Role of XPC in the human cutaneous cancer cells invasion

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ROLE OF XPC IN THE HUMAN CUTANEOUS CANCER CELLS INVASION

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LIST OF ABBREVIATIONS

5-Mc	Methylated cytosine at position C5
6-4 PP	6, 4 pyrimidine-pyrimidone photoproducts
8-oxoG	8-Oxoguanine
AKs	Actinic keratosis
ATM	Ataxia-telangiectasia mutated
ATR	ATM- and Rad3-Related
BrdU	Bromodeoxyuridine
BD	Bowen’s disease
BCC	Basal cell carcinoma
BER	Base excision repair
BHD1	β-hairpin domain 1
BM	Basement membrane
CAMs	Cellular adhesion molecules
CAF	Carcinoma associated fibroblasts
CMM	Cutaneous metastatic melanoma
CP	Committed progenitors
CSF2	Colony stimulating factor 2
CPD	Cyclobutane pyrimidine dimers
CS	Cockayne syndrome
CAK	CDK-activating kinase
DDB	DNA damage binding complex

DDR	DNA damage response
DNA	Deoxy riboneucleic acid
DSBs	Double-stranded DNA breaks
DEJ	Dermal-epidermal junction
ECM	Extracellular matrix components
EGF	Epidermal growth factor
EPU	Epidermal proliferation units
ESCs	Embryonic stem cells
EYFP	Enhanced yellow fluorescent protein
FGF7	Fibroblast growth factors 7
GGR	Global Genome Repair
HD	Hemidesmosomes
HF	Hair follicle
HFSC	Hair follicle stem cells
HGF	Hepatocyte growth factor
HR	Homologous Recombination
IFE	Interfollicular epidermis
IGF	Insulin growth factor
IRS	Inner root sheath
IL-1	Interleukin 1
IPSCs	Induced pluripotent stem cells
ISCC	Invasive SCC
LC	Langerhans cells

LL	Lamina lucida
LRCs	Label retaining cells
MC	Merkel’s cells
MDM2	Mouse double minute 2 homolog protein
MEF	Mouse embryonic fibroblasts
MITF	Microphtalmia-associated transcription factor
MMPs	Matrix metalloproteinase
MMR	Mismatch repair mechanism
MED	Minimal erythema dose
MM	Malignant melanoma
MAPK	Mitogen-activated protein kinases
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
ORS	Outer root sheath
PCNA	Proliferating cell nuclear antigen
ROS	Reactive oxygen species
RPA	Replication protein A
RTKs	Receptor tyrosine kinases
SCs	Stem cells
SB	Suprabasal cells
SCC	Squamous-cell carcinoma
SCCIS	Squamous cell carcinoma in situ
TACs	Transit-amplifying cells

TGFβ	Transforming growth factor-β
TCR	Transcription Coupled Repair
TDG	Thymine DNA glycosylase
TFIIH	Transcription factor IIH
TGD	Transglutaminase domain
TTD	Trichothiodystrophy
UBA	Ubiquitin-Associated domains
UBL	Ubiquitin-like
VEGF	Vascular endothelial growth factor
XRCC1	X-ray repair cross-complementing protein1
XP	Xeroderma pigmentosum

RESUME

Le cancer spinocellulaire (CSC) est le cancer métastatique de la peau le plus fréquent chez l'homme. Son étiologie est liée à l'exposition aux stress génotoxiques, notamment les rayonnements ultraviolets (UV). Le xeroderma pigmentosum de type C (XP-C) est une maladie génétique rare caractérisée par l'absence de la protéine XP-C entrainant une déficience dans le mécanisme de la réparation par excision de nucléotides des lésions à l'ADN induites par les UV. Le maintien de ces lésions chez les patients XP-C entraine l'apparition précoce de CSC particulièrement agressifs. Les fibroblastes cutanés XP-C présentent un phénotype proche de celui de fibroblastes associés aux cellules cancéreuses avec une accumulation des espèces réactives de l'oxygène et une surexpression de la métalloprotéinase matricielle 1 (MMP1).

Nous avons approfondi l'étude du phénotype des fibroblastes XP-C et leurs effets sur l'invasion de cellules de carcinomes. Dans des cultures organotypiques de peau, les fibroblastes XP-C favorisent l'invasion des cellules de CSC. De plus, *in vitro*, la cicatrisation des cellules CSC est plus rapide en présence de surnageants de culture de fibroblastes XP-C et est due à un effet mitogénique des fibroblastes XP-C sur les cellules de CSC dont la proportion la phase G2-M du cycle cellulaire est alors augmentée de 100 %. Nos données montrent que les fibroblastes XP-C surexpriment le facteur de croissance HGF/SF (hepatocytes growth factor/ scatter factor). Le HGF sécrété par les fibroblastes XP-C active son récepteur c-Met et les voies de signalisation p38 et JNK dans les cellules de CSC. Le blocage de HGF entraîne l'inactivation de c-Met, p38 et bloque l'invasion des cellules CSC dans les équivalent de derme de culture de peau organotypiques. Nos résultats ont également montré que les fibroblastes XP-C jouent un rôle de cellules « leader » dans l'invasion des CSC. Mes résultats suggèrent que les fibroblastes XP-C créent un microenvironnement permissif à la prolifération et l'invasion des CSC via la surexpression de HGF. Des thérapies ciblant les fibroblastes XP-C pourraient améliorer le contrôle de l'invasion des CSC chez les patients XP-C.

ABSTRACT

Squamous cell carcinoma (SCC) is the most frequent metastatic skin cancer from epithelial origin. In the majority of cases, the etiology of skin SCC cancer is linked to exposure to genotoxic stress, notably ultraviolet radiation (UVR), in the vast of skin cancers. Xeroderma pigmentosum type C (XP-C) is a rare genetic disorder characterized by a severe susceptibility to aggressive SCCs following minimal exposure to UVR, hence compromising the life expectancy of patients. XP-C cells are deficient in the nucleotide excision repair mechanism (NER) of DNA lesions introduced at bipyrimidine sequences upon UVR exposure. Prior results from the laboratory have shown that XP-C dermal fibroblasts constitutively express a phenotype resembling that of stromal fibroblasts associated to cancer cells with accumulation of reactive oxygen species and over expression of matrix metalloproteinase 1 (MMP1).

In this study, we further explored the phenotype of XP-C primary fibroblasts and their effects on the invasion of SCC cells. Our results show that primary XP-C fibroblasts constitutively overexpress hepatocyte growth factor/scatter factor (HGF/SF). In organotypic skin cultures, XP-C fibroblasts promote the invasion of SCC cells. Also, scratch healing of SCC cells was enhanced in the presence of culture supernatants of XP-C fibroblasts through a mitogenic effect connected to increased ratio of SCC cells in the G2-M phase of the cell cycle. Blockage of c-Met activation by blocking HGF antibodies prevented invasiveness of SCC cells within dermal equivalent presumably through the inhibition of p38 or JNK activation by XP-C fibroblasts culture supernatants. Spheroid assay shows that XP-C fibroblasts led SCC invasions. Our data indicated for the first time that absence of XPC in fibroblasts leads to overexpression of cell growth stimulators such as HGF and other factors (MMP1) responsible for the formation of a microenvironment permissive towards SCC cells proliferation and invasion. Moreover, our findings show that XP-C fibroblasts are leaders cells for SCC cells invasion. Therapies targeting XP-C fibroblasts may be considered as a way to control development of aggressive cancer in XP-C patients.

INTRODUCTION

Chapter one: The skin

The skin is the largest organ of the body and represents 15 % of our body weight. The skin has several important functions. It plays the role of a barrier to protect our body from environmental insults like mechanical constrains, solar radiation, chemical injuries, and bacterial/viral infections (Thomas and Fernandez-Penas 2016). Another role of the skin is to maintain constant the body temperature by regulating fluids exchanges notably through the evaporation of sweat. All these functions are very critical for our wellbeing (Kanitakis 2002).

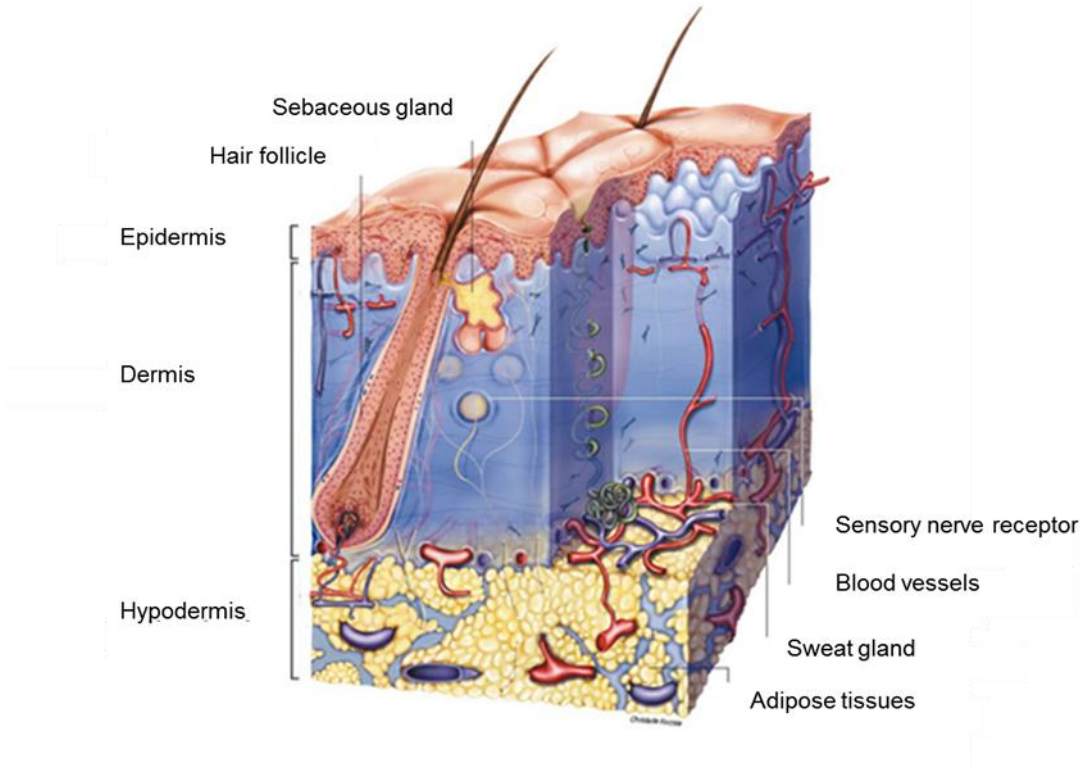

Figure 1: Skin structure. The skin is composed of two compartments, the epidermis in the upper layer and the dermis in the lower layer of the skin. The hypodermis is below the dermis and is composed of fatty acids. The skin contains also the pilosebaceous units and sweats glands.

I. Anatomy of the skin

The skin is composed of two compartments: the epidermis and the dermis (figure 1). The epidermis is a multilayered compartment composed of keratinocytes in direct contact with the external environment. The epidermis is renewing continuously within about three weeks through the proliferation/differentiation of the basal keratinocytes layer (Amberg, Holcmann et al. 2015). The skin contains also specialized structures, called “skin appendages” such as the pilosebaceous units and the sweat glands.

The dermis is located in the deeper layer of the skin, just beneath the epidermis to which it provides a physical and nutritive support. It is composed, of a loose connective tissue layers. Deeper in the skin there is the hypodermis which includes adipocytes and is an important source of fat (Luetteke, Phillips et al. 1994, Sibilia, Fleischmann et al. 2000).

I.1. The epidermis

The epidermis is mainly composed of four types of cells: keratinocytes, melanocytes, Langerhans and Merkel cells. The keratinocytes are of ectodermal origin (Sengel and Mauger 1976) and constitute the majority (about 80 %) of epidermal cells. They synthesize the intermediate filaments of keratins ensuring an important structural role (Simpson, Patel et al. 2011, Loschke, Homberg et al. 2016). Histology of human skin sections shows four main types of epidermal layers: the stratum basale (one cell layer), the stratum spinosum, the stratum granulosum and the stratum corneum (several cell layers) (Candi, Schmidt et al. 2005) (Figure 2). Each layer expresses specific markers. The expression and distribution of the biochemical markers specific of each layer of the skin attest of proper skin homeostasis.

