcinogenesis, 2008. **29**(7): p. 1407-14.
505. Willson, T.M., et al., *The PPARs: from orphan receptors to drug discovery*. J Med Chem, 2000. **43**(4): p. 527-50.
506. Wang, T., et al., *Peroxisome proliferator-activated receptor gamma in malignant diseases*. Crit Rev Oncol Hematol, 2006. **58**(1): p. 1-14.
507. Mansure, J.J., R. Nassim, and W. Kassouf, *Peroxisome proliferator-activated receptor gamma in bladder cancer: a promising therapeutic target*. Cancer Biol Ther, 2009. **8**(7): p. 6-15.
508. DuBois, R.N., et al., *The nuclear eicosanoid receptor, PPARgamma, is aberrantly expressed in colonic cancers*. Carcinogenesis, 1998. **19**(1): p. 49-53.
509. Sarraf, P., et al., *Differentiation and reversal of malignant changes in colon cancer through PPARgamma*. Nat Med, 1998. **4**(9): p. 1046-52.
510. Mueller, E., et al., *Terminal differentiation of human breast cancer through PPAR gamma*. Mol Cell, 1998. **1**(3): p. 465-70.
511. Kubota, T., et al., *Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo*. Cancer Res, 1998. **58**(15): p. 3344-52.
512. Alarcon de la Lastra, C., et al., *New pharmacological perspectives and therapeutic potential of PPAR-gamma agonists*. Curr Pharm Des, 2004. **10**(28): p. 3505-24.
513. Schoonjans, K., B. Staels, and J. Auwerx, *The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation*. Biochim Biophys Acta, 1996. **1302**(2): p. 93-109.
514. Sarraf, P., et al., *Loss-of-function mutations in PPAR gamma associated with human colon cancer*. Mol Cell, 1999. **3**(6): p. 799-804.
515. Ikezoe, T., et al., *Mutational analysis of the peroxisome proliferator-activated receptor gamma gene in human malignancies*. Cancer Res, 2001. **61**(13): p. 5307-10.

516. Ogino, S., et al., *Colorectal cancer expression of peroxisome proliferator-activated receptor gamma (PPARG, PPARgamma) is associated with good prognosis*. *Gastroenterology*, 2009. **136**(4): p. 1242-50.
517. McAlpine, C.A., et al., *Intestinal-specific PPARgamma deficiency enhances tumorigenesis in ApcMin/+ mice*. *Int J Cancer*, 2006. **119**(10): p. 2339-46.
518. Patel, L., et al., *Tumor suppressor and anti-inflammatory actions of PPARgamma agonists are mediated via upregulation of PTEN*. *Curr Biol*, 2001. **11**(10): p. 764-8.
519. Yamada, K.M. and M. Araki, *Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis*. *J Cell Sci*, 2001. **114**(Pt 13): p. 2375-82.
520. Dai, Y., et al., *Loss of XIAP sensitizes rosiglitazone-induced growth inhibition of colon cancer in vivo*. *Int J Cancer*, 2008. **122**(12): p. 2858-63.
521. Wang, D., et al., *Peroxisome proliferator-activated receptor delta confers resistance to peroxisome proliferator-activated receptor gamma-induced apoptosis in colorectal cancer cells*. *Oncogene*, 2012. **31**(8): p. 1013-23.
522. Altieri, D.C., *Validating survivin as a cancer therapeutic target*. *Nat Rev Cancer*, 2003. **3**(1): p. 46-54.
523. Kawasaki, H., et al., *Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer*. *Cancer Res*, 1998. **58**(22): p. 5071-4.
524. Sarela, A.I., et al., *Immunohistochemical detection of the anti-apoptosis protein, survivin, predicts survival after curative resection of stage II colorectal carcinomas*. *Ann Surg Oncol*, 2001. **8**(4): p. 305-10.
525. Qiao, L., et al., *Down-regulation of X-linked inhibitor of apoptosis synergistically enhanced peroxisome proliferator-activated receptor gamma ligand-induced growth inhibition in colon cancer*. *Mol Cancer Ther*, 2008. **7**(7): p. 2203-11.
526. Kaufmann, T., A. Strasser, and P.J. Jost, *Fas death receptor signalling: roles of Bid and XIAP*. *Cell Death Differ*, 2012. **19**(1): p. 42-50.
527. Lee, C.J., et al., *Pioglitazone, a synthetic ligand for PPARgamma, induces apoptosis in RB-deficient human colorectal cancer cells*. *Apoptosis*, 2006. **11**(3): p. 401-11.
528. Ban, J.O., et al., *Suppression of NF-kappaB and GSK-3beta is involved in colon cancer cell growth inhibition by the PPAR agonist troglitazone*. *Chem Biol Interact*, 2010. **188**(1): p. 75-85.
529. Ghosh, S., M.J. May, and E.B. Kopp, *NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses*. *Annu Rev Immunol*, 1998. **16**: p. 225-60.
530. Pikarsky, E., et al., *NF-kappaB functions as a tumour promoter in inflammation-associated cancer*. *Nature*, 2004. **431**(7007): p. 461-6.

531. Garg, A. and B.B. Aggarwal, *Nuclear transcription factor-kappaB as a target for cancer drug development*. Leukemia, 2002. **16**(6): p. 1053-68.
532. Kim, E., et al., *CDK5 is a novel regulatory protein in PPARgamma ligand-induced antiproliferation*. Int J Oncol, 2006. **28**(1): p. 191-4.
533. Kitamura, S., et al., *PPARgamma agonists inhibit cell growth and suppress the expression of cyclin D1 and EGF-like growth factors in ras-transformed rat intestinal epithelial cells*. Int J Cancer, 2001. **94**(3): p. 335-42.
534. Chen, A. and J. Xu, *Activation of PPAR{gamma} by curcumin inhibits Moser cell growth and mediates suppression of gene expression of cyclin D1 and EGFR*. Am J Physiol Gastrointest Liver Physiol, 2005. **288**(3): p. G447-56.
535. Chen, F. and L.E. Harrison, *Ciglitazone-induced cellular anti-proliferation increases p27kip1 protein levels through both increased transcriptional activity and inhibition of proteasome degradation*. Cell Signal, 2005. **17**(7): p. 809-16.
536. Chen, F., et al., *Ciglitazone-induced p27 gene transcriptional activity is mediated through Sp1 and is negatively regulated by the MAPK signaling pathway*. Cell Signal, 2005. **17**(12): p. 1572-7.
537. Lu, S., et al., *Cyclooxygenase-2 overexpression in MCF-10F human breast epithelial cells inhibits proliferation, apoptosis and differentiation, and causes partial transformation*. Int J Cancer, 2005. **116**(6): p. 847-52.
538. Gately, S. and W.W. Li, *Multiple roles of COX-2 in tumor angiogenesis: a target for antiangiogenic therapy*. Semin Oncol, 2004. **31**(2 Suppl 7): p. 2-11.
539. Chen, Z.Y. and C.C. Tseng, *15-deoxy-Delta12,14 prostaglandin J2 up-regulates Kruppel-like factor 4 expression independently of peroxisome proliferator-activated receptor gamma by activating the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase signal transduction pathway in HT-29 colon cancer cells*. Mol Pharmacol, 2005. **68**(5): p. 1203-13.
540. Dang, D.T., J. Pevsner, and V.W. Yang, *The biology of the mammalian Kruppel-like family of transcription factors*. Int J Biochem Cell Biol, 2000. **32**(11-12): p. 1103-21.
541. Bieker, J.J., *Kruppel-like factors: three fingers in many pies*. J Biol Chem, 2001. **276**(37): p. 34355-8.
542. Chen, Z.Y., J. Shie, and C. Tseng, *Up-regulation of gut-enriched kruppel-like factor by interferon-gamma in human colon carcinoma cells*. FEBS Lett, 2000. **477**(1-2): p. 67-72.
543. Dang, D.T., et al., *Overexpression of Kruppel-like factor 4 in the human colon cancer cell line RKO leads to reduced tumorigenicity*. Oncogene, 2003. **22**(22): p. 3424-30.

544. Rageul, J., et al., *KLF4-dependent, PPARgamma-induced expression of GPA33 in colon cancer cell lines*. Int J Cancer, 2009. **125**(12): p. 2802-9.
545. Chen, G.G., et al., *15-hydroxy-eicosatetraenoic acid arrests growth of colorectal cancer cells via a peroxisome proliferator-activated receptor gamma-dependent pathway*. Int J Cancer, 2003. **107**(5): p. 837-43.
546. Chen, G.G., et al., *Apoptosis induced by activation of peroxisome-proliferator activated receptor-gamma is associated with Bcl-2 and NF-kappaB in human colon cancer*. Life Sci, 2002. **70**(22): p. 2631-46.
547. Toaldo, C., et al., *PPARgamma ligands inhibit telomerase activity and hTERT expression through modulation of the Myc/Mad/Max network in colon cancer cells*. J Cell Mol Med, 2010. **14**(6A): p. 1347-57.
548. Kawamata, H., et al., *Differentiation-inducing therapy for solid tumors*. Curr Pharm Des, 2006. **12**(3): p. 379-85.
549. Yoshizumi, T., et al., *Thiazolidinedione, a peroxisome proliferator-activated receptor-gamma ligand, inhibits growth and metastasis of HT-29 human colon cancer cells through differentiation-promoting effects*. Int J Oncol, 2004. **25**(3): p. 631-9.
550. Risau, W., *Mechanisms of angiogenesis*. Nature, 1997. **386**(6626): p. 671-4.
551. Shen, D., C. Deng, and M. Zhang, *Peroxisome proliferator-activated receptor gamma agonists inhibit the proliferation and invasion of human colon cancer cells*. Postgrad Med J, 2007. **83**(980): p. 414-9.
552. Sternlicht, M.D. and Z. Werb, *How matrix metalloproteinases regulate cell behavior*. Annu Rev Cell Dev Biol, 2001. **17**: p. 463-516.
553. Aggarwal, B.B., et al., *Inflammation and cancer: how hot is the link?* Biochem Pharmacol, 2006. **72**(11): p. 1605-21.
554. Giri, S., et al., *The 15-deoxy-delta12,14-prostaglandin J2 inhibits the inflammatory response in primary rat astrocytes via down-regulating multiple steps in phosphatidylinositol 3-kinase-Akt-NF-kappaB-p300 pathway independent of peroxisome proliferator-activated receptor gamma*. J Immunol, 2004. **173**(8): p. 5196-208.
555. Petrova, T.V., K.T. Akama, and L.J. Van Eldik, *Cyclopentenone prostaglandins suppress activation of microglia: down-regulation of inducible nitric-oxide synthase by 15-deoxy-Delta12,14-prostaglandin J2*. Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4668-73.
556. Chatterjee, P.K., et al., *The cyclopentenone prostaglandin 15-deoxy-Delta(12,14)-prostaglandin J2 ameliorates ischemic acute renal failure*. Cardiovasc Res, 2004. **61**(3): p. 630-43.