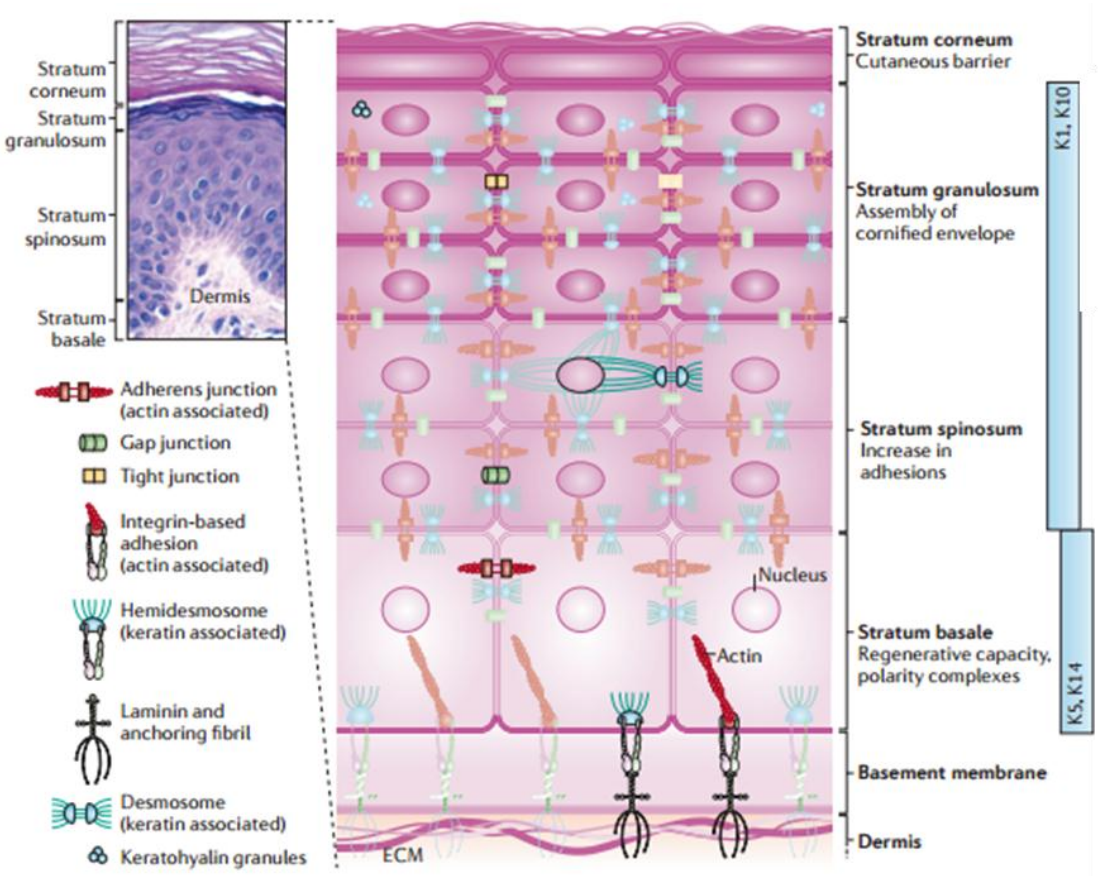

Figure 2: schematic architecture of the epidermis. The epidermis is composed of four cell layers. The basal proliferating cell layer in the stratum basale is in contact with the dermis through hemidesomsomes and integrin-based adhesions. During keratinocytes differentiation, basal keratinocytes detach from the DEJ (basement membrane) and migrate outwards to form successively the stratum spinosum, the stratum granulosum and the stratum corneum. In each layer a unique cytoarchitecture is elaborated supporting their specific functions (adapted from Simpson et al. 2011).

I.1.a. Stratum basale

The stratum basale, also said basal layer, is the deeper layer of the epidermis. Keratinocytes in this layer are brought together with the dermis through a specialized basement membrane which constitutes the central structure of the “dermal-epidermal junction” (DEJ). Some keratinocytes of the basal layer have a high proliferative capacity (Fuchs and Raghavan 2002, Mascre, Dekoninck et al. 2012). Cells in the basal layer of the epidermis remain in contact with the basement membrane through hemidesmosomes and integrin-based adhesions. Intracellular cytoskeleton of basal keratinocytes is made of an extensive network of K5 and K14 keratin filaments (Nelson and Sun 1983). Keratins are essential components of the keratinocytes cytoskeleton in the epidermis. They play a key role in the structure of the epidermis via their interaction with the desmosomes that link two adjacent keratinocytes and the hemidesmosomes that link the basal keratinocytes to the DEJ. When keratinocytes in the basal layer are triggered to differentiate, they lose their proliferative capacity, and detach from the basal layer to fuel the stratum spinosum (Fuchs and Green 1980, Moll, Franke et al. 1982).

I.1.b. Stratum spinosum

In the stratum spinosum, keratinocytes acquire a spindle morphology (Yardley and Goldstein 1976). Spinous cells no longer synthesize the K5/K14 keratins pair and instead synthesize K1/K10 keratins. This transition represents one of the earliest change illustrating triggering of keratinocytes towards terminal differentiation (Fuchs and Green 1980, Rao, Babu et al. 1996). As cells reach the stratum spinosum, they lose some of their lipids and water content. Assembly of intermediate filaments *in vitro* showed that K1 and K10 keratins self-aggregate more than the K5/K14 keratins, and that they are less soluble. These properties are thought to confer a protective role to suprabasal cells against external constraints and aggressions (Fuchs 1990).

I.1.c. Stratum granulosum

In the stratum granulosum, cells flatten and produce keratohyalin granules. The cells keep their anabolic properties and start their destructive phase to eliminate cytoplasmic organelles as well as nuclei (Hoober and Bernstein 1966, Lavker and

Matoltsy 1970). Concentration of intracellular calcium increases, promoting the formation of a dense protein and lipid envelope, called “horny envelop” (cornified envelop). This process is due to the covalent bonding of several proteins including loricrin, involucrin and pro-filaggrin by the specific calcium-dependent membrane bound transglutaminase (Kalinin, Marekov et al. 2001, Candi, Schmidt et al. 2005).

I.1.d. Stratum corneum

Formation of the stratum corneum is the last phase of the differentiation of keratinocytes in the epidermis. It is composed of dead cells called corneocytes. During this terminal step of epidermal differentiation, keratin organize and “line up” together through their interaction with filaggrin, leading to the condensation of keratin filaments into tight bundles (Palmer, Irvine et al. 2006). The profilaggrin present in the keratohyalin granules of the stratum granulosum is converted by proteolysis to mature filaggrin. Filaggrin makes a complex with keratins in the lower layers of the stratum corneum, resulting in morphological flattening of corneocytes. These “polygonal” flat cells lacking a nucleus are linked together by lipids and specific desmosomes called “corneodesmosomes” to form a brick-and-mortar like structure (Plewig 1970). When corneocytes migrate towards the outer skin surface, they change in structure, composition and function. In the deeper layers of the stratum corneum cells are thicker and have high density of keratins (Fuchs and Green 1980). The mechanical behavior of the stratum corneum is influenced by the deeper part of this layer. The outer cells of this layer become highly hydrophobic and their desmosomes undergo proteolytic degradation. Even if the corneocytes are considered as dead cells, they are still fully functional, particularly in the terms of “barrier properties and metabolic functions”.

I.1.e. Other cells of the epidermis

- **Melanocytes** are pigmentary cells derived from the neural crest upon embryonic development. They are found in the basal layer of epidermis (Joly-Tonetti, Wibawa et al. 2016). In human’s skin, there is about 1 melanocyte for 36 keratinocytes (Seiberg 2001). Melanocytes extend their dendrites to adjacent keratinocytes of both the basal layer and the suprabasal layers (Yamaguchi, Brenner et al. 2007, Brenner and Hearing 2008) (figure 3). Melanocytes produce pigment molecules called melanins that

accumulate in granules called melanosomes. Melanosomes are transferred to keratinocytes to provide skin pigmentation. There are two types of melanins: eumelanin, a brown-black polymer, and pheomelanin, a reddish-yellow hue (Rawles 1947). All human skins contain both types of melanin, but the ratio of eumelanin/pheomelanin determines the skin typology (or, more classically, phototype) (Wakamatsu, Kavanagh et al. 2006). The main role of melanins produced by the melanocytes is to protect skin cells against UV radiations and, hence, development of cutaneous cancers.

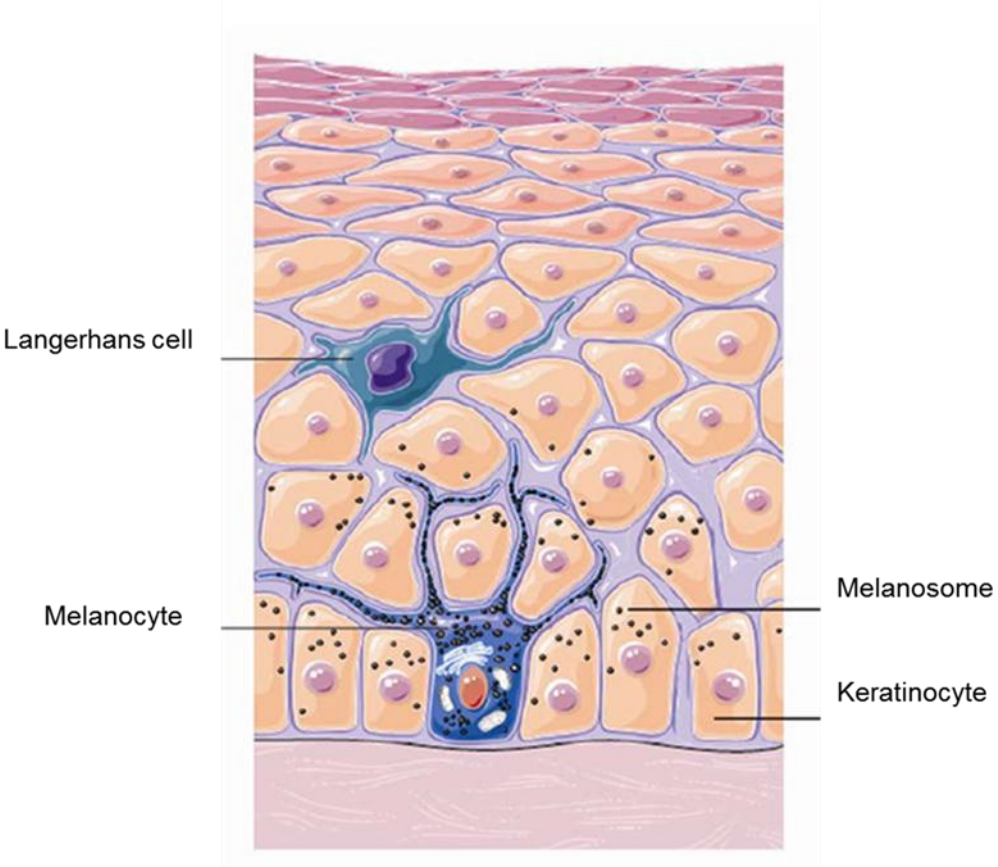

Figure 3: Melanization. Adapted from *http://oerpub.github.io/epubjs-demo-book/content/m46060.xhtml*. Melanin pigments produced by the melanocytes accumulate in the melanosome to be transferred at the end to the adjacent keratinocytes.

- **Langerhans cells (LC)** are dendritic cells found in the skin (Merad, Ginhoux et al. 2008). These cells are considered as leucocyte cells derived from the bone marrow. LC represent about 3-5 % epidermal cells; LC are linked to keratinocytes through E-cadherin-dependent adherent junctions (Tang, Amagai et al. 1993). LC are considered as the first line of immune defense against infectious pathogens in the skin, notably in the epidermis.
- **Merkel cells (MC)** are neuroendocrine cells principally found in touch sensitive areas such as the fingertips and lips. In the epidermis, MC are present in the basal layer where they interact with nerve fibers crossing the DEJ (Fitzpatrick and Breathnach 1963, Boulais and Misery 2007).

I.2.The dermal-epidermal junction (DEJ)

The dermal-epidermal junction (DEJ) is located between the epidermis and the underlying dermis to tightly attach them together (McMillan, Akiyama et al. 2003). Electron microscopic analysis shows that the DEJ contains three zones (figure 4):
1) The hemidesmosomes **(HD)** provide a link between the keratin filaments of the basal keratinocytes and the other components of the DEJ thanks to transmembrane integrin α6β4. 2) The lamina lucida **(LL)** composed of anchoring filaments, such as laminin 332 (laminin 5) and collagen XVII. 3) The lamina densa **(LD)** composed of a dense network of small adhesion molecules (Nidogen, fibronectin) connecting the anchoring filaments to anchoring fibrils (collagen IV and collagen VII) present in the dermis (Ellison and Garrod 1984). Collagen VII is important in the formation of an extensive network in the superficial dermis, under the DEJ (McMillan, Akiyama et al. 2003). The synthesis and the organization of the components of the DEJ are very important for cell adhesion, migration and signal transduction from the extracellular matrix into the epidermal keratinocytes (Burgeson and Christiano 1997). The DEJ allows the circulation between the dermal and the epidermal compartment of substances like cytokines and growth factors and even, cells (lymphocytes and immune cells) (Timpl and Brown 1996).

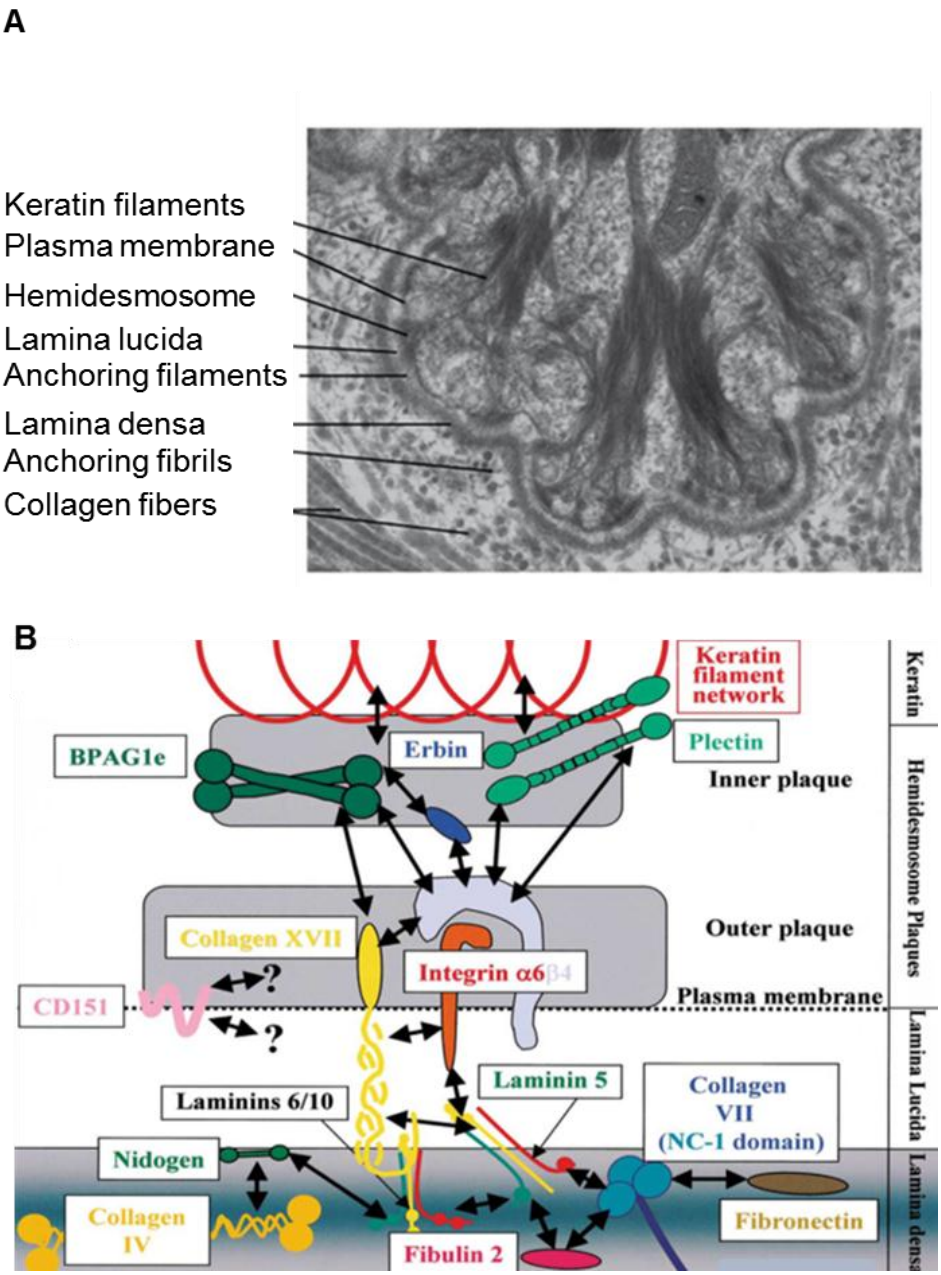

Figure 4: Dermal epidermal junction. A) Under electron microscopy shows: hemidesmosome (HD), lamina lucida (LL) and lamina densa (LD) *(According to Dr. M. Démarchez - Biologie de la peau, http://biologiedelapeau.fr/spip.php?article47&lang=fr).* **(B)** Schema showing the intermolecular interactions between HD and LL/LD. In HD, plectin and BPAG1 link the keratin filaments to the transmembrane collagen XVII and the integrin $\alpha6\beta4$. Collagen XVII and integrin $\alpha6\beta4$ interact with Laminin 5 anchoring filaments in the LL that in turn binds the anchoring fibrils of Collagen VII and the Laminins 6/10. (According to (McMillan, Akiyama et al. 2003).

I.3. The dermis

The dermis is a complex tissue underlying the epidermis. It contains blood and lymphatic vessels, nerves and nerve endings, glands, and, except on glabrous skin, hair follicles (figure 1). The majority of cells populating the dermis are fibroblasts from mesodermal origin (Sengel and Mauger 1976). Dermis varies in thickness as a function of its location in the body ranging from 0.6 mm on the eyelids to 3 mm on the back, palms and soles (Arda, Goksugur et al. 2014). The dermis is composed of two zones: a superficial layer that interdigitates with the epidermis, called the “papillary dermis”, and a deeper zone called “reticular dermis” (figure 5). The papillary dermis is thinner than the reticular dermis and represents about 10 % of the dermis. The density and the proliferative capacity of papillary fibroblasts are higher than reticular fibroblasts (Sorrell, Baber et al. 2004). Papillary and reticular layers are mainly composed of extracellular matrix components (ECM) produced by the fibroblasts (Sorrell, Baber et al. 2004, Driskell, Lichtenberger et al. 2013). They differ in both the composition and the organization of their ECM (table 1).

The dermis is characterized by the loose assembly of collagen and elastin fibers embedded in the extracellular matrix (ECM). In the papillary dermis, “papillary fibers” are arranged vertically and connect to the dermal epidermal junction, while fibers of the reticular dermis, tend to arrange horizontally. Dermal fibroblasts mainly produce collagen type I and III, elastin, structural proteoglycans (versican, decorin), glycoproteins (fibronectin and tenascins) and collagen VII, a major actor of dermis-epidermis junction (table 1).

Dermal fibroblasts act together with epidermal keratinocytes in the organization of the dermis-epidermis junction. They produce collagen IV and VII, two components present in the lamina densa and the anchoring fibrils. Fibroblasts also modulate the activity of keratinocytes in the epidermis by producing growth factors and cytokines. It has been shown that the proliferation and differentiation capacities of keratinocytes are regulated by dermal fibroblasts notably by the expression of the fibroblast growth factor 7 (FGF7) and the colony-stimulating factor 2 (CSF2). FGF7 stimulates keratinocytes proliferation and CSF2 is necessary to their differentiation (Maas-Szabowski, Shimotoyodome et al. 1999, Szabowski, Maas-Szabowski et al. 2000). The expression of these proteins is regulated by interleukin1, a regulatory cytokine 1 expressed by keratinocytes (Carr, Li et al. 2016). Moreover, fibroblasts play important roles in

wounds healing and the balanced production of type I and type III collagens (Smola, Stark et al. 1998). The dermis also contains dermal dendritic cells, macrophages and mast cells that represent the resident immune system in the dermis.

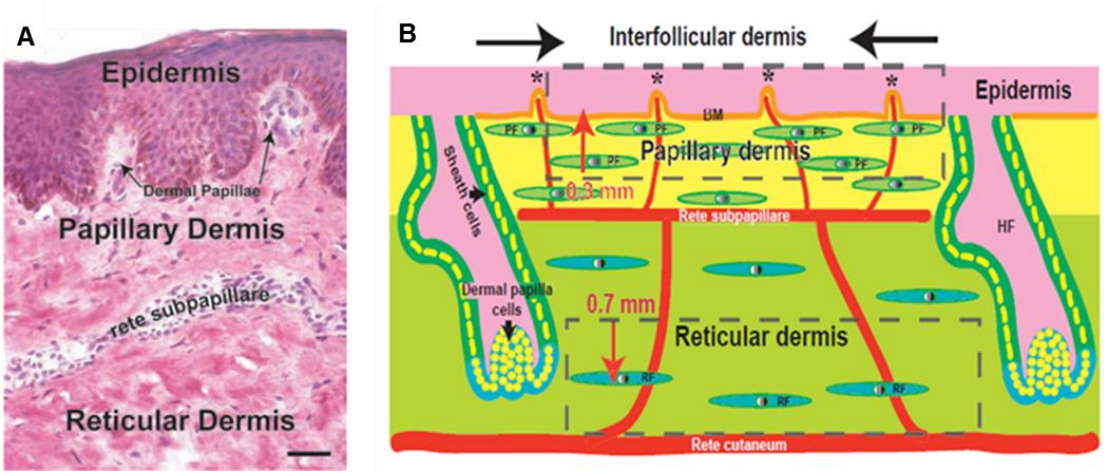

Figure 5: Histology of the skin: A) the papillary and the reticular dermis are separated by vascularized rete subpapillare. Dermal papillae interdigitates within the epidermis, scale bar 45 µm. **B)** Schema of adult human skin. Two different populations of dermal fibroblasts can be cultured from the interfollicular dermis, the papillary fibroblasts cultured from skin located at a depth of 0.3 mm and the reticular fibroblasts cultured from the skin located at 0.7 mm (Sorrell and Caplan 2004).

Matrix component	Papillary dermis	Reticular dermis
Collagens I and III	High ratio of types III to I	Low ratio of type III to I
Collagen IV	Present in basement membrane	Absent
Collagen VI	Present at dermal-epidermal junction (DEJ)	Weakly present
Collagen XII	Present	Low to absent
Collagen XIV	Low to absent	Present
Collagen XVI	Present in DEJ-region	Absent
Tenascin-C	Present in DEJ-region	Absent
Tenascin-X	Weak in DEJ-region	Present
Versican	Diffuse in DEJ-region, present in matrix fibrils	Present in association with elastic fibers
Decorin	Present	Present

Table 1: Distribution of extracellular matrix components in dermal layer (Sorrell, 2004)

I.4. The skin appendages

Upon embryonic development, embryonic mesodermal stem cells receive signals from ectodermal cells to instruct them to commit to a particular differentiation program and generate a stratified epidermis and skin appendages such as hair follicles and sebaceous glands (Fuchs 2007).

I.4.a. The pilosebaceous follicle

Pilosebaceous follicles are found in the hairy part of the body. They include hair follicles, sebaceous glands and the arrector pili muscle which causes the hair to stand up when it contracts (pilo-arrection). In the dermis, the hair follicles consist of the hair shaft surrounded by an inner root sheath (IRS) and an outer root sheath (ORS) (figure 6-A). In the adult human skin, the ORS is in continuation with the basal layer of the inter-follicular epidermis. At the deep end of the hair follicle, the ORS forms a germinal matrix (hair bulb) where cells proliferate and differentiate to form the different cells of the internal sheaths and the hair shaft (Fuchs 2007). In the mouse, hair follicle stem

cells reside in a niche that is located at the bottom of the non-cycling portion of the ORS, called the bulge (Taylor, Lehrer et al. 2000, Oshima, Rochat et al. 2001). Hair follicle stem cells are multipotent cells (Majo, Rochat et al. 2008, Bonfanti, Claudinot et al. 2010). They possess the potential to proliferate to allow hair regeneration, but they possess also the potential to make epidermis and sebaceous glands (Blanpain and Fuchs 2006).

The growth of hair follicles is cyclic and is subdivided in 3 phases (figure 6-B): 1) The anagen phase is an active growth phase of the hair cells in the bulb. 2) In the catagen phase, hair growth stops. The follicle enters a destructive phase, leading to its degeneration; in contrast, the bulge region that contains the stem cells remains intact. 3) The telogen phase is a quiescent phase (Blanpain and Fuchs 2009). In the human hair follicle, the average duration of each phase is 3 years, 3 weeks and 3 months, respectively (Bernard 2006). Under “normal hair conditions” about 85% of follicles are in anagen phase and 15 % in telogen phase. At the end of the telogen phase, the follicles regenerate from the germinative cells and enter a new anagen phase.

Sebaceous glands (SG) are exocrine glands attached to the hair follicle. SG produce and secrete sebum, a liquid rich in lipids. Secretion of sebum into the hair canal helps lubrification and growth of the hair shaft.

I.4.b. Sweat glands

Sweat glands are exocrine glands that play an essential role in thermoregulation. Eccrine sweat glands are localized on the entire surface of the skin but they are particularly numerous on the palms of hands, soles of feet and forehead.

The secretion by the sweat glands represent a primary form of refreshing in humans. The secretory portion of these glands is found in the dermis and the excretory duct opens directly at the skin surface. The sweat glands are found in the armpits and perianal areas. They are not significant for cooling in humans, but rather act as scent glands. Their excretory duct opens into hair follicle.

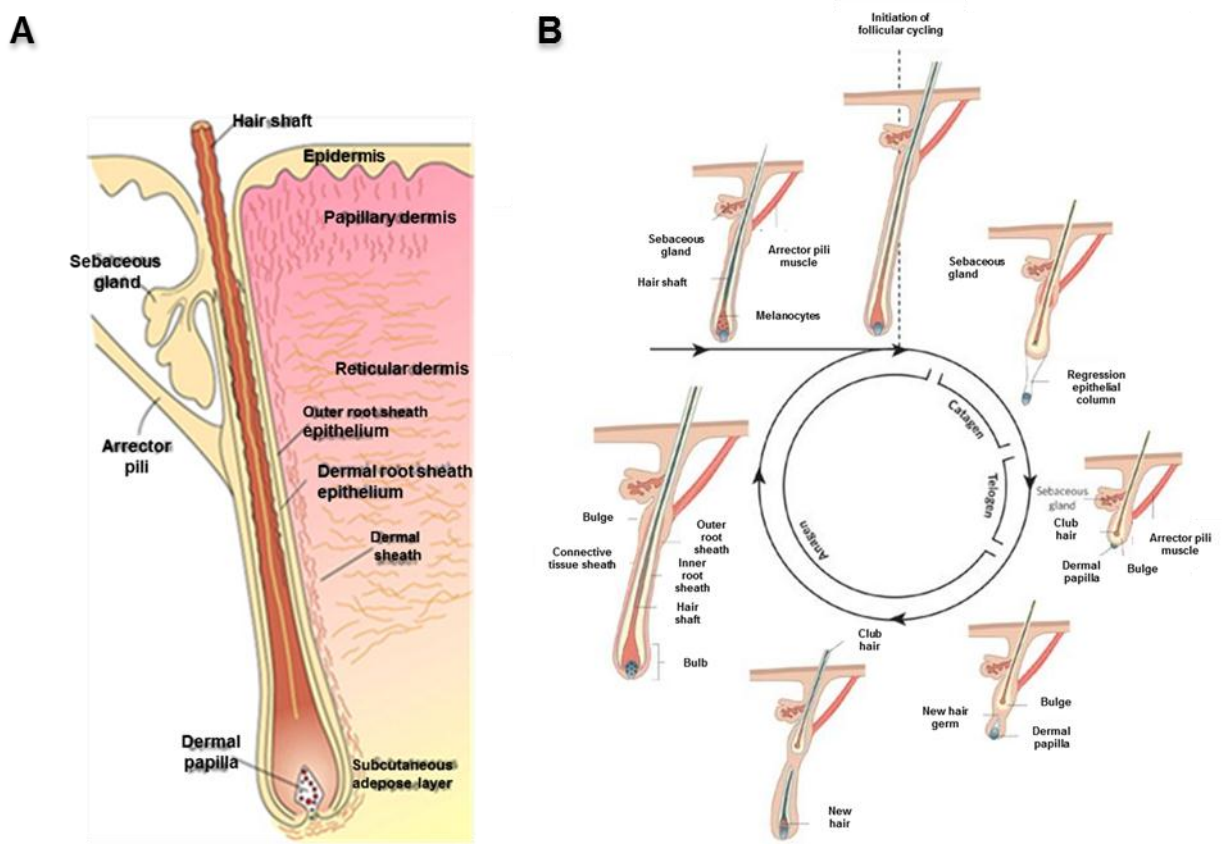

Figure 6: the hair follicule. A) Haired skin is composed of a pilosebaceous unit that contains a hair follicle (HF) and its surrounding interfollicular epidermis (IFE), which is responsible for skin barrier function. HFs are composed of different concentric layers of cells. At the base of the HF, the matrix contain proliferative cells and then embark concentric terminal differentiation programs that generate the mature follicle. In mice, hair follicle stem cells (HFSCs) reside in a niche that is located at the bottom of the non-cycling portion of the outer root sheath (ORS), called the bulge. HFs also contain sebaceous glands, which form from and are replenished by resident SCs located in the outer root sheet (ORS). These SCs differentiate to form the sebocytes which release oils that lubricate the hair channel and skin surface following degeneration (Jahoda and Reynolds 2001). **B)** Schema of hair cycle stages, starting from the first postnatal anagen, hair shaft is growing and arising through the skin surface. Hair follicles progress to the destructive (catagen) phase, during which the lower two-thirds of the follicle undergo apoptosis and regress. The dermal papilla is brought to rest below the bulge-stem-cell compartment, and after the resting (telogen) phase, a critical threshold of activating factors is reached and the stem cells become activated to regrow the hair (Fuchs 2007).