557. Inoue, H., T. Tanabe, and K. Umesono, *Feedback control of cyclooxygenase-2 expression through PPARgamma*. J Biol Chem, 2000. **275**(36): p. 28028-32.
558. Fahmi, H., et al., *15d-PGJ(2) is acting as a 'dual agent' on the regulation of COX-2 expression in human osteoarthritic chondrocytes*. Osteoarthritis Cartilage, 2002. **10**(11): p. 845-8.
559. Subbaramaiah, K., et al., *Peroxisome proliferator-activated receptor gamma ligands suppress the transcriptional activation of cyclooxygenase-2. Evidence for involvement of activator protein-1 and CREB-binding protein/p300*. J Biol Chem, 2001. **276**(15): p. 12440-8.
560. Kim, E.H. and Y.J. Surh, *15-deoxy-Delta12,14-prostaglandin J2 as a potential endogenous regulator of redox-sensitive transcription factors*. Biochem Pharmacol, 2006. **72**(11): p. 1516-28.
561. Minuzzo, S., et al., *Angiogenesis meets immunology: cytokine gene therapy of cancer*. Mol Aspects Med, 2007. **28**(1): p. 59-86.
562. Azuma, Y., et al., *15-Deoxy-delta(12,14)-prostaglandin J2 is a negative regulator of macrophage functions*. Int Immunopharmacol, 2001. **1**(12): p. 2101-8.
563. Rao, C.V. and B.S. Reddy, *NSAIDs and chemoprevention*. Curr Cancer Drug Targets, 2004. **4**(1): p. 29-42.
564. Feagins, L.A. and B.L. Cryer, *Do non-steroidal anti-inflammatory drugs cause exacerbations of inflammatory bowel disease?* Dig Dis Sci, 2010. **55**(2): p. 226-32.
565. Matuk, R., et al., *The spectrum of gastrointestinal toxicity and effect on disease activity of selective cyclooxygenase-2 inhibitors in patients with inflammatory bowel disease*. Inflamm Bowel Dis, 2004. **10**(4): p. 352-6.
566. Prinz-Langenohl, R., I. Fohr, and K. Pietrzik, *Beneficial role for folate in the prevention of colorectal and breast cancer*. Eur J Nutr, 2001. **40**(3): p. 98-105.
567. Swinson, C.M., et al., *Role of sulphasalazine in the aetiology of folate deficiency in ulcerative colitis*. Gut, 1981. **22**(6): p. 456-61.
568. Thorlacius, H. and E. Toth, *Role of chromoendoscopy in colon cancer surveillance in inflammatory bowel disease*. Inflamm Bowel Dis, 2007. **13**(7): p. 911-7.
569. Koelink, P.J., et al., *5-Aminosalicylic acid inhibits colitis-associated but not sporadic colorectal neoplasia in a novel conditional Apc mouse model*. Carcinogenesis, 2009. **30**(7): p. 1217-24.
570. Girnun, G.D., et al., *APC-dependent suppression of colon carcinogenesis by PPARgamma*. Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13771-6.
571. Issemann, I. and S. Green, *Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators*. Nature, 1990. **347**(6294): p. 645-50.
572. Tyagi, S., et al., *The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases*. J Adv Pharm Technol Res, 2011. **2**(4): p. 236-40.

573. Zhu, Y., et al., *Cloning and identification of mouse steroid receptor coactivator-1 (mSRC-1), as a coactivator of peroxisome proliferator-activated receptor gamma*. *Gene Expr*, 1996. **6**(3): p. 185-95.
574. Zhu, Y., et al., *Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR gamma) gene: alternative promoter use and different splicing yield two mPPAR gamma isoforms*. *Proc Natl Acad Sci U S A*, 1995. **92**(17): p. 7921-5.
575. Fajas, L., et al., *The organization, promoter analysis, and expression of the human PPARgamma gene*. *J Biol Chem*, 1997. **272**(30): p. 18779-89.
576. Fajas, L., J.C. Fruchart, and J. Auwerx, *PPARgamma3 mRNA: a distinct PPARgamma mRNA subtype transcribed from an independent promoter*. *FEBS Lett*, 1998. **438**(1-2): p. 55-60.
577. Sundvold, H. and S. Lien, *Identification of a novel peroxisome proliferator-activated receptor (PPAR) gamma promoter in man and transactivation by the nuclear receptor RORalpha1*. *Biochem Biophys Res Commun*, 2001. **287**(2): p. 383-90.
578. Chen, Y., A.R. Jimenez, and J.D. Medh, *Identification and regulation of novel PPAR-gamma splice variants in human THP-1 macrophages*. *Biochim Biophys Acta*, 2006. **1759**(1-2): p. 32-43.
579. Bishop-Bailey, D. and J. Wray, *Peroxisome proliferator-activated receptors: a critical review on endogenous pathways for ligand generation*. *Prostaglandins Other Lipid Mediat*, 2003. **71**(1-2): p. 1-22.
580. Schupp, M. and M.A. Lazar, *Endogenous ligands for nuclear receptors: digging deeper*. *J Biol Chem*, 2010. **285**(52): p. 40409-15.
581. Benoit, G., et al., *International Union of Pharmacology. LXVI. Orphan nuclear receptors*. *Pharmacol Rev*, 2006. **58**(4): p. 798-836.
582. Berger, J.P., T.E. Akiyama, and P.T. Meinke, *PPARs: therapeutic targets for metabolic disease*. *Trends Pharmacol Sci*, 2005. **26**(5): p. 244-51.
583. Berger, J., et al., *Novel peroxisome proliferator-activated receptor (PPAR) gamma and PPARdelta ligands produce distinct biological effects*. *J Biol Chem*, 1999. **274**(10): p. 6718-25.
584. Duval, C., et al., *The role of PPARs in atherosclerosis*. *Trends Mol Med*, 2002. **8**(9): p. 422-30.
585. Martens, F.M., et al., *Metabolic and additional vascular effects of thiazolidinediones*. *Drugs*, 2002. **62**(10): p. 1463-80.
586. Zoete, V., A. Grosdidier, and O. Michielin, *Peroxisome proliferator-activated receptor structures: ligand specificity, molecular switch and interactions with regulators*. *Biochim Biophys Acta*, 2007. **1771**(8): p. 915-25.
587. Yu, S. and J.K. Reddy, *Transcription coactivators for peroxisome proliferator-activated receptors*. *Biochim Biophys Acta*, 2007. **1771**(8): p. 936-51.

588. Varga, T., Z. Czimmerer, and L. Nagy, *PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation*. *Biochim Biophys Acta*, 2011. **1812**(8): p. 1007-22.
589. Kilgore, K.S. and A.N. Billin, *PPARbeta/delta ligands as modulators of the inflammatory response*. *Curr Opin Investig Drugs*, 2008. **9**(5): p. 463-9.
590. Peters, J.M., J.E. Foreman, and F.J. Gonzalez, *Dissecting the role of peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta) in colon, breast, and lung carcinogenesis*. *Cancer Metastasis Rev*, 2011. **30**(3-4): p. 619-40.
591. Peters, J.M., J.L. Morales, and F.J. Gonzalez, *Modulation of gastrointestinal inflammation and colorectal tumorigenesis by peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta)*. *Drug Discov Today Dis Mech*, 2011. **8**(3-4): p. e85-e93.
592. Pascual, G., et al., *A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma*. *Nature*, 2005. **437**(7059): p. 759-63.
593. Adhikary, T., et al., *Genomewide analyses define different modes of transcriptional regulation by peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta)*. *PLoS One*, 2011. **6**(1): p. e16344.
594. Choi, J.H., et al., *Antidiabetic actions of a non-agonist PPARgamma ligand blocking Cdk5-mediated phosphorylation*. *Nature*, 2011. **477**(7365): p. 477-81.
595. Escher, P., et al., *Rat PPARs: quantitative analysis in adult rat tissues and regulation in fasting and refeeding*. *Endocrinology*, 2001. **142**(10): p. 4195-202.
596. Pyper, S.R., et al., *PPARalpha: energy combustion, hypolipidemia, inflammation and cancer*. *Nucl Recept Signal*, 2010. **8**: p. e002.
597. Hashimoto, T., et al., *Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting*. *J Biol Chem*, 2000. **275**(37): p. 28918-28.
598. Kersten, S., et al., *Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting*. *J Clin Invest*, 1999. **103**(11): p. 1489-98.
599. Leone, T.C., C.J. Weinheimer, and D.P. Kelly, *A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders*. *Proc Natl Acad Sci U S A*, 1999. **96**(13): p. 7473-8.
600. Martin, G., et al., *Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators*. *J Biol Chem*, 1997. **272**(45): p. 28210-7.

601. Sato, O., et al., *Dual promoter structure of mouse and human fatty acid translocase/CD36 genes and unique transcriptional activation by peroxisome proliferator-activated receptor alpha and gamma ligands*. J Biol Chem, 2002. **277**(18): p. 15703-11.
602. Guerre-Millo, M., et al., *Peroxisome proliferator-activated receptor alpha activators improve insulin sensitivity and reduce adiposity*. J Biol Chem, 2000. **275**(22): p. 16638-42.
603. Crisafulli, C., et al., *PPAR-alpha contributes to the anti-inflammatory activity of 17beta-estradiol*. J Pharmacol Exp Ther, 2009. **331**(3): p. 796-807.
604. Lemberger, T., et al., *Expression of the peroxisome proliferator-activated receptor alpha gene is stimulated by stress and follows a diurnal rhythm*. J Biol Chem, 1996. **271**(3): p. 1764-9.
605. Rotman, N. and W. Wahli, *Fatty acid synthesis and PPARalpha hand in hand*. Chem Biol, 2009. **16**(8): p. 801-2.
606. Chakravarthy, M.V., et al., *Identification of a physiologically relevant endogenous ligand for PPARalpha in liver*. Cell, 2009. **138**(3): p. 476-88.
607. Yessoufou, A., et al., *Peroxisome proliferator-activated receptor alpha deficiency increases the risk of maternal abortion and neonatal mortality in murine pregnancy with or without diabetes mellitus: Modulation of T cell differentiation*. Endocrinology, 2006. **147**(9): p. 4410-8.
608. Hashimoto, F., et al., *PPARalpha agonists clofibrate and gemfibrozil inhibit cell growth, down-regulate hCG and up-regulate progesterone secretions in immortalized human trophoblast cells*. Biochem Pharmacol, 2004. **68**(2): p. 313-21.
609. Michalik, L., et al., *PPAR expression and function during vertebrate development*. Int J Dev Biol, 2002. **46**(1): p. 105-14.
610. Wang, Q., H. Fujii, and G.T. Knipp, *Expression of PPAR and RXR isoforms in the developing rat and human term placentas*. Placenta, 2002. **23**(8-9): p. 661-71.
611. Kota, B.P., T.H. Huang, and B.D. Roufogalis, *An overview on biological mechanisms of PPARs*. Pharmacol Res, 2005. **51**(2): p. 85-94.
612. Mandard, S., M. Muller, and S. Kersten, *Peroxisome proliferator-activated receptor alpha target genes*. Cell Mol Life Sci, 2004. **61**(4): p. 393-416.
613. Kersten, S., et al., *The peroxisome proliferator-activated receptor alpha regulates amino acid metabolism*. FASEB J, 2001. **15**(11): p. 1971-8.
614. Uhlen, M., et al., *Towards a knowledge-based Human Protein Atlas*. Nat Biotechnol, 2010. **28**(12): p. 1248-50.