Chapter two: Stem cells

“Epidermal” stem cells are located in the epidermal basal layer and in the outer root sheath of the hair follicle. They play important role in the maintenance of skin homeostasis, renewal and hair regeneration through cycling regeneration of hair follicles. Epidermal stem cells also contribute to epidermal regeneration during wound healing after epidermal injuries such as burns, cuts, abrasion or pathology-led epidermal injuries (Blanpain and Fuchs 2006). Stem cells are known for their ability to self-renew to generate additional stem cells, and the capacity to generate progeny that are fated to differentiate into at least one type of highly differentiated descendant (Lajtha 1979).

I. Embryonic stem cells (ESCs)

ESCs are isolated from the inner cells mass (ICM) of blastocyst. They are pluripotent cells that can give rise to all cell types of the body under specific circumstances such as the presence of tissue-specific growth factors. ESCs may then give rise to one or more progenitor cell lineage involved in the functional organization of a given tissue.

II. Induced pluripotent stem cells (IPSCs)

Studies showed that human induced pluripotent stem cells (IPSCs) could be generated from adult somatic cells by introducing genes encoding the transcription factors Oct3/4, Sox2, c-Myc, and Klf4. IPSCs exhibit the morphology and growth properties of ESCs and express ESCs marker genes (Yamanaka and Takahashi 2006). They can be propagated indefinitely and are able to differentiate into virtually every cell type which “theoretically” make them viable for transplantation and experimental disease modeling (Wu, Doorenbos et al. 2016).

To avoid inappropriate recruitment and differentiation triggering, stem cells are thought to be maintained in a very specific microenvironment, called a “niche”. The stem cell niche constitutes a tissular microenvironmental context. In the niche, stem cells are undifferentiated; they self-renew by dividing very rarely to preserve the integrity of their genetic and epigenetic information comprising the program of tissue morphogenesis (Morrison, Shah et al. 1997, Morrison and Spradling 2008).w

III. Keratinocyte stem cells ex vivo

A major difficulty in the study of stem cells is to obtain an efficient model for preserving their presence and functionality *ex vivo*, for example, when taken out from their natural niche. After many failures to reliably isolate living embryonic and somatic stem cells, the idea emerged that development of appropriate culture systems should allow preserving both long-term potential of renewal and stem cells differentiation potential. In 1975, Rheinwald and Green reported that epidermal keratinocytes from a skin biopsy could be grown for a very long-term so long as a feeder layer of lethally irradiated mouse embryonic fibroblasts was seeded few hours prior primary keratinocyte seeding (3T3-J2 fibroblasts are gamma irradiated in order to stop their growth, but not the growth of keratinocytes, and hence play the role of feeder cells) (Rheinwald and Green 1975). In the presence of epidermal growth factor (EGF) keratinocytes can be maintained in culture for more than 100 populations doublings (Rheinwald and Green 1977). Under appropriate conditions, a single stem keratinocyte can give not only the entire epidermis of an individual (≈ 10^{11} cells), but also of all individuals on the planet (≈ 10^{21} cells) (Warrick, Garcia et al. 2012).

Under adequate culture conditions, human epidermal keratinocytes clonogenic cells may initiate three types of colonies: the holoclones, the meroclones and the paraclones (Barrandon and Green 1987) (figure 7). The holoclones are large colonies with smooth perimeter. 95 % holoclones progeny is clonogenic. The paraclones are small, terminally differentiating colonies, with a very limited proliferative potential (<15 divisions). Less than 5 % of the cells initiated by a paraclone are themselves clonogenic. The meroclones are medium-sized colonies with irregular contour. The clonogenic potential of their progeny is very heterogeneous, ranging from 5 to 95 %. Along serial propagation, the percentage of meroclones and of paraclones progressively increases at the expense of holoclones. The proportion of epidermal stem cells (holoclones) present in a keratinocytes culture is a good indicator of the quality of the culture.

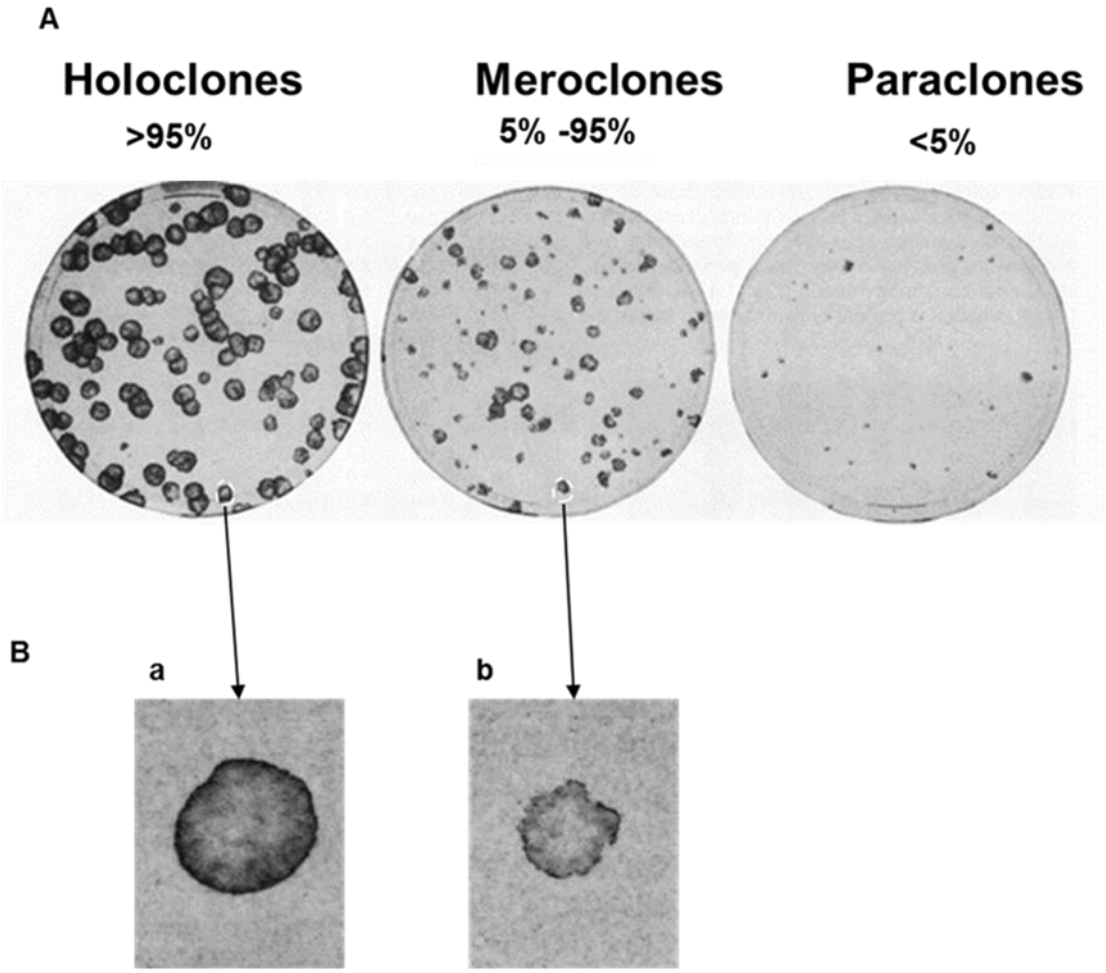

Figure 7: The 3 types of colonies formed by keratinocytes in culture. A) Colony forming efficiency of three different clones isolated from primary cultures of keratinocytes. Holoclones have high proliferative potential and correspond to epidermal stem cells. Paraclones are composed of cells with a limited growth potential (<15 divisions). Meroclones are intermediate and constitute a transitional state between holoclones and paraclones. B) Macroscopic colony types formed by keratinocytes. Twelve days' colonies were fixed and stained with rhodamine. Note the smooth perimeter of holoclones (a) and the wrinkled perimeter in meroclones (b) (Barrandon and Green 1987).

The most important evidence of the presence and the maintenance of stem cells in culture comes from the success of the transplantation of human cultured epithelium autografts on patients with severe burns. Renewal for very long term (over 20 years) of an epidermis with normal characteristics, despite the absence of skin appendages, shows that stem cells in culture were preserved and retained all their potentials of proliferation and differentiation after transplantation (Gallico, O'Connor et al. 1984, Pellegrini, Ranno et al. 1999, Ronfard, Rives et al. 2000).

Ex vivo, reconstructed skin models composed of human keratinocytes growing on a collagen lattice containing dermal fibroblasts were also developed to reproduce the processes of stratification and epidermal differentiation (Bell, Ehrlich et al. 1981, Asselineau, Bernard et al. 1986). The reconstructed skins can be grafted on the immunodeficient mouse to regenerate human skin *in vivo* (Demarchez, Hartmann et al. 1992, Del Rio, Larcher et al. 2002). Human skin can also be regenerated in the long term from single layered epithelial sheets grafted onto the immunocompromised mouse. Following the seminal paper of Marcela Del Rio (Del Rio, Larcher et al. 2002), my host laboratory demonstrated that stem keratinocytes can be enriched from mass culture of genetically corrected human primary keratinocytes (Bergoglio, Larcher et al. 2007, Warrick, Garcia et al. 2012).

IV. Localization of stem cells *in vivo*

IV.1. Stem cells in the basal layer

Although the existence of a population of stem cells in the basal layer of the epidermis has been evoked since several years, their number and the precise location of their niche remains a subject of debates. According estimations, epidermal stem cells would represent between 0.1 % and 1 % of keratinocytes present in the basal layer (Schneider, Barland et al. 2003). Recent studies show that there are mutual influences between skin stem cells and their niches (Hsu, Li et al. 2014). Stem cells and their progeny are thought to interact with components of the niche. Stem cells and their progeny can coexist within a niche, suggesting that niche signals alone are not sufficient to maintain ‘stemness’ (Hsu, Pasolli et al. 2011, Sato, van Es et al. 2011). These studies suggest that “there is an exchange in the regulation between the parents (stem cells) and their progeny cells” (Hsu and Fuchs 2012, Hsu, Li et al. 2014).

According to first observations described by Potten and his team in the late seventeens, progeny of epidermal stem cell is organized as columns, called epidermal proliferation units (EPU) (Potten 1974). Using retroviral transfer of a tracing gene (beta-galactosidase), it has been shown that each column comprises about ten basal keratinocytes subsequently giving rise to the suprabasal cell layers (Mackenzie 1997). Cells of each EPU are thought to be the progeny of a single stem cell. This progeny would possess a limited proliferative potential and are called transit amplifying cells (TACs). TACs divide several times before leaving the basal layer to embark the stepwise process of differentiation (figure 8) (Jones and Simons 2008, Hsu, Li et al. 2014). The existence of TACs would present two advantages: 1) The rapid regeneration of the skin in case of injury; 2) The limitation of stem cells divisions, which preserves their pool and limits the possibility of genotoxic mutagenesis, and hence cancer initiation.

In 2007, Clayton *et al.* proposed another model of epidermal stem cells regulation in the mouse. In brief, using cell tracing strategies, the authors suggested that recruitment of stem cells in the basal layer of the mouse tail epidermis i) is stochastic, ii) does not give rise to TACs (Clayton, Doupe et al. 2007). No report generalizing this model to human interfollicular epidermis has been published yet.