615. Krey, G., et al., *Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay.* Mol Endocrinol, 1997. **11**(6): p. 779-91.
616. Leibowitz, M.D., et al., *Activation of PPARdelta alters lipid metabolism in db/db mice.* FEBS Lett, 2000. **473**(3): p. 333-6.
617. Oliver, W.R., Jr., et al., *A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5306-11.
618. Sprecher, D.L., et al., *Triglyceride:high-density lipoprotein cholesterol effects in healthy subjects administered a peroxisome proliferator activated receptor delta agonist.* Arterioscler Thromb Vasc Biol, 2007. **27**(2): p. 359-65.
619. Tanaka, T., et al., *Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15924-9.
620. Wang, Y.X., et al., *Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity.* Cell, 2003. **113**(2): p. 159-70.
621. Wang, Y.X., et al., *Regulation of muscle fiber type and running endurance by PPARdelta.* PLoS Biol, 2004. **2**(10): p. e294.
622. Schuler, M., et al., *PGC1alpha expression is controlled in skeletal muscles by PPARbeta, whose ablation results in fiber-type switching, obesity, and type 2 diabetes.* Cell Metab, 2006. **4**(5): p. 407-14.
623. Hansen, J.B., et al., *Peroxisome proliferator-activated receptor delta (PPARdelta)-mediated regulation of preadipocyte proliferation and gene expression is dependent on cAMP signaling.* J Biol Chem, 2001. **276**(5): p. 3175-82.
624. Barish, G.D., V.A. Narkar, and R.M. Evans, *PPAR delta: a dagger in the heart of the metabolic syndrome.* J Clin Invest, 2006. **116**(3): p. 590-7.
625. Lim, H.J., et al., *PPARdelta ligand L-165041 ameliorates Western diet-induced hepatic lipid accumulation and inflammation in LDLR-/- mice.* Eur J Pharmacol, 2009. **622**(1-3): p. 45-51.
626. Liu, S., et al., *Role of peroxisome proliferator-activated receptor {delta}/{beta} in hepatic metabolic regulation.* J Biol Chem, 2011. **286**(2): p. 1237-47.
627. Nagasawa, T., et al., *Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPARdelta agonist, on development of steatohepatitis in mice fed a methionine- and choline-deficient diet.* Eur J Pharmacol, 2006. **536**(1-2): p. 182-91.
628. Shan, W., et al., *Peroxisome proliferator-activated receptor-beta/delta protects against chemically induced liver toxicity in mice.* Hepatology, 2008. **47**(1): p. 225-35.

629. Shan, W., et al., *Ligand activation of peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) attenuates carbon tetrachloride hepatotoxicity by downregulating proinflammatory gene expression*. *Toxicol Sci*, 2008. **105**(2): p. 418-28.
630. Lim, H. and S.K. Dey, *PPAR delta functions as a prostacyclin receptor in blastocyst implantation*. *Trends Endocrinol Metab*, 2000. **11**(4): p. 137-42.
631. Peters, J.M., et al., *Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta)*. *Mol Cell Biol*, 2000. **20**(14): p. 5119-28.
632. Mano, H., et al., *Cloning and function of rabbit peroxisome proliferator-activated receptor delta/beta in mature osteoclasts*. *J Biol Chem*, 2000. **275**(11): p. 8126-32.
633. Burdick, A.D., et al., *The role of peroxisome proliferator-activated receptor-beta/delta in epithelial cell growth and differentiation*. *Cell Signal*, 2006. **18**(1): p. 9-20.
634. Tontonoz, P., et al., *mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer*. *Genes Dev*, 1994. **8**(10): p. 1224-34.
635. Yu, S., et al., *Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARGgamma1) overexpression*. *J Biol Chem*, 2003. **278**(1): p. 498-505.
636. Wahli, W., *PPAR gamma: ally and foe in bone metabolism*. *Cell Metab*, 2008. **7**(3): p. 188-90.
637. Akune, T., et al., *PPARGgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors*. *J Clin Invest*, 2004. **113**(6): p. 846-55.
638. Cock, T.A., et al., *Enhanced bone formation in lipodystrophic PPARgamma(hyp/hyp) mice relocates haematopoiesis to the spleen*. *EMBO Rep*, 2004. **5**(10): p. 1007-12.
639. Barak, Y., et al., *PPAR gamma is required for placental, cardiac, and adipose tissue development*. *Mol Cell*, 1999. **4**(4): p. 585-95.
640. Nadra, K., et al., *Differentiation of trophoblast giant cells and their metabolic functions are dependent on peroxisome proliferator-activated receptor beta/delta*. *Mol Cell Biol*, 2006. **26**(8): p. 3266-81.
641. Semple, R.K., V.K. Chatterjee, and S. O'Rahilly, *PPAR gamma and human metabolic disease*. *J Clin Invest*, 2006. **116**(3): p. 581-9.
642. Attakpa, E., et al., *Docosahexaenoic acid modulates the expression of T-bet and GATA-3 transcription factors, independently of PPARalpha, through suppression of MAP kinase activation*. *Biochimie*, 2009. **91**(11-12): p. 1359-65.
643. Delerive, P., et al., *Peroxisome proliferator-activated receptor alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1*. *J Biol Chem*, 1999. **274**(45): p. 32048-54.

644. Takano, H., et al., *The role of PPARgamma-dependent pathway in the development of cardiac hypertrophy*. *Drugs Today (Barc)*, 2003. **39**(5): p. 347-57.
645. Shah, Y.M., K. Morimura, and F.J. Gonzalez, *Expression of peroxisome proliferator-activated receptor-gamma in macrophage suppresses experimentally induced colitis*. *Am J Physiol Gastrointest Liver Physiol*, 2007. **292**(2): p. G657-66.
646. Adachi, M., et al., *Peroxisome proliferator activated receptor gamma in colonic epithelial cells protects against experimental inflammatory bowel disease*. *Gut*, 2006. **55**(8): p. 1104-13.
647. Dubuquoy, L., et al., *PPARgamma as a new therapeutic target in inflammatory bowel diseases*. *Gut*, 2006. **55**(9): p. 1341-9.
648. Kielian, T. and P.D. Drew, *Effects of peroxisome proliferator-activated receptor-gamma agonists on central nervous system inflammation*. *J Neurosci Res*, 2003. **71**(3): p. 315-25.
649. Lovett-Racke, A.E., et al., *Peroxisome proliferator-activated receptor alpha agonists as therapy for autoimmune disease*. *J Immunol*, 2004. **172**(9): p. 5790-8.
650. Dubuquoy, L., et al., *[Peroxisome proliferator-activated receptor (PPAR) gamma: a new target for the treatment of inflammatory bowel disease]*. *Gastroenterol Clin Biol*, 2000. **24**(8-9): p. 719-24.
651. Okamoto, H., et al., *Inhibition of NF-kappaB signaling by fenofibrate, a peroxisome proliferator-activated receptor-alpha ligand, presents a therapeutic strategy for rheumatoid arthritis*. *Clin Exp Rheumatol*, 2005. **23**(3): p. 323-30.
652. Madej, A., et al., *Effects of fenofibrate on plasma cytokine concentrations in patients with atherosclerosis and hyperlipoproteinemia IIb*. *Int J Clin Pharmacol Ther*, 1998. **36**(6): p. 345-9.
653. Michalik, L. and W. Wahli, *PPARs Mediate Lipid Signaling in Inflammation and Cancer*. *PPAR Res*, 2008. **2008**: p. 134059.
654. Reddy, J.K., D.L. Azarnoff, and C.E. Hignite, *Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens*. *Nature*, 1980. **283**(5745): p. 397-8.
655. Peters, J.M., R.C. Cattley, and F.J. Gonzalez, *Role of PPAR alpha in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643*. *Carcinogenesis*, 1997. **18**(11): p. 2029-33.
656. Hays, T., et al., *Role of peroxisome proliferator-activated receptor-alpha (PPARalpha) in bezafibrate-induced hepatocarcinogenesis and cholestasis*. *Carcinogenesis*, 2005. **26**(1): p. 219-27.

657. Cheung, C., et al., *Diminished hepatocellular proliferation in mice humanized for the nuclear receptor peroxisome proliferator-activated receptor alpha*. *Cancer Res*, 2004. **64**(11): p. 3849-54.
658. Peters, J.M., C. Cheung, and F.J. Gonzalez, *Peroxisome proliferator-activated receptor-alpha and liver cancer: where do we stand?* *J Mol Med (Berl)*, 2005. **83**(10): p. 774-85.
659. Panigrahy, D., et al., *PPARalpha agonist fenofibrate suppresses tumor growth through direct and indirect angiogenesis inhibition*. *Proc Natl Acad Sci U S A*, 2008. **105**(3): p. 985-90.
660. Pozzi, A., et al., *Peroxisomal proliferator-activated receptor-alpha-dependent inhibition of endothelial cell proliferation and tumorigenesis*. *J Biol Chem*, 2007. **282**(24): p. 17685-95.
661. Balkwill, F., *TNF-alpha in promotion and progression of cancer*. *Cancer Metastasis Rev*, 2006. **25**(3): p. 409-16.
662. Aoyama, T., et al., *Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha)*. *J Biol Chem*, 1998. **273**(10): p. 5678-84.
663. Koppenol, W.H., P.L. Bounds, and C.V. Dang, *Otto Warburg's contributions to current concepts of cancer metabolism*. *Nat Rev Cancer*, 2011. **11**(5): p. 325-37.
664. Grommes, C., G.E. Landreth, and M.T. Heneka, *Antineoplastic effects of peroxisome proliferator-activated receptor gamma agonists*. *Lancet Oncol*, 2004. **5**(7): p. 419-29.
665. Koeffler, H.P., *Peroxisome proliferator-activated receptor gamma and cancers*. *Clin Cancer Res*, 2003. **9**(1): p. 1-9.
666. Elnemr, A., et al., *PPARGamma ligand (thiazolidinedione) induces growth arrest and differentiation markers of human pancreatic cancer cells*. *Int J Oncol*, 2000. **17**(6): p. 1157-64.
667. Gupta, R.A., et al., *Target genes of peroxisome proliferator-activated receptor gamma in colorectal cancer cells*. *J Biol Chem*, 2001. **276**(32): p. 29681-7.
668. Tontonoz, P., et al., *Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor*. *Proc Natl Acad Sci U S A*, 1997. **94**(1): p. 237-41.
669. Huang, J.W., et al., *Peroxisome proliferator-activated receptor gamma-independent ablation of cyclin D1 by thiazolidinediones and their derivatives in breast cancer cells*. *Mol Pharmacol*, 2005. **67**(4): p. 1342-8.
670. Lapillonne, H., et al., *Activation of peroxisome proliferator-activated receptor gamma by a novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest and apoptosis in breast cancer cells*. *Cancer Res*, 2003. **63**(18): p. 5926-39.