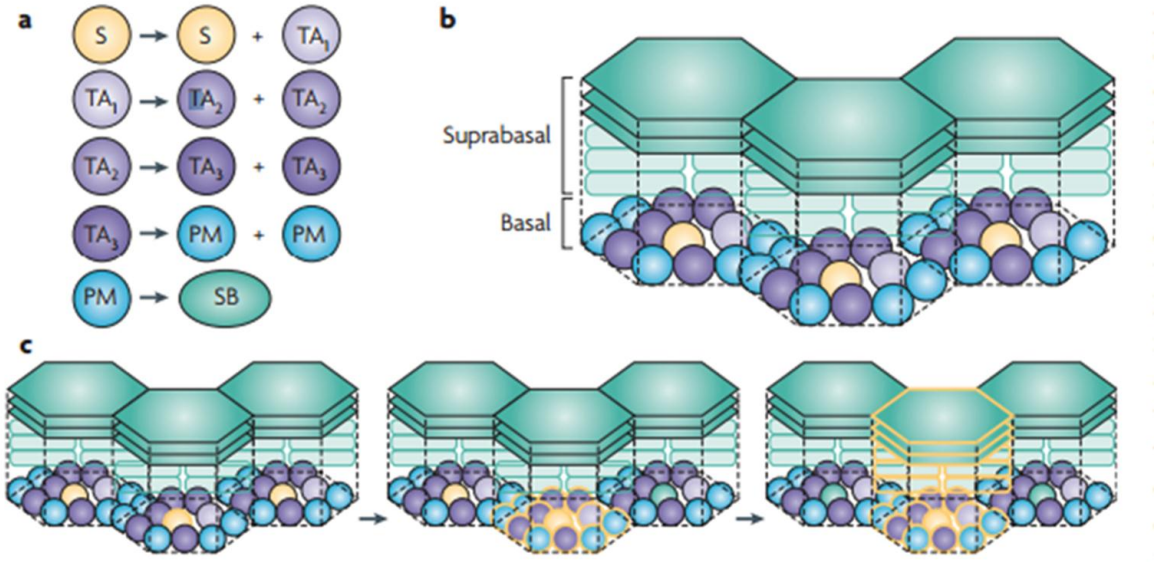

Figure 8: Epidermal proliferative unit (EPU) hypothesis. **a)** Self-renewing stem cells (yellow) generate a transit-amplifying (TA) cell population (purple), which undergoes symmetric division (TA1 to TA3) before their terminal differentiation into post-mitotic (PM) cells (blue). These PM cells migrate from the basal layer and become suprabasal (SB) cells (green). **b)** Schema showing the spatial organization of cells in EPUs. **c)** EPU model in a clonal labelling experiment (Jones and Simons 2008).

IV.2. Stem cells of the hair follicle

One of the first methods used to determine the location of stem cells *in vivo* was based on the use of nucleotide analogs such as bromodeoxyuridine (BrdU) or [3H] thymidine. After peritoneal injection, these DNA precursors are incorporated in the DNA of dividing cells during replication. After a few cell divisions, the marker is rapidly diluted and is no longer detectable in the progeny of stem cells. Based on the fact that stem cells do not divide frequently, a population called «label retaining cells" (LRCs), presumably corresponding to stem cells was identified.

In 1990, Cotsarelis *et al.* showed that the higher number of LRCs of skin reside in the bulge of the mouse hair follicle. The bulge is a region of the outer root sheath beneath the sebaceous gland and the pili arrector muscle. Only few LRCs are observed in the interfollicular murine of epidermis (Cotsarelis, Sun et al. 1990). Using the rat vibrissal follicle as a model, Kobayashi, reported that clonogenic assays showed

that most clonogenic cells (keratinocyte colony-forming cells) are clustered in the bulge. By microdissection of rat vibrissal follicles, the authors reported that about 95 % of clonogenic cell are concentrated in the bulge area. In contrast, less than 5 % clonogenic cells were found located in the matrix area of the follicle (lower part of HF, sometimes called “the bulb”) (Kobayashi, Rochat et al. 1993). The same team showed that clonogenic cells of the human hair follicle are also concentrated in a region below the midpoint of the hair follicle and outside the hair bulb, in the site of insertion of arrector muscles; however it is important to notice that the morphology of the human hair follicle does not include a “bulge like” region (Rochat, Kobayashi et al. 1994).

Later, it has been shown that cells of the bulge are multipotent stem cells with high clonogenic capacity and capable to regenerate the different compartment of the pilosebaceous follicle (Taylor, Lehrer et al. 2000, Blanpain, Lowry et al. 2004, Claudinot, Nicolas et al. 2005). During wound healing, some keratinocyte located in the bulge region participate to regeneration of the intrefollicular epidermis. However, under normal conditions, stem keratinocytes located in the bulge do not seem to participate to homeostasic renewal of the interfollicular epidermis (Ito, Liu et al. 2005, Levy, Lindon et al. 2005) (figure 9).

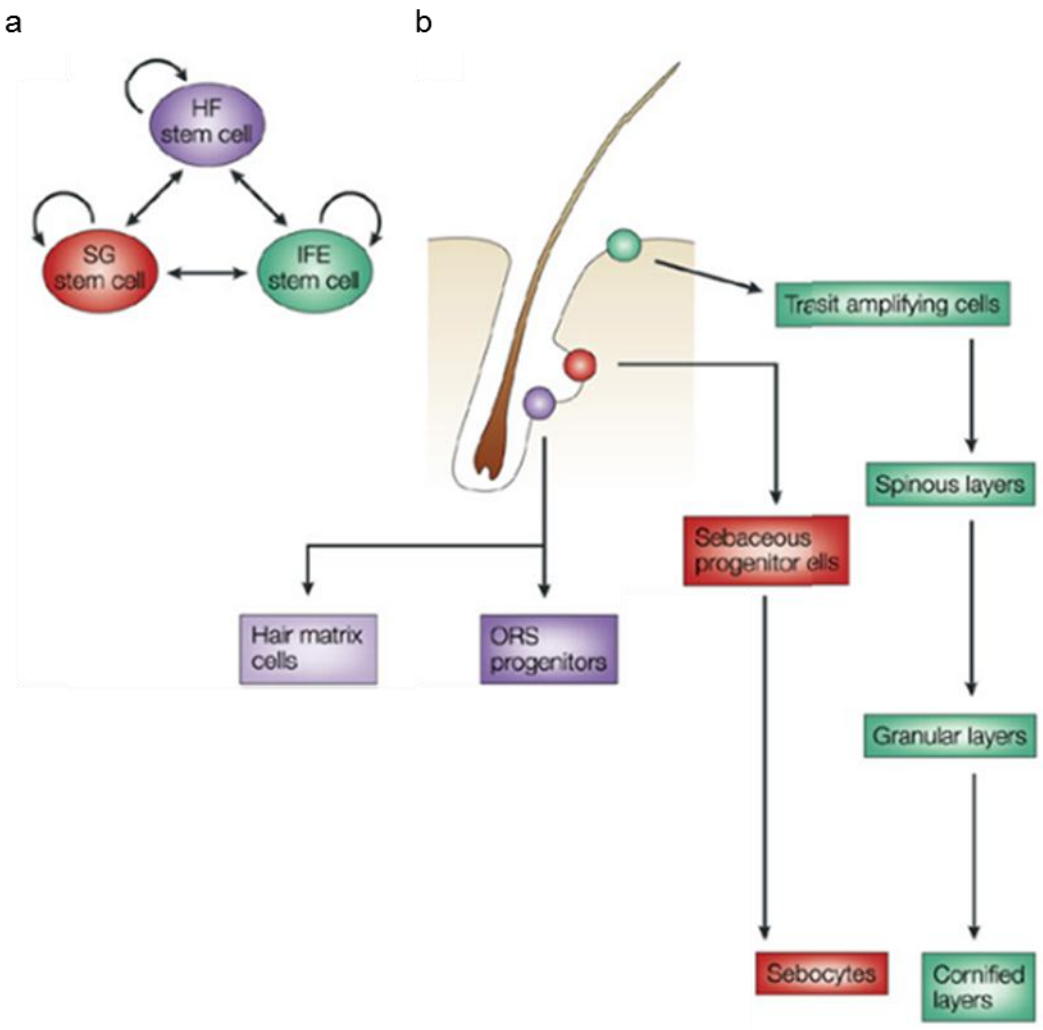

Figure 9 The different compartments of stem cells in the skin. a) Epidermal stem cells of the hair follicle (HF), sebaceous gland (SG) or interfollicular epidermis (IFE) are interchangeable and functionally equivalent. b) The stem cell of the IFE are located in the basal layer and allow permanent renewal of the epidermis. The progenitors of the sebaceous glands (SG) are responsible for the physiological renewal of sebocytes. The HF stem cells form the cells of the outer root sheath (ORS) and inner root sheath (IRS) (Owens and Watt 2003).

Chapter three: Consequences of solar radiations on skin

Exposure of skin to sun radiations leads to many types of biological responses, including immunosuppression, glycation of ECM, peroxidation of lipids and proteins. Schematically, the first category of “immediate” short-term responses to sunlight exposure include suntan (for moderate exposure) as sunburn and erythema (for high UV dose exposure). The second one is a long-term response, characterized by photoaging and the development of skin cancers.

Solar UV are classified into three categories depending of their wavelengths: UVC (200 - 280 nm), UVB (280 - 320 nm), and UVA (320 - 400 nm) (Cerutti and Trump 1991). UVA (95 %) and UVB (5 %) of the UV spectrum reaching the earth’s surface, respectively. UVC are absorbed by the ozone layer of the stratosphere avoiding them to reach the Earth. However, due to the depletion of the ozone in certain regions of the world, sunlight reaching the surface of the Earth tend to be enriched in UVB and UVC radiations (Lloyd 1993). In the human skin, the epidermis and the superficial dermis “absorb” UVB due to their high energy; UVA are less energetic than UVB and penetrate more deeply into the skin (figure 10). UVB are about ~ 10,000 times more carcinogenic than UVA. UVB cause damages in DNA and initiate, and promote skin cancer progression (Bowden 2004).

I. Effects of UVB and UVC on DNA

In the human body, 10^{13} cells receive about 10^{4} DNA lesions every day (Lindahl and Barnes 2000). Accumulation of these lesions is mutagenic. DNA aromatic bases pyrimidines (cytosine (C) or thymine (T)) absorb photons energy at wavelengths ranging from 230 to 290 nm (i.e. from UVC to UVB wavelengths, respectively). The absorption of UV photons in the DNA gives rise to dimeric photoproducts between adjacent pyrimidine bases on the same DNA strand. Two types of bulky DNA lesions that introduce a majXPor DNA distortion are produced: cyclobutane pyrimidine dimers (CPD) and 6, 4 pyrimidine-pyrimidone photoproducts (6-4 PP) (figure 11).

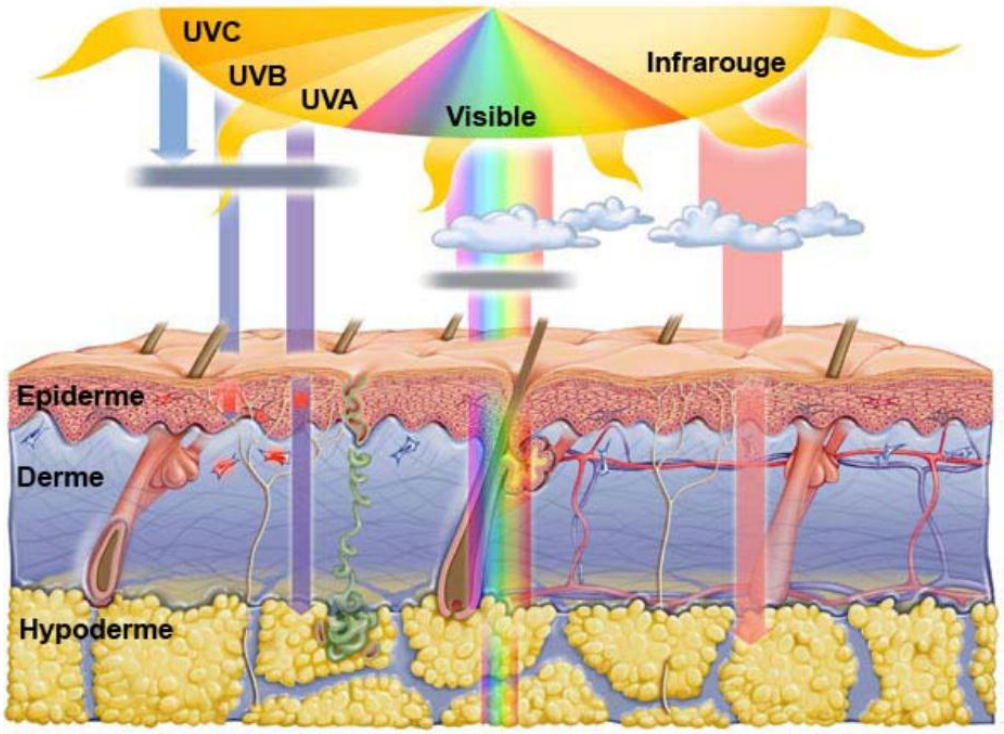

Figure 10: UV penetration in the skin. UVA penetrate deeply into the dermis, while UVB is absorbed in the epidermis and the surface of the dermis. UVC are absorbed by the ozone layer which prevent it these wavelengths to reach the Earth surface.

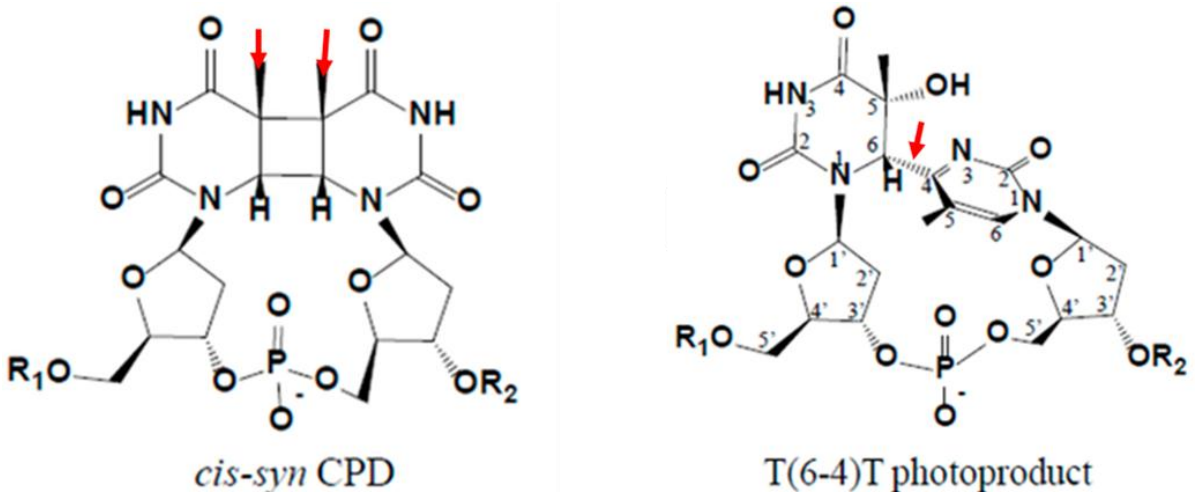

Figure 11: DNA lesions induced by UV. CPD is obtained by the formation of covalent linkage between C5 and C6 atoms in the adjacent pyrimidines on the same DNA strand. 6-4 PP is formed by founding covalent linkage between C6 and C4 atoms between the two adjacent pyrimidines on the same DNA strand (Yokoyama and Mizutani 2014).

I.1. CPD formation

CPD lesions are formed by a covalent link between the C5 and C6 atoms of two adjacent pyrimidine bases (figure 11). Irradiation of cultured cells with UVB leads to the production of about 1-5.10^{-4} lesions / kbp / J.m^2 (Perdiz, Grof et al. 2000, Douki and Cadet 2001). CPD lesions distort the DNA double helix of about 7 to 10° compared to its initial conformation (Kim, Patel et al. 1995, Miaskiewicz and Ornstein 1996). It has also been shown that methylated cytosine at position C5 (5-mC) in CpG promoter regions absorb UV lengths of 273 nm (UVB radiation) (Drouin and Therrien 1997) and thus promote CPD formation in genetic regulatory regions (Pfeifer, You et al. 2005) (figure 12).

I.1. 6-4 PP formation

6-4 PP lesions result from the formation of a covalent bridge between the C6 and C4 carbons of two adjacent pyrimidines on the same DNA strand (Figure 11). 6-4 PP lesions are much more distorting than CPD lesions. They lead to a 44 ° distortion of the DNA (Kim, Patel et al. 1995). The ratio of the number of CPD and 6-4 PP formed for a given UVB dose is estimated between 8 : 1 (Perdiz, Grof et al. 2000) and 3 : 1 respectively (Douki and Cadet 2001). The influence of cytosine methylation on the formation of 6-4 PP has not been clearly established (Pfeifer, Drouin et al. 1991, Mitchell 2000).

CPDs are repaired slower than 6-4 PP (Thomas, Okumoto et al. 1989, Mitchell, Brash et al. 1990). This difference in repair is thought to be related to the highest DNA distortion induced by 6-4 PP lesions which facilitate their recognition by the DNA repair mechanisms (Szymkowski, Lawrence et al. 1993, Kim, Patel et al. 1995, Sugasawa 2010). The CPDs persist longer in DNA and are responsible for at least 80 % of the UVB-induced mutation (You, Lee et al. 2001).

II. Analysis of mutations induced by UVB and UVC

Studies on mutations induced by UVC and UVB radiations demonstrate that, from yeast to mammalian cells, the majority of UV mutations observed in cells are C → T or CC → TT transitions formed at adjacent pyrimidines (figure 12) (Armstrong and Kunz 1990, Pfeifer, You et al. 2005). These mutations are a “signature” of the mutagenesis induced by UVB / UVC radiations (Hutchinson 1994, Brash 2015).

III. Effect of UVA on the skin

DNA bases weakly absorb UVA but UVA can activate photosensitizers in the skin such as melanin, porphyrins and riboflavin. Continuous exposure to sunlight generates reactive oxygen species (ROS), organic free radicals and other toxic products (Wondrak, Jacobson et al. 2006, Ridley, Whiteside et al. 2009).

Accumulation of ROS leads to direct damages of cellular components, including lipid peroxidation, protein oxidation and nucleic acids alteration. These damages may induce both cell death and malignant transformation (Bragado, Armesilla et al. 2007, Liu, Chen et al. 2008).

The modification in skin structural proteins is accompanied by depletion of anti-oxidant enzymes such as catalase and superoxide dismutase (Bernerd and Asselineau 1998, Sander, Chang et al. 2002). *Ex vivo* studies have shown that photooxidative stresses lead to the induction of MMPs which trigger the extracellular matrix proteins (ECM). The induction of MMPs leads to the degradation of the collagen fibres present in the ECM (Dalle Carbonare and Pathak 1992, Adams 2000, Frechet, Warrick et al. 2008). UVA can lead to indirect DNA damages by generating CPD and 8-oxoG lesions due to ROS generation (Douki, Reynaud-Angelin et al. 2003). To attenuate the effect of UV exposure, cells have several mechanisms in place, including antioxidants, DNA repair, and stress signalling pathways (Cadet, Delatour et al. 1999, Birben, Sahiner et al. 2012).

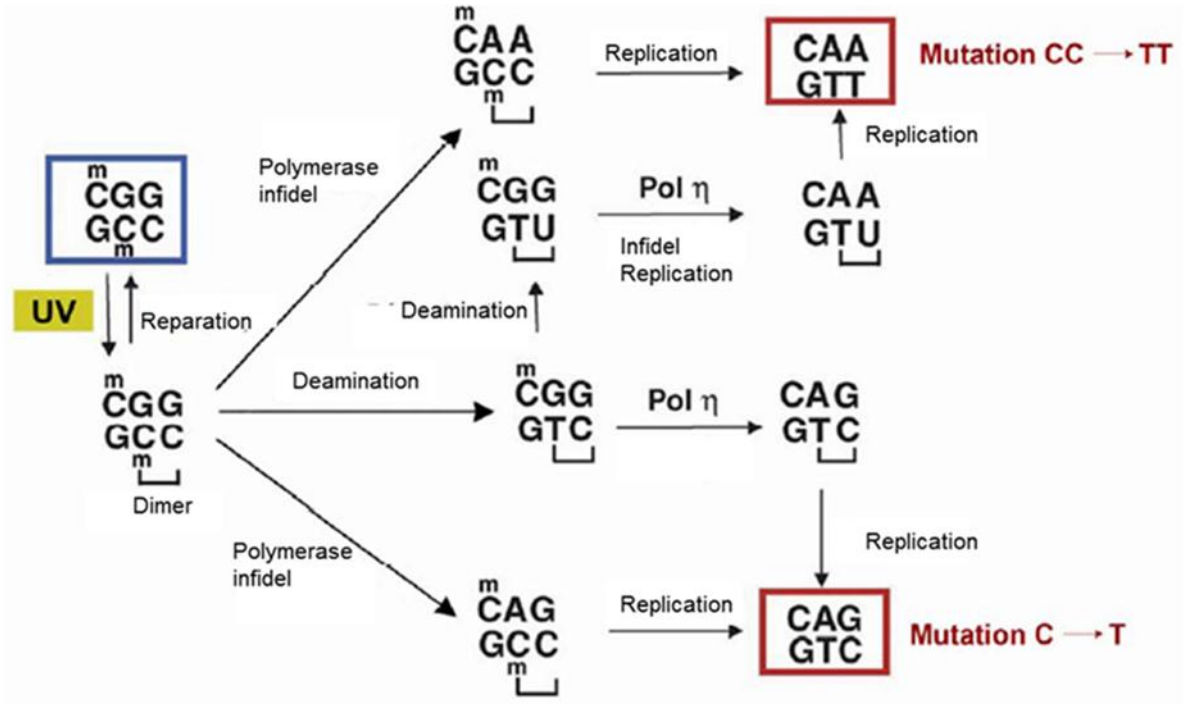

Figure 12: Mutagenesis mechanisms at a cyclobutane pyrimidine dimer (CPD). Scheme shows, 5′-CmCG is particularly prone to CPD formation after irradiation due to the presence of a 5-methylecytosine base (mC). Bypass of the CPD by an error-prone DNA polymerase could produce a C to T or CC to TT mutation. Deamination may occur within the CPD and may affect the 5-methylcytosine alone or the 5-methylcytosine and cytosine (double deamination). During subsequent DNA replication, polymerase η will introduce a C to T or a CC to TT tandem transition mutation (Pfeifer, You et al. 2005).

Chapter Four: Skin cancers

In the Human, 80 % tumors are from epithelial origin (DePinho 2000). The high proliferative potential of epidermal skin cells and the exposition to genotoxic agents, notably UV, both contribute to the development of cutaneous tumors during ageing. There are three types of skin cancers: basal cell carcinoma (BCC) and squamous-cell carcinoma (SCC) that arise from keratinocytes, and melanoma derived from melanocytes. SCC and BCC represent about 20 % and 80 % of nonmelanoma skin cancers, respectively (Kwa, Campana et al. 1992, Brellier, Marionnet et al. 2004, Amberg, Holcmann et al. 2015). Ultraviolet exposure is the major etiological agent of these types of cancers (Fears and Scotto 1983, Preston and Stern 1992).

I. Squamous cell carcinoma (SCC)

SCCs are associated with significant risk of metastasis. Over 250,000 new cases of SCCs appear in US annually. The average incidence rates of SCC in UK was 22.65/100 000 person-years (Lomas, Leonardi-Bee et al. 2012). In addition of factors as age, sex and skin color, two or more painful sun exposures may increase the risk of SCCs skin cancer about 3-fold in Australian population (Green and Battistutta 1990). SCCs development is regarded as a multistep process starting from a precursor lesion called actinic keratosis (AKs). In about 5 % cases, AKs may develop as squamous cell carcinoma in situ (SCCIS) or Bowen's disease, invasive SCC (ISCC) and metastatic SCC (figure 13), while 95 % spontaneously regress (Yanofsky, Mercer et al. 2011, Ratushny, Gober et al. 2012, Chen, Halliday et al. 2013).

I.1. Actinic keratosis (AK)

AK is a scaly hyperkeratosis lesion which develops in skin areas exposed to sun notably the face, ears, and neck (Salasche 2000, Rogers, Weinstock et al. 2010). Histological analysis of AKs shows atypical keratinocytes with enlarged, irregular, and hyperchromatic nuclei (Arciniegas, Carrillo et al. 2015). Abnormal growth of AKs disrupts cell differentiation and results in a thickening of the stratum corneum (Cockerell 2000). AK is a form of intra-epidermal keratinocytic neoplasia. The risk of progression of AK to SCCIS or ISCC has been estimated from 7784 AKs lesions; it is

0.60 % at 1 year, 1.5 % at 2 years, 2.2 % at 3 years, 2.6 % at 4 years, and 3.5 % at 5 years (Criscione, Weinstock et al. 2009).

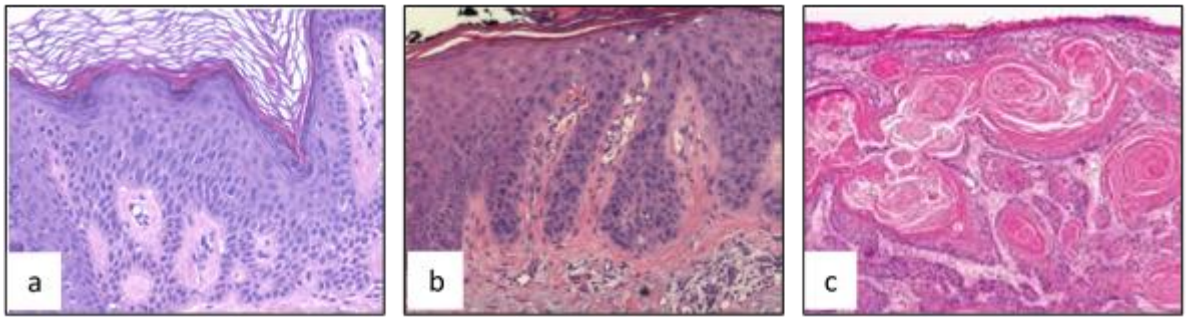

Figure 13: Histologic aspects of squamous Cell Carcinomas and their precursor lesions. a) Actinic keratosis. b) SCC in situ (SCCIS). c) Invasive squamous cell carcinoma (ISCC) (Yanofsky, Mercer et al. 2011, Chen, Halliday et al. 2013).

I.2. Squamous cell carcinoma in situ (SCCIS) or Bowen's disease (BD)

Patients with SCCIS have scaly red lesions in sun-exposed areas. SCCIS is considered as the early stage of the intra-epidermal form of SCC. Histological analysis of SCCIS shows that keratinocytes become pale with abnormal differentiation features and atypical nuclei, contributing to parakeratotic traits (Maione, Errichetti et al. 2016). SCCIS do not invade the dermis (Chen, Halliday et al. 2013) (figure 13). About 3–5 % untreated SCCIS develop into invasive SCCs (Cox, Eedy et al. 2007).

I.3. Invasive SCC (ISSC)

About 70-80 % ISCC is found in photo-exposed areas. ISCC incidence increases in photo-exposed areas (Iversen and Tretli 1999). Histologically, early stage ISCCs look like AK lesion; AK may however be distinguished from ISCCs by the presence of invasive cells passing through the basement membrane into the dermis. In the later stages of invasion, carcinoma cells are characterized by the formation of nests of atypical tumor cells in the dermis (figure 13) (Kelder, Ebrahimi et al. 2012, Chen, Halliday et al. 2013). Cells in this stage exhibit a clinical and histological spectrum of aggressiveness and metastatic potential, depending on tumor thickness and depth of invasion (Dinehart, Nelson-Adesokan et al. 1997).