671. Wang, C., et al., *Inhibition of cellular proliferation through I κ B kinase-independent and peroxisome proliferator-activated receptor gamma-dependent repression of cyclin D1*. Mol Cell Biol, 2001. **21**(9): p. 3057-70.
672. Yin, F., et al., *Troglitazone inhibits growth of MCF-7 breast carcinoma cells by targeting G1 cell cycle regulators*. Biochem Biophys Res Commun, 2001. **286**(5): p. 916-22.
673. Koga, H., et al., *Involvement of p21(WAF1/Cip1), p27(Kip1), and p18(INK4c) in troglitazone-induced cell-cycle arrest in human hepatoma cell lines*. Hepatology, 2001. **33**(5): p. 1087-97.
674. Wei, S., et al., *Thiazolidinediones modulate the expression of beta-catenin and other cell-cycle regulatory proteins by targeting the F-box proteins of Skp1-Cul1-F-box protein E3 ubiquitin ligase independently of peroxisome proliferator-activated receptor gamma*. Mol Pharmacol, 2007. **72**(3): p. 725-33.
675. Palakurthi, S.S., et al., *Anticancer effects of thiazolidinediones are independent of peroxisome proliferator-activated receptor gamma and mediated by inhibition of translation initiation*. Cancer Res, 2001. **61**(16): p. 6213-8.
676. Bae, M.A. and B.J. Song, *Critical role of c-Jun N-terminal protein kinase activation in troglitazone-induced apoptosis of human HepG2 hepatoma cells*. Mol Pharmacol, 2003. **63**(2): p. 401-8.
677. Zander, T., et al., *Induction of apoptosis in human and rat glioma by agonists of the nuclear receptor PPARgamma*. J Neurochem, 2002. **81**(5): p. 1052-60.
678. Shiau, C.W., et al., *Thiazolidinediones mediate apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 functions independently of PPARgamma*. Cancer Res, 2005. **65**(4): p. 1561-9.
679. Yan, K.H., et al., *The synergistic anticancer effect of troglitazone combined with aspirin causes cell cycle arrest and apoptosis in human lung cancer cells*. Mol Carcinog, 2010. **49**(3): p. 235-46.
680. Mantovani, A., et al., *Cancer-related inflammation*. Nature, 2008. **454**(7203): p. 436-44.
681. Glass, C.K. and K. Saijo, *Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells*. Nat Rev Immunol, 2010. **10**(5): p. 365-76.
682. Demetri, G.D., et al., *Induction of solid tumor differentiation by the peroxisome proliferator-activated receptor-gamma ligand troglitazone in patients with liposarcoma*. Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3951-6.
683. Debrock, G., et al., *A phase II trial with rosiglitazone in liposarcoma patients*. Br J Cancer, 2003. **89**(8): p. 1409-12.

684. Smith, M.R., et al., *Rosiglitazone versus placebo for men with prostate carcinoma and a rising serum prostate-specific antigen level after radical prostatectomy and/or radiation therapy*. *Cancer*, 2004. **101**(7): p. 1569-74.
685. Hisatake, J.I., et al., *Down-Regulation of prostate-specific antigen expression by ligands for peroxisome proliferator-activated receptor gamma in human prostate cancer*. *Cancer Res*, 2000. **60**(19): p. 5494-8.
686. Mueller, E., et al., *Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer*. *Proc Natl Acad Sci U S A*, 2000. **97**(20): p. 10990-5.
687. Schwartz, G.K., et al., *Phase I and pharmacokinetic study of LY293111, an orally bioavailable LTB4 receptor antagonist, in patients with advanced solid tumors*. *J Clin Oncol*, 2005. **23**(23): p. 5365-73.
688. Burstein, H.J., et al., *Use of the peroxisome proliferator-activated receptor (PPAR) gamma ligand troglitazone as treatment for refractory breast cancer: a phase II study*. *Breast Cancer Res Treat*, 2003. **79**(3): p. 391-7.
689. Kulke, M.H., et al., *A phase II study of troglitazone, an activator of the PPARgamma receptor, in patients with chemotherapy-resistant metastatic colorectal cancer*. *Cancer J*, 2002. **8**(5): p. 395-9.
690. Hau, P., et al., *Low-dose chemotherapy in combination with COX-2 inhibitors and PPAR-gamma agonists in recurrent high-grade gliomas - a phase II study*. *Oncology*, 2007. **73**(1-2): p. 21-5.
691. Kebebew, E., et al., *A phase II trial of rosiglitazone in patients with thyroglobulin-positive and radioiodine-negative differentiated thyroid cancer*. *Surgery*, 2006. **140**(6): p. 960-6; discussion 966-7.
692. Tepmongkol, S., et al., *Rosiglitazone effect on radioiodine uptake in thyroid carcinoma patients with high thyroglobulin but negative total body scan: a correlation with the expression of peroxisome proliferator-activated receptor-gamma*. *Thyroid*, 2008. **18**(7): p. 697-704.
693. Lefebvre, A.M., et al., *Activation of the peroxisome proliferator-activated receptor gamma promotes the development of colon tumors in C57BL/6J-APCMin/+ mice*. *Nat Med*, 1998. **4**(9): p. 1053-7.
694. Saez, E., et al., *Activators of the nuclear receptor PPARgamma enhance colon polyp formation*. *Nat Med*, 1998. **4**(9): p. 1058-61.
695. Lubet, R.A., et al., *Rosiglitazone, a PPAR gamma agonist: potent promoter of hydroxybutyl(butyl)nitrosamine-induced urinary bladder cancers*. *Int J Cancer*, 2008. **123**(10): p. 2254-9.

696. Piccinni, C., et al., *Assessing the association of pioglitazone use and bladder cancer through drug adverse event reporting*. *Diabetes Care*, 2011. **34**(6): p. 1369-71.
697. Fuchs, E. and S. Raghavan, *Getting under the skin of epidermal morphogenesis*. *Nat Rev Genet*, 2002. **3**(3): p. 199-209.
698. Matsuura, H., et al., *Correlation between expression of peroxisome proliferator-activated receptor beta and squamous differentiation in epidermal and tracheobronchial epithelial cells*. *Mol Cell Endocrinol*, 1999. **147**(1-2): p. 85-92.
699. Westergaard, M., et al., *Modulation of keratinocyte gene expression and differentiation by PPAR-selective ligands and tetradecylthioacetic acid*. *J Invest Dermatol*, 2001. **116**(5): p. 702-12.
700. Rosenfield, R.L., et al., *Rat preputial sebocyte differentiation involves peroxisome proliferator-activated receptors*. *J Invest Dermatol*, 1999. **112**(2): p. 226-32.
701. Kim, D.J., et al., *PPARbeta/delta selectively induces differentiation and inhibits cell proliferation*. *Cell Death Differ*, 2006. **13**(1): p. 53-60.
702. Wahli, W., *Peroxisome proliferator-activated receptors (PPARs): from metabolic control to epidermal wound healing*. *Swiss Med Wkly*, 2002. **132**(7-8): p. 83-91.
703. Hanley, K., et al., *Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPARalpha*. *J Invest Dermatol*, 1998. **110**(4): p. 368-75.
704. Kang, H.Y., et al., *Expression and function of peroxisome proliferator-activated receptors in human melanocytes*. *Br J Dermatol*, 2004. **150**(3): p. 462-8.
705. Schmuth, M., et al., *Peroxisome proliferator-activated receptor (PPAR)-beta/delta stimulates differentiation and lipid accumulation in keratinocytes*. *J Invest Dermatol*, 2004. **122**(4): p. 971-83.
706. Komuves, L.G., et al., *Stimulation of PPARalpha promotes epidermal keratinocyte differentiation in vivo*. *J Invest Dermatol*, 2000. **115**(3): p. 353-60.
707. Hanley, K., et al., *Farnesol stimulates differentiation in epidermal keratinocytes via PPARalpha*. *J Biol Chem*, 2000. **275**(15): p. 11484-91.
708. Mao-Qiang, M., et al., *Peroxisome-proliferator-activated receptor (PPAR)-gamma activation stimulates keratinocyte differentiation*. *J Invest Dermatol*, 2004. **123**(2): p. 305-12.
709. Rivier, M., et al., *Differential expression of peroxisome proliferator-activated receptor subtypes during the differentiation of human keratinocytes*. *J Invest Dermatol*, 1998. **111**(6): p. 1116-21.
710. Rivier, M., et al., *Peroxisome proliferator-activated receptor-alpha enhances lipid metabolism in a skin equivalent model*. *J Invest Dermatol*, 2000. **114**(4): p. 681-7.