I.4. Genetics of skin SCC carcinogenesis

The *TP53* gene is considered as the “Guardian of the genome” due to its key role in the control of cycle and apoptosis. *TP53* is the most frequently mutated gene in human cancers (Olivier, Eeles et al. 2002) and disruption of the normal p53 function is generally accepted as an important step in human carcinogenesis (Greenblatt, Bennett et al. 1994, Ziegler, Jonason et al. 1994). The analysis of p53 mutations in skin and cutaneous SCC have been extensively studied in human and murine tumors including skin and other organs such as lung, colon and liver.

In the epidermis, p53 is constitutively expressed in small quantities. UV radiation induces p53 stabilization after phosphorylation by ATR and p38 protein kinases allowing its contribution to repair processes of DNA damages and/or the induction of apoptosis (Campbell, Quinn et al. 1993, Hall, McKee et al. 1993). In sun-exposed skin, numerous clones/clusters of p53 immunoreactive keratinocytes have been identified (Urano, Oura et al. 1992, Ponten, Berne et al. 1995, Jonason, Kunala et al. 1996, Ren, Ahmadian et al. 1997). DNA sequencing of these clones have shown that 30-70 % harbor a *TP53*-mutated gene (Ren, Ponten et al. 1996, Ponten, Berg et al. 1997, Tabata, Nagano et al. 1999). Furthermore, *TP53* is mutated in 75-80 % AK lesions. The presence of *TP53* gene mutations in SCCIS and SCC has been found to be 40 %, and 41-90 %, respectively. These results suggest that AK lesions have acquired the genetic mutations prior becoming cutaneous SCC (Ziegler, Jonason et al. 1994, Ortonne 2002). These results also indicate that p53 loss is important to skin SCC progression (Campbell, Quinn et al. 1993, Ren, Ponten et al. 1996, Ziegler, Jonason et al. 1996, Ren, Ahmadian et al. 1997). The vast majority of p53 mutations found in AK, SCCIS, and SCC most often display the typical UV (C > T or CC > TT) signature, thus being indicative of UV-radiation-induced mutations.

Several other mutations in tumor suppressor genes or oncogenes, such as *RAS* or *p16* (CDKN2A), have been implicated in carcinogenesis. Ras is a member of GTPase proteins family encoded by *RAS* oncogenes. Ras activity activates wide a set of downstream effectors pathways involved in cell growth, differentiation and cell survival. Mutations of *RAS* oncogenes have been reported to occur in 10-40 % non-melanoma skin cancers (SCC and BCC) (Ananthaswamy and Pierceall 1990). Data from the Catalog Of Somatic Mutations In Cancer (COSMIC; Sanger Institute) indicate

that 21 % cutaneous SCC harbor activating RAS mutations (9 % Hras, 7 % Nras, 5 % Kras) (Bamford, Dawson et al. 2004).

p16 is a tumor suppressor protein, which contributes to the regulation of cell cycle by inhibiting entry in the S phase. p16 is downregulated in high numbers of tumors and is frequently dysfunctional in SCCs (Mortier, Marchetti et al. 2002, Pacifico, Goldberg et al. 2008, Romagosa, Simonetti et al. 2011). Studies by Hodges showed that p16 is expressed at higher levels in AK and SCCI than in SCC (Hodges and Smoller 2002, Salama, Mahmood et al. 2003) suggesting that disruption of the *p16* gene is associated with AKs progression to SCCs.

AK also often shows frequent loss of heterozygosity (LOH) in *p16* in chromosomes (3p, 9p, 9q, 13q, 17p and 17q) (Rehman, Takata et al. 1996). *p16* LOH in AK leads to uncontrolled G1 to S transition, then promoting the progression of AK premalignant lesions to SCCs (Rehman, Quinn et al. 1994, Mortier, Marchetti et al. 2002, Yoo, Cho et al. 2004).

II. Basal cell carcinoma (BCC)

Basal cell carcinoma (BCC) is the most common malignancy in humans and its incidence has increased rapidly over the last 50 years. The rising incidence of BCC is probably due to increased sun exposures and longevity of the population (Tourli, Langner et al. 2016). BCC are thought to be the consequence of acute UV sun burns in early age (Rubin, 2005). BCC are predominantly diagnosed in sun exposed areas as head, neck, and back of the hands (Rubin, Chen et al. 2005), but they can also be found in sun-protected skin (Crowson and Magro 1996). The risk of BCC is higher in elderly persons and in persons with fair skin with blonde/reddish hair (Green and Battistutta 1990).

BCC is thought to arise from epidermal basal keratinocytes (Preston and Stern 1992) and has been histologically subdivided into several subtypes by Crowson in 2006: The “indolent-growth” BCCs that include superficial BCC and nodular BCC, and “aggressive-growth” BCCs that include sclerosing BCC, infiltrative BCC, and metatypical BCC (Crowson 2006). Superficial BCC represents 10-15 % of BCCs (http://www.med.muni.cz/biomedjournal/pdf/2006/05/261_270.pdf). Superficial BCC is characterized by a proliferation of atypical basaloid cells that invade the dermis and

form an axis parallel to the epidermal surface. In addition, clefts between palisadic basal cells (epidermal) and the subjacent (dermal) stroma are observed (Crowson and Magro 1996, Ulrich, Roewert-Huber et al. 2011) (figure 14). This observation suggests a role of the stroma in tumor development (Bhowmick, Chytil et al. 2004, Valin, Barnay-Verdier et al. 2009, Gache, Brellier et al. 2015).

- **Nodular BCC** account for about 30-75 % BCCs (Sexton, Jones et al. 1990). BCC is composed of cell islets of basaloid keratinocytes in the papillary or reticular dermis.
- **Sclerosing (morpheaform) BCC** represents 1-5 % BCCs. Sclerosing BCC is characterized by columns of basaloid cells in a dense mesh of collagen. BCC show widespread invasion in the reticular dermis and the subcutis (Goldberg 1996).
- **Infiltrative BCC** represents about 10 % BCCs. It is characterized by extended growth pattern and long, thin strands of tumor cells penetrating deeply in the dermis ECM. Tumor cells have frequent mitotic activity and individual cell necrosis (Crowson and Magro 1996).
- **Metatypical BCC** is a form of aggressive growth BCC with infiltrating tongues of tumor cells and/or admixed nodular BCC components. Metatypical BCCs show intercellular bridge formation and/or cytoplasmic keratinization (de Faria 1985).

The incidence of metastatic BCC ranges from 0.003 % to 0.55 % (Robinson and Dahiya 2003). Metastasis of BCC may depend on the size of the original tumor. Original tumors greater than 3 cm in diameter have a 2 % incidence of metastasis. The incidence increases to 25 % in tumors greater than 5 cm and to 50 % in lesions of 10 cm (Snow, Sahl et al. 1994). The stromal dependence of the tumor provides also an explanation for the low metastatic potential of BCC. Metastasis and death related with BCC is uncommon but BCC can cause significant morbidity due to destructive local spread (Robinson and Dahiya 2003). Activation of SHH / PATCHED pathway, a signaling pathway essential in development, morphogenesis and stem cell maintenance (notably in hair follicles), is observed in the majority of sporadic BCC and could be an early event of tumor formation (Dahmane, Lee et al. 1997).

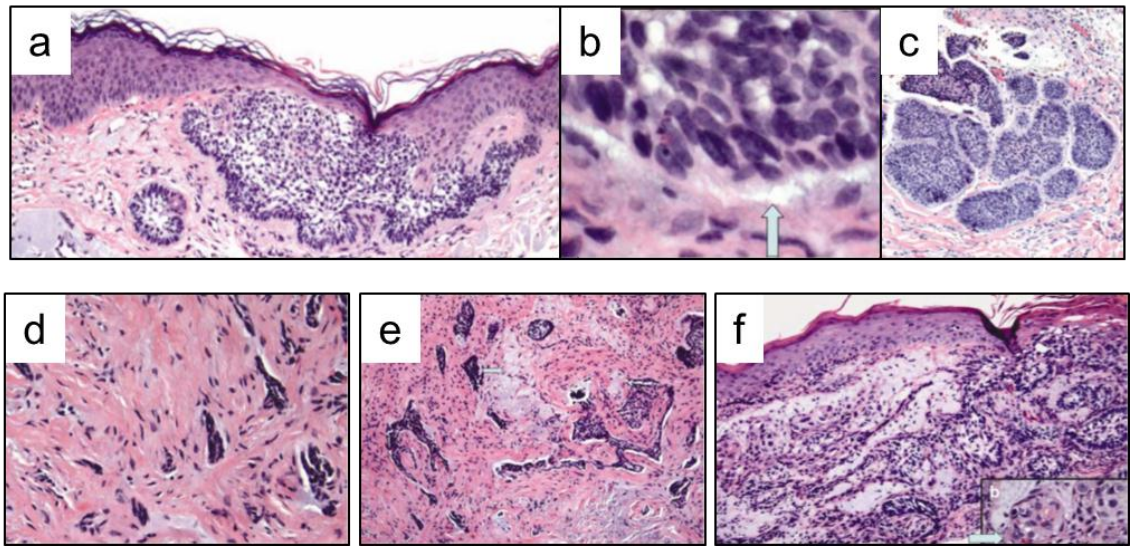

Figure 14: Histomorphology of BCC variants. a) Superficial BCC. b) Clefts between the palisadic basal cells and the subjacent stroma. c) Nodular BCC. d) Sclerosing BCC. e) Infiltrative BCC. f) Metatypical BCC (adapted from Crowson, 2006).

III. Melanoma

Cutaneous metastatic melanoma (CMM) derives from melanocyte transformation in 75 % cases and from pre-existing nevi in 25 % cases. Melanoma accounts for less than 4-6 % skin cancers but is responsible for 80 % skin cancer related deaths due to its aggressiveness and high metastatic potential. Melanoma incidence is doubling every 10-20 years in worldwide Caucasian population.

Melanomagenesis results from a complex combination of constitutional predispositions and environmental factors. CMM is hereditary in 10 % cases and major predispositions include 1) skin phototype (patients with fair hair/eyes/skin); 2) patients with high numbers of nevi; 3) patients with a familial history of melanoma. The main environmental factor to the development of melanoma is the chronic and intense sun exposures in the childhood (Oliveria, Saraiya et al. 2006, Bertolotto 2013).

In familial melanoma, rare deleterious germinal mutations of *CDKN2A* and *CDK4* confer a high propensity towards the risk of CMM (Hussussian, Struewing et al. 1994, Zuo, Weger et al. 1996). The products of these genes are involved in cell cycle arrest and cellular senescence. A germinal mutation in the microphtalmia-associated transcription factor (MITF), a regulator of melanocyte differentiation, has also been

shown to increase the risk of developing melanoma and is also known to be implicated in kidney cancer development (Bertolotto, Lesueur et al. 2011). Sporadic melanoma is mostly associated with activating mutations in BRAF (40–50 %) and NRAS (15 %) (Smalley 2003, Hocker and Tsao 2007, Liu, Kelly et al. 2007, Devitt, Liu et al. 2011) and/or mutations in PTEN (Russo, Torrisi et al. 2009).

IV. Cancer and microenvironment

The initiation, invasion and metastasis of cancer cells are linked to their structural and cellular environments and do not depend solely on a cancer cell-autonomous mechanism. The basement membrane (BM) can be a barrier in the progression of the tumor cells. In the late 70th, studies have shown alteration of the basement membrane at the areas in contact with tumor cells. These studies suggested a proteolytic enzymatic activity of neoplastic epithelial cells towards the surrounding BM components in order to provide space for tumor extension (McKinney and Singh 1977, Liotta, Tryggvason et al. 1980). Later on, it has been shown that epithelial BM molecules synthesized by carcinoma cells, in particular Laminin 322/Laminin 5 or collagen VII that play central roles in epidermal-dermal adhesion, can act as potent driving forces to promote invasion and tumorigenesis for a review: (Marinkovich 2007).

Fibroblasts are also major modifiers of cancer progression (Elenbaas and Weinberg 2001, Tlsty and Hein 2001). Cancer cells are surrounded by fibroblasts at all stages of disease progression (Kalluri 2016). Fibroblasts in association with tumor cells acquire an activated phenotype called carcinoma associated fibroblasts (CAF). CAF fibroblasts express high amount of ECM molecules such as collagen type I and III, tenascin C, fibronectin. Furthermore, CAF express α-smooth-muscle actin. They also express metalloproteinase (MMPs) that degrade the ECM and facilitate the migration and invasion of cancer cells as well as growth factors transforming growth factor-β (TGFβ), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) that provide oncogenic signals for tumor progression. CAF also mediate an inflammatory response by secreting various chemokines. Their production of growth factors, chemokines and extracellular matrix degrading enzymes facilitates the angiogenic recruitment of endothelial cells (Kalluri and Zeisberg 2006).

Chapter five: DNA repair mechanisms and XP-C

The human body is the target of numerous DNA attacks every day, among which are the UV-induced radiations contained in the solar light. Some of these lesions (notably DNA adducts) are distorting and can block genome replication and transcription. Saturation of either DNA repair mechanisms, or their constitutive defects, may cause mutagenesis and may alter cell viability and cancer development (Jackson and Bartek 2009). A few hours exposure to sunlight can induce more than 10^6 photoproducts in exposed keratinocytes (Mouret, Baudouin et al. 2006). DNA repair mechanisms are highly conserved from bacteria to complex eukaryotes. Five repair mechanisms dedicated to the repair of specific DNA damages have been identified up to now (figure 15):

1) The nucleotide excision repair (NER) removes lesions distorting the DNA double helix.

2) The base excision repair (BER) eliminates the damaged nucleotides including oxidized bases, abasic sites and single strand breaks.

3) The mismatch repair mechanism (MMR) removes incorrect nucleotides incorporated by DNA polymerases.

4) Homologous Recombination (HR) repairs DNA double strand breaks. HR requires extensive homology for repair, and thus is primarily used in late S and G2 phases of the cell cycle. HR is a faithful mechanism.