711. Hanley, K., et al., *Activators of the nuclear hormone receptors PPARalpha and FXR accelerate the development of the fetal epidermal permeability barrier*. J Clin Invest, 1997. **100**(3): p. 705-12.
712. Lee, S.S., et al., *Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators*. Mol Cell Biol, 1995. **15**(6): p. 3012-22.
713. Schmuth, M., et al., *Role of peroxisome proliferator-activated receptor alpha in epidermal development in utero*. J Invest Dermatol, 2002. **119**(6): p. 1298-303.
714. Yang, Q., et al., *Alterations in skin and stratified epithelia by constitutively activated PPARalpha*. J Invest Dermatol, 2006. **126**(2): p. 374-85.
715. Michalik, L., et al., *Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR)alpha and PPARbeta mutant mice*. J Cell Biol, 2001. **154**(4): p. 799-814.
716. Rosen, E.D., et al., *PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro*. Mol Cell, 1999. **4**(4): p. 611-7.
717. Tan, N.S., et al., *Critical roles of PPAR beta/delta in keratinocyte response to inflammation*. Genes Dev, 2001. **15**(24): p. 3263-77.
718. Di-Poi, N., et al., *Antiapoptotic role of PPARbeta in keratinocytes via transcriptional control of the Akt1 signaling pathway*. Mol Cell, 2002. **10**(4): p. 721-33.
719. Di-Poi, N., et al., *The anti-apoptotic role of PPARbeta contributes to efficient skin wound healing*. J Steroid Biochem Mol Biol, 2003. **85**(2-5): p. 257-65.
720. Billoni, N., et al., *Expression of peroxisome proliferator activated receptors (PPARs) in human hair follicles and PPAR alpha involvement in hair growth*. Acta Derm Venereol, 2000. **80**(5): p. 329-34.
721. Di-Poi, N., et al., *Epithelium-mesenchyme interactions control the activity of peroxisome proliferator-activated receptor beta/delta during hair follicle development*. Mol Cell Biol, 2005. **25**(5): p. 1696-712.
722. Zheng, Y., et al., *Scd1 is expressed in sebaceous glands and is disrupted in the asebia mouse*. Nat Genet, 1999. **23**(3): p. 268-70.
723. Rosenfield, R.L., et al., *Mechanisms of androgen induction of sebocyte differentiation*. Dermatology, 1998. **196**(1): p. 43-6.
724. Chen, W., et al., *Expression of peroxisome proliferator-activated receptor and CCAAT/enhancer binding protein transcription factors in cultured human sebocytes*. J Invest Dermatol, 2003. **121**(3): p. 441-7.
725. Grimaldi, P.A., *The roles of PPARs in adipocyte differentiation*. Prog Lipid Res, 2001. **40**(4): p. 269-81.

726. Trivedi, N.R., et al., *Peroxisome proliferator-activated receptors increase human sebum production*. J Invest Dermatol, 2006. **126**(9): p. 2002-9.
727. Downie, M.M., et al., *Peroxisome proliferator-activated receptor and farnesoid X receptor ligands differentially regulate sebaceous differentiation in human sebaceous gland organ cultures in vitro*. Br J Dermatol, 2004. **151**(4): p. 766-75.
728. Strauss, J.S., P.E. Pochi, and E.N. Whitman, *Suppression of sebaceous gland activity with eicosa-5:8:11:14-tetraenoic acid*. J Invest Dermatol, 1967. **48**(5): p. 492-3.
729. Westergaard, M., et al., *Expression and localization of peroxisome proliferator-activated receptors and nuclear factor kappaB in normal and lesional psoriatic skin*. J Invest Dermatol, 2003. **121**(5): p. 1104-17.
730. Romanowska, M., et al., *Activation of PPARbeta/delta causes a psoriasis-like skin disease in vivo*. PLoS One, 2010. **5**(3): p. e9701.
731. Mossner, R., et al., *Variations in the genes encoding the peroxisome proliferator-activated receptors alpha and gamma in psoriasis*. Arch Dermatol Res, 2004. **296**(1): p. 1-5.
732. Sheu, M.Y., et al., *Topical peroxisome proliferator activated receptor-alpha activators reduce inflammation in irritant and allergic contact dermatitis models*. J Invest Dermatol, 2002. **118**(1): p. 94-101.
733. Ellis, C.N., et al., *Troglitazone improves psoriasis and normalizes models of proliferative skin disease: ligands for peroxisome proliferator-activated receptor-gamma inhibit keratinocyte proliferation*. Arch Dermatol, 2000. **136**(5): p. 609-16.
734. Demerjian, M., et al., *Topical treatment with thiazolidinediones, activators of peroxisome proliferator-activated receptor-gamma, normalizes epidermal homeostasis in a murine hyperproliferative disease model*. Exp Dermatol, 2006. **15**(3): p. 154-60.
735. Kuenzli, S. and J.H. Saurat, *Peroxisome proliferator-activated receptors in cutaneous biology*. Br J Dermatol, 2003. **149**(2): p. 229-36.
736. Kuenzli, S. and J.H. Saurat, *Effect of topical PPARbeta/delta and PPARgamma agonists on plaque psoriasis. A pilot study*. Dermatology, 2003. **206**(3): p. 252-6.
737. Varani, J., et al., *Thiazolidinediones: potential as therapeutics for psoriasis and perhaps other hyperproliferative skin disease*. Expert Opin Investig Drugs, 2006. **15**(11): p. 1453-68.
738. Clark, R.B., et al., *The nuclear receptor PPAR gamma and immunoregulation: PPAR gamma mediates inhibition of helper T cell responses*. J Immunol, 2000. **164**(3): p. 1364-71.
739. Ruhl, R., et al., *Inhibition of IgE-production by peroxisome proliferator-activated receptor ligands*. J Invest Dermatol, 2003. **121**(4): p. 757-64.
740. Dahten, A., S. Mergemeier, and M. Worm, *PPARGamma expression profile and its cytokine driven regulation in atopic dermatitis*. Allergy, 2007. **62**(8): p. 926-33.

741. Dahten, A., et al., *Systemic PPARgamma ligation inhibits allergic immune response in the skin*. J Invest Dermatol, 2008. **128**(9): p. 2211-8.
742. Behshad, R., K.D. Cooper, and N.J. Korman, *A retrospective case series review of the peroxisome proliferator-activated receptor ligand rosiglitazone in the treatment of atopic dermatitis*. Arch Dermatol, 2008. **144**(1): p. 84-8.
743. De Belilovsky, C., et al., *Natural peroxisome proliferator-activated receptor-alpha agonist cream demonstrates similar therapeutic response to topical steroids in atopic dermatitis*. J Dermatolog Treat, 2011. **22**(6): p. 359-65.
744. Tan, N.S., et al., *Essential role of Smad3 in the inhibition of inflammation-induced PPARbeta/delta expression*. EMBO J, 2004. **23**(21): p. 4211-21.
745. Ashcroft, G.S., et al., *Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response*. Nat Cell Biol, 1999. **1**(5): p. 260-6.
746. Tan, N.S., et al., *Genetic- or transforming growth factor-beta 1-induced changes in epidermal peroxisome proliferator-activated receptor beta/delta expression dictate wound repair kinetics*. J Biol Chem, 2005. **280**(18): p. 18163-70.
747. Kippenberger, S., et al., *Activators of peroxisome proliferator-activated receptors protect human skin from ultraviolet-B-light-induced inflammation*. J Invest Dermatol, 2001. **117**(6): p. 1430-6.
748. Lo Verme, J., et al., *The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide*. Mol Pharmacol, 2005. **67**(1): p. 15-9.
749. Dubrac, S., et al., *Peroxisome proliferator-activated receptor-alpha activation inhibits Langerhans cell function*. J Immunol, 2007. **178**(7): p. 4362-72.
750. Blanquart, C., et al., *Peroxisome proliferator-activated receptors: regulation of transcriptional activities and roles in inflammation*. J Steroid Biochem Mol Biol, 2003. **85**(2-5): p. 267-73.
751. Jiang, C., A.T. Ting, and B. Seed, *PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines*. Nature, 1998. **391**(6662): p. 82-6.
752. Jozkowicz, A., et al., *Ligands of peroxisome proliferator-activated receptor-gamma increase the generation of vascular endothelial growth factor in vascular smooth muscle cells and in macrophages*. Acta Biochim Pol, 2000. **47**(4): p. 1147-57.
753. Chawla, A., et al., *PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation*. Nat Med, 2001. **7**(1): p. 48-52.

754. Sertznig, P., et al., *Present concepts and future outlook: function of peroxisome proliferator-activated receptors (PPARs) for pathogenesis, progression, and therapy of cancer*. J Cell Physiol, 2007. **212**(1): p. 1-12.
755. Kim, D.J., et al., *Peroxisome proliferator-activated receptor beta (delta)-dependent regulation of ubiquitin C expression contributes to attenuation of skin carcinogenesis*. J Biol Chem, 2004. **279**(22): p. 23719-27.
756. Kim, D.J., et al., *Peroxisome proliferator-activated receptor-beta/delta inhibits epidermal cell proliferation by down-regulation of kinase activity*. J Biol Chem, 2005. **280**(10): p. 9519-27.
757. Zhang, Q., et al., *Epidermal peroxisome proliferator-activated receptor gamma as a target for ultraviolet B radiation*. J Biol Chem, 2005. **280**(1): p. 73-9.
758. Nicol, C.J., et al., *PPARgamma influences susceptibility to DMBA-induced mammary, ovarian and skin carcinogenesis*. Carcinogenesis, 2004. **25**(9): p. 1747-55.
759. He, G., et al., *The effect of PPARgamma ligands on UV- or chemically-induced carcinogenesis in mouse skin*. Mol Carcinog, 2005. **43**(4): p. 198-206.
760. Thuillier, P., et al., *Activators of peroxisome proliferator-activated receptor-alpha partially inhibit mouse skin tumor promotion*. Mol Carcinog, 2000. **29**(3): p. 134-42.
761. Muller-Decker, K., et al., *Prostaglandin-H-synthase isozyme expression in normal and neoplastic human skin*. Int J Cancer, 1999. **82**(5): p. 648-56.
762. Nijsten, T., et al., *Cyclooxygenase-2 expression and angiogenesis in squamous cell carcinoma of the skin and its precursors: a paired immunohistochemical study of 35 cases*. Br J Dermatol, 2004. **151**(4): p. 837-45.
763. Nijsten, T., et al., *Peroxisome proliferator-activated receptors in squamous cell carcinoma and its precursors*. J Cutan Pathol, 2005. **32**(5): p. 340-7.
764. Michalik, L., B. Desvergne, and W. Wahli, *Peroxisome-proliferator-activated receptors and cancers: complex stories*. Nat Rev Cancer, 2004. **4**(1): p. 61-70.
765. Berwick, M. and C. Wiggins, *The current epidemiology of cutaneous malignant melanoma*. Front Biosci, 2006. **11**: p. 1244-54.
766. Eigentler, T.K., et al., *Palliative therapy of disseminated malignant melanoma: a systematic review of 41 randomised clinical trials*. Lancet Oncol, 2003. **4**(12): p. 748-59.
767. Botton, T., et al., *In vitro and in vivo anti-melanoma effects of ciglitazone*. J Invest Dermatol, 2009. **129**(5): p. 1208-18.
768. Mossner, R., et al., *Agonists of peroxisome proliferator-activated receptor gamma inhibit cell growth in malignant melanoma*. J Invest Dermatol, 2002. **119**(3): p. 576-82.

769. Sertznig, P., et al., *Cross-talk between vitamin D receptor (VDR)- and peroxisome proliferator-activated receptor (PPAR)-signaling in melanoma cells*. *Anticancer Res*, 2009. **29**(9): p. 3647-58.
770. Grabacka, M., et al., *Peroxisome proliferator-activated receptor alpha activation decreases metastatic potential of melanoma cells in vitro via down-regulation of Akt*. *Clin Cancer Res*, 2006. **12**(10): p. 3028-36.
771. Grabacka, M., et al., *Inhibition of melanoma metastases by fenofibrate*. *Arch Dermatol Res*, 2004. **296**(2): p. 54-8.
772. Rubins, H.B., et al., *Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group*. *N Engl J Med*, 1999. **341**(6): p. 410-8.
773. Freeman, S.R., et al., *Statins, fibrates, and melanoma risk: a systematic review and meta-analysis*. *J Natl Cancer Inst*, 2006. **98**(21): p. 1538-46.
774. Liu, Y., et al., *Growth inhibition and differentiation induced by peroxisome proliferator activated receptor gamma ligand rosiglitazone in human melanoma cell line A375*. *Med Oncol*, 2006. **23**(3): p. 393-402.
775. Denkins, Y., et al., *Role of omega-3 polyunsaturated fatty acids on cyclooxygenase-2 metabolism in brain-metastatic melanoma*. *J Lipid Res*, 2005. **46**(6): p. 1278-84.
776. Cario-Andre, M., et al., *Polyunsaturated fatty acids partially reproduce the role of melanocytes in the epidermal melanin unit*. *Exp Dermatol*, 2005. **14**(3): p. 194-201.
777. Badawi, A.F., et al., *Inhibition of rat mammary gland carcinogenesis by simultaneous targeting of cyclooxygenase-2 and peroxisome proliferator-activated receptor gamma*. *Cancer Res*, 2004. **64**(3): p. 1181-9.
778. Reichle, A., et al., *Targeted combined anti-inflammatory and angiostatic therapy in advanced melanoma: a randomized phase II trial*. *Melanoma Res*, 2007. **17**(6): p. 360-4.
779. Wang, Y., et al., *Synthesis and preliminary antitumor activity evaluation of a DHA and doxorubicin conjugate*. *Bioorg Med Chem Lett*, 2006. **16**(11): p. 2974-7.
780. Fracasso, P.M., et al., *Phase 1 and pharmacokinetic study of weekly docosahexaenoic acid-paclitaxel, Taxoprexin, in resistant solid tumor malignancies*. *Cancer Chemother Pharmacol*, 2009. **63**(3): p. 451-8.
781. Bradley, M.O., et al., *Tumor targeting by covalent conjugation of a natural fatty acid to paclitaxel*. *Clin Cancer Res*, 2001. **7**(10): p. 3229-38.
782. Zouboulis, C.C., *Is acne vulgaris a genuine inflammatory disease?* *Dermatology*, 2001. **203**(4): p. 277-9.
783. Zouboulis, C.C., *Acne and sebaceous gland function*. *Clin Dermatol*, 2004. **22**(5): p. 360-6.

784. Zouboulis, C.C. and K. Degitz, *Androgen action on human skin -- from basic research to clinical significance*. *Exp Dermatol*, 2004. **13 Suppl 4**: p. 5-10.
785. Kim, M.J., et al., *Limited cooperation between peroxisome proliferator-activated receptors and retinoid X receptor agonists in sebocyte growth and development*. *Mol Genet Metab*, 2001. **74**(3): p. 362-9.
786. Rosenfield, R.L., D. Deplewski, and M.E. Greene, *Peroxisome proliferator-activated receptors and skin development*. *Horm Res*, 2000. **54**(5-6): p. 269-74.
787. Delerive, P., J.C. Fruchart, and B. Staels, *Peroxisome proliferator-activated receptors in inflammation control*. *J Endocrinol*, 2001. **169**(3): p. 453-9.
788. Alestas, T., et al., *Enzymes involved in the biosynthesis of leukotriene B4 and prostaglandin E2 are active in sebaceous glands*. *J Mol Med (Berl)*, 2006. **84**(1): p. 75-87.
789. Zouboulis, C.C., et al., *A new concept for acne therapy: a pilot study with zileuton, an oral 5-lipoxygenase inhibitor*. *Arch Dermatol*, 2003. **139**(5): p. 668-70.
790. Devchand, P.R., et al., *The PPARalpha-leukotriene B4 pathway to inflammation control*. *Nature*, 1996. **384**(6604): p. 39-43.
791. Zouboulis, C.C., *Zileuton, a new efficient and safe systemic anti-acne drug*. *Dermatoendocrinol*, 2009. **1**(3): p. 188-92.
792. Jimenez, S.A. and C.T. Derk, *Following the molecular pathways toward an understanding of the pathogenesis of systemic sclerosis*. *Ann Intern Med*, 2004. **140**(1): p. 37-50.
793. Varga, J. and D. Abraham, *Systemic sclerosis: a prototypic multisystem fibrotic disorder*. *J Clin Invest*, 2007. **117**(3): p. 557-67.
794. Selman, M. and A. Pardo, *Idiopathic pulmonary fibrosis: an epithelial/fibroblastic cross-talk disorder*. *Respir Res*, 2002. **3**: p. 3.
795. Thannickal, V.J., et al., *Mechanisms of pulmonary fibrosis*. *Annu Rev Med*, 2004. **55**: p. 395-417.
796. Burgess, H.A., et al., *PPARgamma agonists inhibit TGF-beta induced pulmonary myofibroblast differentiation and collagen production: implications for therapy of lung fibrosis*. *Am J Physiol Lung Cell Mol Physiol*, 2005. **288**(6): p. L1146-53.
797. Duan, S.Z., M.G. Usher, and R.M. Mortensen, *Peroxisome proliferator-activated receptor-gamma-mediated effects in the vasculature*. *Circ Res*, 2008. **102**(3): p. 283-94.
798. Abraham, D.J., et al., *New developments in fibroblast and myofibroblast biology: implications for fibrosis and scleroderma*. *Curr Rheumatol Rep*, 2007. **9**(2): p. 136-43.
799. Kapoor, M., et al., *Loss of peroxisome proliferator-activated receptor gamma in mouse fibroblasts results in increased susceptibility to bleomycin-induced skin fibrosis*. *Arthritis Rheum*, 2009. **60**(9): p. 2822-9.

800. Wu, M., et al., *Rosiglitazone abrogates bleomycin-induced scleroderma and blocks profibrotic responses through peroxisome proliferator-activated receptor-gamma*. Am J Pathol, 2009. **174**(2): p. 519-33.
801. Shi-wen, X., et al., *Rosiglitazone alleviates the persistent fibrotic phenotype of lesional skin scleroderma fibroblasts*. Rheumatology (Oxford), 2010. **49**(2): p. 259-63.
802. Shah, R.R., *Can pharmacogenetics help rescue drugs withdrawn from the market?* Pharmacogenomics, 2006. **7**(6): p. 889-908.
803. Maggi, A., et al., *Techniques: reporter mice - a new way to look at drug action*. Trends Pharmacol Sci, 2004. **25**(6): p. 337-42.
804. Shih, C.M., et al., *Usefulness of chest single photon emission computed tomography with technetium-99m methoxyisobutylisonitrile to predict taxol based chemotherapy response in advanced non-small cell lung cancer*. Cancer Lett, 2003. **199**(1): p. 99-105.
805. Rohren, E.M., T.G. Turkington, and R.E. Coleman, *Clinical applications of PET in oncology*. Radiology, 2004. **231**(2): p. 305-32.
806. Padhani, A.R. and L. Ollivier, *The RECIST (Response Evaluation Criteria in Solid Tumors) criteria: implications for diagnostic radiologists*. Br J Radiol, 2001. **74**(887): p. 983-6.
807. Hunter, D.J. and P.G. Conaghan, *Imaging outcomes and their role in determining outcomes in osteoarthritis and rheumatoid arthritis*. Curr Opin Rheumatol, 2006. **18**(2): p. 157-62.
808. Lespessailles, E., et al., *Imaging techniques for evaluating bone microarchitecture*. Joint Bone Spine, 2006. **73**(3): p. 254-61.
809. Maggi, A. and P. Ciana, *Reporter mice and drug discovery and development*. Nat Rev Drug Discov, 2005. **4**(3): p. 249-55.
810. Yu, Y., et al., *Quantification of target gene expression by imaging reporter gene expression in living animals*. Nat Med, 2000. **6**(8): p. 933-7.
811. Ray, P., et al., *Monitoring gene therapy with reporter gene imaging*. Semin Nucl Med, 2001. **31**(4): p. 312-20.
812. de Wet, J.R., et al., *Firefly luciferase gene: structure and expression in mammalian cells*. Mol Cell Biol, 1987. **7**(2): p. 725-37.
813. Spibey, C.A., P. Jackson, and K. Herick, *A unique charge-coupled device/xenon arc lamp based imaging system for the accurate detection and quantitation of multicolour fluorescence*. Electrophoresis, 2001. **22**(5): p. 829-36.
814. Zhang, W., et al., *Rapid in vivo functional analysis of transgenes in mice using whole body imaging of luciferase expression*. Transgenic Res, 2001. **10**(5): p. 423-34.
815. Ciana, P., et al., *In vivo imaging of transcriptionally active estrogen receptors*. Nat Med, 2003. **9**(1): p. 82-6.

816. Lipshutz, G.S., et al., *In utero delivery of adeno-associated viral vectors: intraperitoneal gene transfer produces long-term expression*. *Mol Ther*, 2001. **3**(3): p. 284-92.
817. Matthews, J.C., K. Hori, and M.J. Cormier, *Purification and properties of Renilla reniformis luciferase*. *Biochemistry*, 1977. **16**(1): p. 85-91.
818. Loening, A.M., et al., *Consensus guided mutagenesis of Renilla luciferase yields enhanced stability and light output*. *Protein Eng Des Sel*, 2006. **19**(9): p. 391-400.
819. Venisnik, K.M., et al., *Bifunctional antibody-Renilla luciferase fusion protein for in vivo optical detection of tumors*. *Protein Eng Des Sel*, 2006. **19**(10): p. 453-60.
820. Bhaumik, S. and S.S. Gambhir, *Optical imaging of Renilla luciferase reporter gene expression in living mice*. *Proc Natl Acad Sci U S A*, 2002. **99**(1): p. 377-82.
821. Tannous, B.A., et al., *Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo*. *Mol Ther*, 2005. **11**(3): p. 435-43.
822. Yang, M., et al., *Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases*. *Proc Natl Acad Sci U S A*, 2000. **97**(3): p. 1206-11.
823. Hoffman, R.M., *The multiple uses of fluorescent proteins to visualize cancer in vivo*. *Nat Rev Cancer*, 2005. **5**(10): p. 796-806.
824. Baird, G.S., D.A. Zacharias, and R.Y. Tsien, *Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral*. *Proc Natl Acad Sci U S A*, 2000. **97**(22): p. 11984-9.
825. Bevis, B.J. and B.S. Glick, *Rapidly maturing variants of the Discosoma red fluorescent protein (DsRed)*. *Nat Biotechnol*, 2002. **20**(1): p. 83-7.
826. Shaner, N.C., et al., *Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein*. *Nat Biotechnol*, 2004. **22**(12): p. 1567-72.
827. Bullok, K. and D. Piwnica-Worms, *Synthesis and characterization of a small, membrane-permeant, caspase-activatable far-red fluorescent peptide for imaging apoptosis*. *J Med Chem*, 2005. **48**(17): p. 5404-7.
828. Hsu, A.R., et al., *In vivo near-infrared fluorescence imaging of integrin alphavbeta3 in an orthotopic glioblastoma model*. *Mol Imaging Biol*, 2006. **8**(6): p. 315-23.
829. Ehrin, E., et al., *Preparation of 11C-labelled Raclopride, a new potent dopamine receptor antagonist: preliminary PET studies of cerebral dopamine receptors in the monkey*. *Int J Appl Radiat Isot*, 1985. **36**(4): p. 269-73.
830. MacLaren, D.C., et al., *Repetitive, non-invasive imaging of the dopamine D2 receptor as a reporter gene in living animals*. *Gene Ther*, 1999. **6**(5): p. 785-91.

831. Gambhir, S.S., et al., *A mutant herpes simplex virus type 1 thymidine kinase reporter gene shows improved sensitivity for imaging reporter gene expression with positron emission tomography*. Proc Natl Acad Sci U S A, 2000. **97**(6): p. 2785-90.
832. Tjuvajev, J.G., et al., *Imaging the expression of transfected genes in vivo*. Cancer Res, 1995. **55**(24): p. 6126-32.
833. Tjuvajev, J.G., et al., *Noninvasive imaging of herpes virus thymidine kinase gene transfer and expression: a potential method for monitoring clinical gene therapy*. Cancer Res, 1996. **56**(18): p. 4087-95.
834. Tjuvajev, J.G., et al., *Imaging herpes virus thymidine kinase gene transfer and expression by positron emission tomography*. Cancer Res, 1998. **58**(19): p. 4333-41.
835. Namavari, M., et al., *Synthesis of 8-[(18)F]fluoroguanine derivatives: in vivo probes for imaging gene expression with positron emission tomography*. Nucl Med Biol, 2000. **27**(2): p. 157-62.
836. Liang, Q., et al., *Noninvasive, quantitative imaging in living animals of a mutant dopamine D2 receptor reporter gene in which ligand binding is uncoupled from signal transduction*. Gene Ther, 2001. **8**(19): p. 1490-8.
837. Mankoff, D.A., A.F. Shields, and K.A. Krohn, *PET imaging of cellular proliferation*. Radiol Clin North Am, 2005. **43**(1): p. 153-67.
838. Issa, W., et al., *Synthesis and evaluation of a thymidine analog for positron emission tomography study of tumor DNA proliferation in vivo*. Nucl Med Biol, 2004. **31**(7): p. 839-49.
839. Glaser, M., et al., *Iodine-124 labelled annexin-V as a potential radiotracer to study apoptosis using positron emission tomography*. Appl Radiat Isot, 2003. **58**(1): p. 55-62.
840. Vergote, J., et al., *Could 99mTc-MIBI be used to visualize the apoptotic MCF7 human breast cancer cells?* Cell Mol Biol (Noisy-le-grand), 2001. **47**(3): p. 467-71.
841. Delbeke, D. and W.H. Martin, *Metabolic imaging with FDG: a primer*. Cancer J, 2004. **10**(4): p. 201-13.
842. Chatziioannou, A., et al., *Detector development for microPET II: a 1 microl resolution PET scanner for small animal imaging*. Phys Med Biol, 2001. **46**(11): p. 2899-910.
843. Nair, D.G., *About being BOLD*. Brain Res Brain Res Rev, 2005. **50**(2): p. 229-43.
844. Van der Linden, A., et al., *Current status of functional MRI on small animals: application to physiology, pathophysiology, and cognition*. NMR Biomed, 2007. **20**(5): p. 522-45.
845. Louie, A.Y., et al., *In vivo visualization of gene expression using magnetic resonance imaging*. Nat Biotechnol, 2000. **18**(3): p. 321-5.
846. Weissleder, R., et al., *In vivo magnetic resonance imaging of transgene expression*. Nat Med, 2000. **6**(3): p. 351-5.

847. Rice, B.W., M.D. Cable, and M.B. Nelson, *In vivo imaging of light-emitting probes*. J Biomed Opt, 2001. **6**(4): p. 432-40.
848. Schmidt-Ullrich, R., et al., *NF-kappaB activity in transgenic mice: developmental regulation and tissue specificity*. Development, 1996. **122**(7): p. 2117-28.
849. Carlsen, H., et al., *In vivo imaging of NF-kappa B activity*. J Immunol, 2002. **168**(3): p. 1441-6.
850. Huang, C., et al., *Blocking activator protein-1 activity, but not activating retinoic acid response element, is required for the antitumor promotion effect of retinoic acid*. Proc Natl Acad Sci U S A, 1997. **94**(11): p. 5826-30.
851. Bruning, J.C., et al., *Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles*. Cell, 1997. **88**(4): p. 561-72.
852. Su, L.K., et al., *Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene*. Science, 1992. **256**(5057): p. 668-70.
853. DiMasi, J.A., R.W. Hansen, and H.G. Grabowski, *The price of innovation: new estimates of drug development costs*. J Health Econ, 2003. **22**(2): p. 151-85.
854. Preziosi, P., *Science, pharmacoeconomics and ethics in drug R&D: a sustainable future scenario?* Nat Rev Drug Discov, 2004. **3**(6): p. 521-6.
855. Rawlins, M.D., *Cutting the cost of drug development?* Nat Rev Drug Discov, 2004. **3**(4): p. 360-4.
856. Freireich, E.J., et al., *Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man*. Cancer Chemother Rep, 1966. **50**(4): p. 219-44.
857. Xie, W. and R.M. Evans, *Pharmaceutical use of mouse models humanized for the xenobiotic receptor*. Drug Discov Today, 2002. **7**(9): p. 509-15.
858. Kenakin, T., *Predicting therapeutic value in the lead optimization phase of drug discovery*. Nat Rev Drug Discov, 2003. **2**(6): p. 429-38.
859. Houten, S.M., et al., *In vivo imaging of farnesoid X receptor activity reveals the ileum as the primary bile acid signaling tissue*. Mol Endocrinol, 2007. **21**(6): p. 1312-23.
860. Han, Z., et al., *Nuclear transfer in mouse oocytes and embryos*. Methods Enzymol, 2010. **476**: p. 171-84.
861. Ciana, P., et al., *A novel peroxisome proliferator-activated receptor responsive element-luciferase reporter mouse reveals gender specificity of peroxisome proliferator-activated receptor activity in liver*. Mol Endocrinol, 2007. **21**(2): p. 388-400.
862. Nakajima, T., et al., *Sex-dependent regulation of hepatic peroxisome proliferation in mice by trichloroethylene via peroxisome proliferator-activated receptor alpha (PPARalpha)*. Carcinogenesis, 2000. **21**(4): p. 677-82.

863. Djouadi, F., et al., *A gender-related defect in lipid metabolism and glucose homeostasis in peroxisome proliferator-activated receptor alpha-deficient mice*. J Clin Invest, 1998. **102**(6): p. 1083-91.
864. Costet, P., et al., *Peroxisome proliferator-activated receptor alpha-isoform deficiency leads to progressive dyslipidemia with sexually dimorphic obesity and steatosis*. J Biol Chem, 1998. **273**(45): p. 29577-85.
865. Lewitt, M.S. and K. Brismar, *Gender difference in the leptin response to feeding in peroxisome-proliferator-activated receptor-alpha knockout mice*. Int J Obes Relat Metab Disord, 2002. **26**(10): p. 1296-300.
866. Tai, E.S., et al., *Hepatic expression of PPARalpha, a molecular target of fibrates, is regulated during inflammation in a gender-specific manner*. FEBS Lett, 2003. **546**(2-3): p. 237-40.
867. Jalouli, M., et al., *Sex difference in hepatic peroxisome proliferator-activated receptor alpha expression: influence of pituitary and gonadal hormones*. Endocrinology, 2003. **144**(1): p. 101-9.
868. Kostadinova, R., W. Wahli, and L. Michalik, *PPARs in diseases: control mechanisms of inflammation*. Curr Med Chem, 2005. **12**(25): p. 2995-3009.

RESUME

HOMEOSTASIE DE L'INTESTIN ET DE LA PEAU : CIBLES ET MODELES POUR ETUDIER L'INFLAMMATION ET LA CARCINOGENESE.

L'homéostasie des muqueuses intestinale et cutanée dépend des interactions complexes entre le microbiote, l'épithélium et le système immunitaire de l'hôte. Des mécanismes régulateurs divers coopèrent afin de maintenir l'équilibre physiologique, et un défaut dans ces mécanismes entraînent des situations pathologiques. Le glucagon like peptide 2 (GLP-2) est un neuropeptide caractérisé par des propriétés prolifératives et anti-inflammatoires. Le potentiel thérapeutique des analogues de GLP-2 est actuellement évalué dans des essais cliniques pour des maladies digestives. Les effets du GLP-2 dans l'intestin sont médiés par son récepteur GLP-2 receptor (GLP-2R). Malgré la présence des nombreuses études évaluant l'expression cellulaire et la distribution tissulaire du GLP-2R, celles-ci restent controversées, que ce soit chez l'homme ou les rongeurs. Il est admis que l'expression du GLP-2R était confinée au tube digestif, principalement à l'intestin proximal, malgré des études évoquant une expression extra-intestinale. Une meilleure compréhension de l'expression et de la distribution de GLP-2R est nécessaire pour élucider les fonctions biologiques de GLP-2. Nous avons réalisé une cartographie de l'expression de GLP-2R dans différents organes murins ainsi que dans des lignées immortalisées humaines et murines. Nous avons également évalué l'expression intestinale du GLP-2R dans 2 modèles de colites induites chez la souris et dans des biopsies intestinales des patients atteints de maladies inflammatoires chroniques de l'intestin (MICI). Nous avons démontré que GLP-2R était exprimé en dehors du tube digestif notamment dans la vessie, le système nerveux central, le mésentère, les ganglions mésentériques, la rate et le foie. Nous avons également montré que la plus forte expression de GLP-2R dans le tube digestif était détectée dans le colon proximal et le rectum. Dans les modèles murins de colites et chez les patients atteints de MICI, l'expression du GLP-2R était diminuée, notamment dans les zones inflammatoires. Ceci pose la question le rôle physiopathologique des analogues de GLP-2 dans les maladies inflammatoires digestives. En conclusion, les hypothèses précédentes considérant une expression du GLP-2R majoritaire dans le tube proximal doivent être

reconsidérées. Les fonctions physiologiques extra-intestinales du GLP-2 doivent être explorés afin d'anticiper des effets indésirables extra-digestifs des analogues de GLP2.

Nous nous sommes intéressés au 5-aminosalicylic acid (5-ASA), autre molécule avec une relevance clinique pour le maintien de l'homéostasie intestinale. En effet, grâce à ses propriétés anti-inflammatoires, le 5-ASA est le traitement de choix dans les MICI. Les patients atteints de maladie de Crohn et de la rectocolite hémorragique ont un risque élevé de développer un cancer colorectal par rapport au reste de la population. Des études cliniques ont montré qu'un usage régulier du 5-ASA était associé à un risque faible de survenue de cancer colorectal. Néanmoins, les mécanismes moléculaires responsables des effets antinéoplasiques du 5-ASA sont mal connus. Dans cette étude, nous avons démontré que les effets anti-cancérigènes du 5-ASA étaient médiés par le peroxisome proliferator activated recepteur-gamma (PPAR γ). En utilisant des concentrations cliniquement pertinentes, nous avons confirmé in vitro, par comptage cellulaires, immunohistochimie (Ki-67) et TUNEL que le 5-ASA présentait des propriétés anti-prolifératives and proapoptotiques. L'emploi du GW9662, antagoniste spécifique de PPAR γ , abolissait les effets du 5-ASA. Dans un modèle in vivo de tumeur implanté chez la souris, une forte réduction du développement de la tumeur était détectée grâce à un effet topique du 5-ASA qui était inhibé par le GW9662. Finalement, dans un modèle de carcinogenèse du colon induite par l'azoxymethane (AOM), le 5-ASA était capable de réduire les lésions néoplasiques grâce à un mécanisme PPAR γ dépendant. Cette étude établie le rôle clé de PPAR γ médiant les effets anti-néoplasiques du 5-ASA. Ceci ouvre une perspective pour le développement de ligands de PPAR γ avec une meilleure efficacité ou pour leur usage en association avec d'autres traitements.

L'action de PPAR γ , et des PPARs en général, n'est pas limitée au tube digestif. Les PPARs ont été montré comme important dans différents systèmes physiologiques. Par exemple, dans la peau, les PPARs sont capables de réguler de nombreuses fonctions telles que la prolifération et la différenciation des cellules ainsi que les réponses inflammatoires. Les PPARs et leurs ligands sont donc considérés comme des cibles thérapeutiques particulièrement intéressantes dans certaines maladies de la peau comme les maladies inflammatoires et les mélanomes. Par conséquent, des modèles validés pour la

caractérisation et le développement de nouveaux ligands des PPARs sont nécessaires. Le développement de nouvelles molécules est un processus assez long qui nécessite des années de recherche préclinique pour démontrer l'absence de toxicité. Dans ce but, nous avons développé au sein du laboratoire un modèle de souris qui permet l'évaluation qualitative et quantitative de l'activité transcriptionnelle des PPARs. Ces souris (PPRE-Luc) ont été générées par intégration génomique d'une construction contenant le gène de la Firefly luciférase en aval d'éléments de réponse aux PPARs (PPRE). Dans ce travail, nous avons défini et validé les conditions expérimentales qui permettront d'utiliser les souris PPRE-Luc en tant qu'outil non-invasif pour l'évaluation in vivo de l'activité des PPARs dans la peau. Nous avons démontré par imagerie optique que l'application topique de 40mM de luciférine pendant 10 minutes était suffisante pour révéler l'activité optimale de la luciférase dans la peau des souris. Le traitement de la peau des souris avec des agonistes de PPAR γ et PPAR α , pioglitazone et WY14643, induisait une augmentation significative de l'émission des photons au cours du temps avec un pic 6 heures après le traitement. Nous avons également réalisé une étude de dose-réponse avec un large panel de concentrations de pioglitazone et WY14643, appliquées sur la peau des souris. La spécificité de la bioluminescence induite par la pioglitazone et le WY14643 a été évaluée par l'administration des antagonistes de PPAR γ et PPAR α , GW9662 et GW6471, respectivement. Cette approche a révélé que la spécificité des agonistes de PPARs diminuait quand la concentration de ceux-ci augmentait. Ces résultats ont été confirmés in vitro par la quantification de l'activité luciférase dans les extraits protéiques réalisés à partir de la peau de nos souris. En conclusion, cette étude montre que les souris PPRE-Luc représentent un modèle valide et spécifique pour étudier le profil pharmacologique des nouveaux agonistes de PPARs dans la peau.

SUMMARY

INTESTINAL AND SKIN HOMEOSTASIS: TARGETING INFLAMMATION AND CANCER

Intestinal and skin physiologies are quite similar as far as the homeostasis in both depends on complex interactions between the microbiota, the epithelium and the host immune system. Diverse regulatory mechanisms cooperate to maintain the equilibrium, and a breakdown in these pathways may precipitate pathological conditions. Glucagon like peptide 2 (GLP-2) represents one of the hot topics in research in intestinal physiology. Its dual function as an anti-inflammatory agent and growth factor has led to its consideration in therapeutic strategies and GLP-2 analogs are currently in clinical trials for several digestive diseases. The integrative responses to GLP-2 are mediated via the GLP-2 receptor (GLP-2R). Despite extensive research, precise tissue distribution of GLP-2R expression remains controversial both in rodents and humans. It is widely believed that GLP-2R expression is restricted to the gastrointestinal tract, mainly to the proximal bowel, despite the presence of few studies reporting extra-intestinal expression. Thus, to enhance our knowledge concerning the potential functions of GLP-2 analogs, a better understanding for GLP-2R expression is considered necessary. We therefore realized a panel of GLP-2R expression in mice tissues and in several human, murine and rat cells lines. Given the therapeutic beneficial effects of GLP-2 analogs in intestinal disorders, we investigated the intestinal expression of GLP-2R in mice models of chemically-induced colitis and in inflammatory bowel disease (IBD) patients. We demonstrated that GLP-2R is more widely expressed than expected with significant expression in several mice tissues including bladder, central nervous system, mesenteric adipose tissue, mesenteric lymph nodes, spleen, and liver. We also showed that the expression of GLP-2R in the gastrointestinal tract follows an increasing gradient toward the distal gut with highest expression in the colon and rectum. Interestingly, the intestinal expression of GLP-2R is significantly decreased in experimental mice models of colitis and in IBD patients which raised the point of the physiological role of GLP-2 analogs in digestive disease patients. Overall, previous hypotheses limiting GLP-2R expression and function to proximal bowel need to be revisited, and further studies should address the extra-intestinal biological function of GLP-2.

Along with GLP-2, another important element with significant clinical relevance to the intestinal homeostasis is the 5-aminosalicylic acid (5-ASA) and its potential in preventing colorectal cancer (CRC) in IBD patients. 5-ASA was opted since decades as the anti-inflammatory of choice of in IBD. In addition to its efficacy and safety, 5-ASA was also believed to have unique chemopreventive properties. Patients suffering from Crohn's disease and ulcerative colitis displayed an increased risk of developing CRC. Several studies have shown that regular usage of 5-ASA prevented the development of CRC in IBD patients. However, the mechanisms sustaining 5-ASA chemopreventive properties remain largely unknown. In this work, we demonstrated that the anti-neoplastic effects of 5-ASA are mediated via peroxisome proliferator activated receptor-gamma (PPAR γ). Using clinically-relevant concentrations, we first showed by *in vitro* cell count, Ki67 immunostaining, and TUNEL assay that 5-ASA displayed both anti-proliferating and pro-apoptotic properties. Subsequent to GW9662 (PPAR γ antagonist) application, evidence that PPAR γ mediated the chemopreventive effects of 5-ASA was established. In an *in vivo* model of xenograft tumor, we showed a strong reduction in tumor development due to topic effect of 5-ASA through a PPAR γ -dependent mechanism. Finally, in a model of azoxymethane (AOM)-induced colon carcinogenesis, 5-ASA significantly suppressed the number of both aberrant crypt foci and aberrant crypts via PPAR γ signaling pathway. This work establishing the key role of PPAR γ in mediating 5-ASA anti-neoplastic properties, may lead to the development of more effective PPAR γ ligands with higher efficiency or for use in association with agents of additive or synergic effects.

The role of PPAR γ , and more generally PPARs, is not restricted to the gastrointestinal tract. PPARs have been shown to mediate important regulatory functions in different physiological systems. In the skin, PPARs have been demonstrated to regulate important cellular functions, including cell proliferation and differentiation, as well as inflammatory responses. These functions identified PPARs and corresponding ligands as potential therapeutic targets for a broad variety of skin disorders, including inflammatory diseases and skin malignancies. Thus, major efforts should be made to generate valid models for the characterization and development of new PPAR ligands as therapeutics in skin diseases. However, the development of novel drugs is a long process requiring years of preclinical research to ensure that the molecule of interest can be administered to humans with a

minimal risk of toxic effects. To this aim, we have recently developed a reporter mouse for the ubiquitous and quantitative measurement of the transcriptional activity of PPARs. These mice (PPRE-Luc) were generated by genomic integration of a transgene flanked by insulators and carrying a reporter gene, the *Firefly luciferase* gene, under the control of PPAR responsive regulatory element. In this study, we have defined and validated experimental conditions to establish PPRE-Luc mice as a valuable tool for *in vivo* non invasive evaluation of PPARs activation in the skin. We demonstrated by optical imaging that topical application of 40mM of Luciferin for 10 minutes was enough to reveal the optimal luciferase activity in mice skin. The treatment of mice skin with the PPAR γ and PPAR α agonists, pioglitazone and WY14643, was associated with significant increase in photons emission reaching maximal signaling at 6 hours. We have performed dose response studies by testing a large range of pioglitazone and WY14643 concentrations on mouse skin. The specificity of bioluminescence signal induced pioglitazone and WY14643 was assessed using PPAR γ and PPAR α antagonists, GW9662 and GW6471, respectively. This approach revealed that the isoform specificity of PPARs agonists decreased when high ligand concentrations were applied on mouse skin. These results were further confirmed by *in vitro* measurement of luciferase activity in skin extracts. Taken together, our results demonstrated that PPRE-Luc mice represent a valuable reporter mouse model for the *in vivo* pharmacological profiling of drugs targeting PPARs in the skin.