5) The non-homologous end joining mechanism (NHEJ) is based on re-ligation of the two broken DNA ends, in some cases after DNA polymerization or shortening by exonuclease activity. NHEJ is not considered as a faithful mechanism. NHEJ is active throughout the entire cell cycle except in the S/G2 cell cycle phase (Sonoda, Hochegger et al. 2006).

Alterations in these repair mechanisms, notably the NER, have important consequences for the genome integrity and the cellular homeostasis. Germinal mutations in the genes implicated in DNA repair are associated with cancer prone syndromes, as in the case for the rare genetic disease xeroderma pigmentosum (XP).

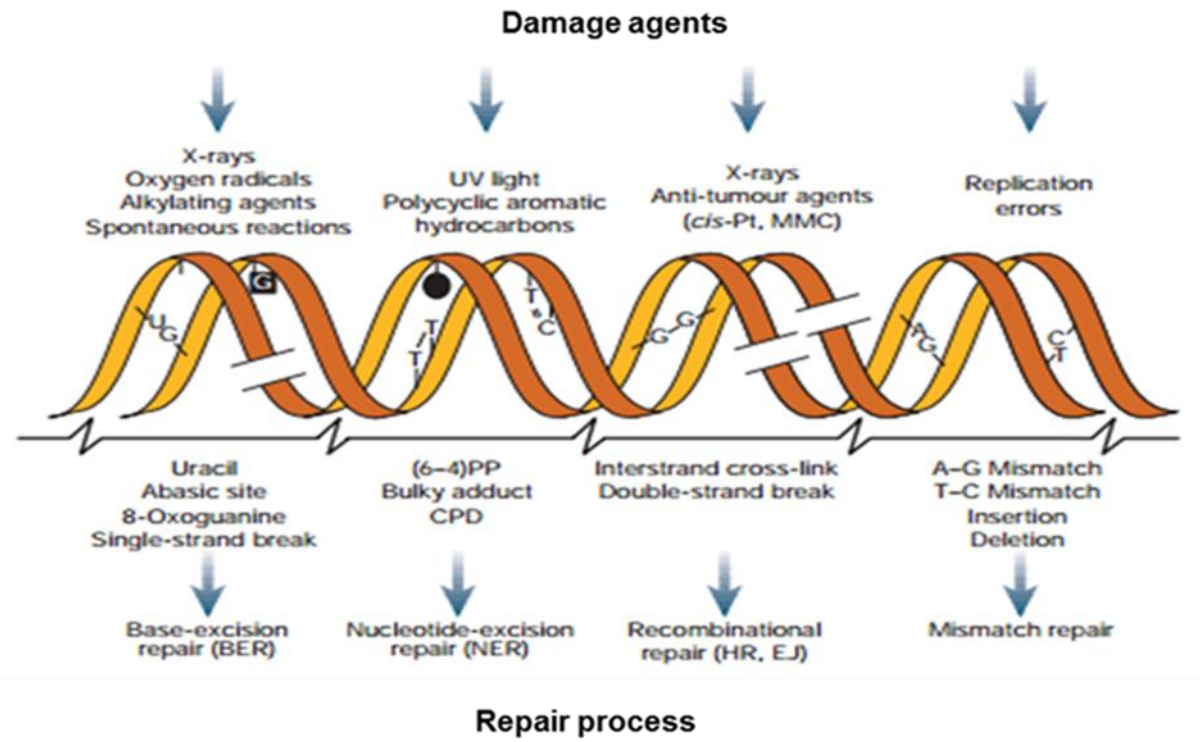

Figure 15: Genotoxic stress, DNA damages and repair mechanisms. Common DNA damaging agents as X-ray, oxygen radicals, alkylating agents, UV light, polycyclic aromatic hydrocarbons. These agents cause DNA damages as uracil abasic site, 8-Oxoguanine, single strand break; these damages are repaired by base excision repair (BER). Damages as pyrimidine dimers (CPD and 6-4PP) are repaired by nucleotide excision repair. The interstrand cross-link and the double strand break damages are repaired by Homologous Recombination repair (HR), non-homologous end joining repair (NHEJ). The insertion or deletion errors repair by mismatch repair (MMR) (Hoeijmakers 2001).

I. The nucleotide excision repair (NER)

The nucleotide excision repair **(NER)** is a versatile system that repairs a wide variety of lesions causing distortion of the structure of double helix of DNA. The NER removes damages including bulky adducts in the DNA due to exposure to chemical and environmental stresses (Wogan, Hecht et al. 2004, Hanahan and Weinberg 2011). In the skin, the main substrates for NER, and the most important from “a clinical point view” are pyrimidines dimers induced by UV (CPD and 6-4 PP).

The NER mechanism includes the coordinated interaction of about thirty proteins and is thought to take place in a sequential manner: **(1)** Recognition of the lesion; **(2)** Formation and stabilization of a multiprotein complex comprising the transcription factor TFIIH; **(3)** Unwinding of the DNA double helix around the lesion by specific helicases; **(4)** Excision of damaged DNA strand; **(5)** DNA replication from the uninjured strand; **(6)** Ligation of the new synthesized strand (de Laat, Jaspers et al. 1999) (figure 16).

In the 1980th, Hanawalt and colleagues observed that the transcriptionally active regions of the genome were repaired by the NER faster than the silent regions (Bohr, Smith et al. 1985, Mellon, Spivak et al. 1987). From this observation, the existence of two NER pathways have been demonstrated: Global Genome Repair (GGR), which removes the lesions present on the non-transcribed strand (Mellon, Spivak et al. 1987, Gillet and Scharer 2006, Hanawalt and Spivak 2008) and the Transcription Coupled Repair (TCR), which repairs lesions present on the transcribed strand of active genes.

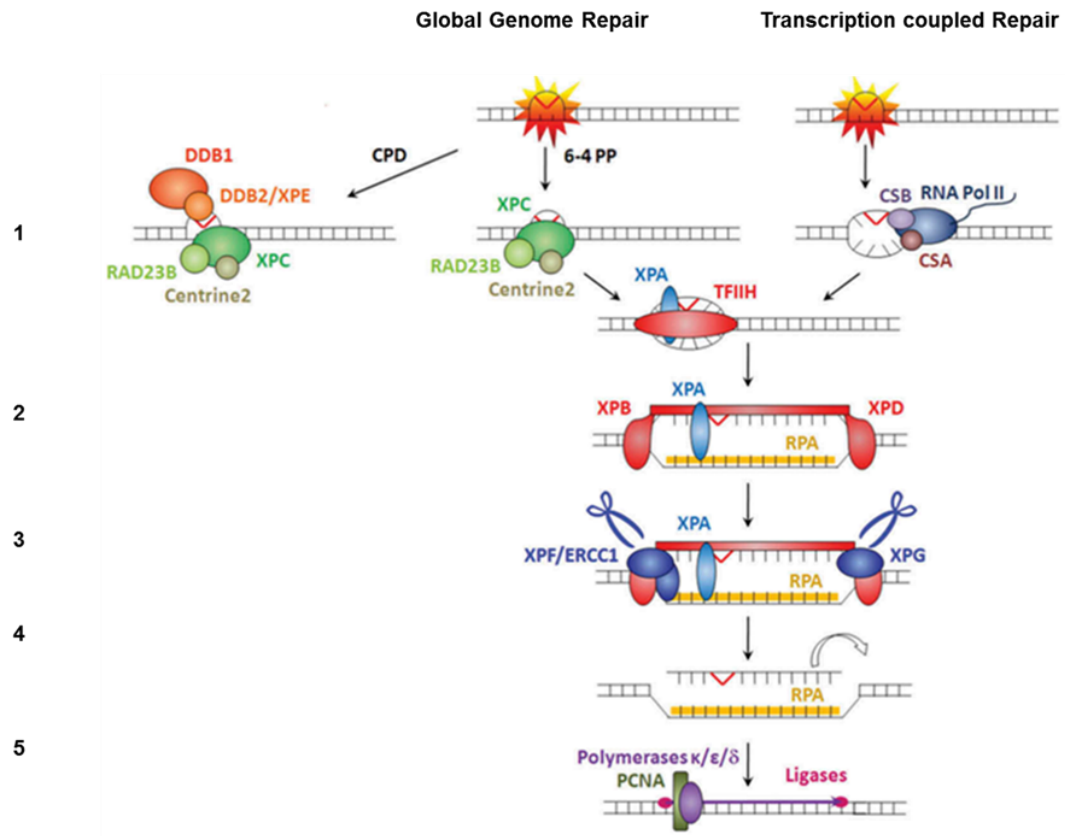

Figure 16: Scheme for the nucleotide excision repair (NER). Global genome repair (top left):**1)** Recognition of lesion: the XPC/RAD23B and/or DDB1/DDB2 (XPE) complexes recognize the distortion in DNA helix due to UVR. **2)** Opening the DNA: Recruitment of TFIIH transcription factor at lesion level and opening of the DNA by the XPB and XPD helicases, while RPA binds to the single strand to protect it. **3)** The 3' incision is made by XPG followed by the 5' incision by the ERCC1/XPF. **4)** Excision of a 28–30 mer containing the lesion. **5)** Filling the gap and the ligation are carried out by polymerases □□or δ in the presence of replicative factors and ligase I. **Transcription-coupled repair** (top right): recognition of a DNA lesion is supposed to be due to the blockage of RNA polymerase II at the site of the lesion distortion. CSB and, probably CSA, in conjunction with XPG, seem to be able to remove the stalled RNA pol II and to recruit the TFIIH factor. Then, the rest of this pathway is identical to the GGR steps. During transcription-coupled repair, XPC and XPE are not necessary for a full repair, and during GGR, CSB and CSA are not necessary for a full repair. Adaptedfrom http://www.tandfonline.com/doi/pdf/10.1080/21678707.2017.1256770?needAccess=true.

I.1. Recognition of the lesions

I.1.a. Global genome repair (GGR)

The xeroderma pigmentosum group C (XPC) protein plays a central role in recognizing UV-induced DNA distortions during the GGR mechanism (Batty, Rapic'-Otrin et al. 2000, Sugasawa 2016). XPC initiates NER by binding to the DNA damage sites, causing local modification of DNA conformation, and facilitating the recruitment of the other repair factors RAD23B/RAD23A and Centrin-2 (Masutani, Sugasawa et al. 1994, Araki, Masutani et al. 2001).

I.1.a.a) XPC role in recognizing DNA damages

XPC has a high affinity to damaged DNA (Batty, Rapic'-Otrin et al. 2000). Biochemical studies *in vitro* have shown that XPC has a higher affinity for 6-4 PP than CPD lesions. In a more general manner, the affinity of XPC for damaged DNA depends on the lesion-induced distortion (Kim, Patel et al. 1995).

In 2007, experiments of crystallization of Rad4 (the yeast orthologue of XPC) in presence of a DNA fragment containing a CPD lesion have revealed that Rad4 binds to the DNA strand opposite to the damaged DNA and not to the lesion itself (figure 17). Rad4 bears a transglutaminase domain (TGD) and 3 β-hairpin domains (BHD1-3). TGD and BDH1 form a C-shaped structure that interacts with an 11-bp segment on the 3’ side of the damage DNA, while BHD2 and BHD3 form van der Waals contacts with the DNA substrate near the damaged sequence. The insertion of BHD3 into the DNA leads to bend the DNA backbone (Min and Pavletich 2007). The binding of Rad4 at the vicinity of the damaged DNA strand induces change in DNA conformation in order to facilitate the repair (Janicijevic, Sugasawa et al. 2003). Destabilized DNA damaged zone is illustrated in (figure 18) (Mu, Geacintov et al. 2015).

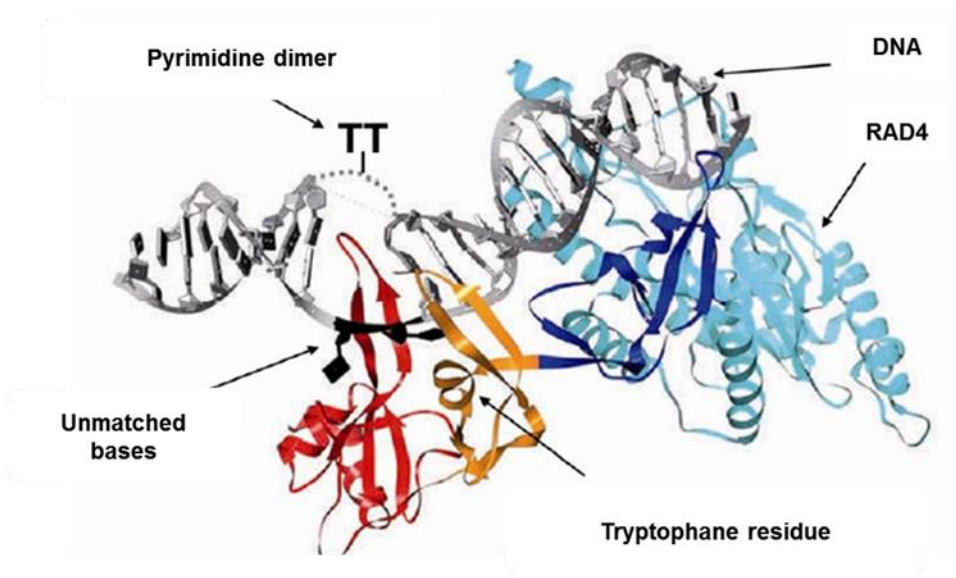

Figure 17: Crystallographic structure of the binding of Rad4 (XPC ortholog in the yeast) to the DNA. The Rad4 protein binds to the DNA in front of the lesion (TT) (in gray color), causing the expulsion of unmatched complementary residues (black) out of the double helix. The tryptophan residue gives the protein its affinity for damaged DNA regions. (Adapted from: (Min and Pavletich 2007).

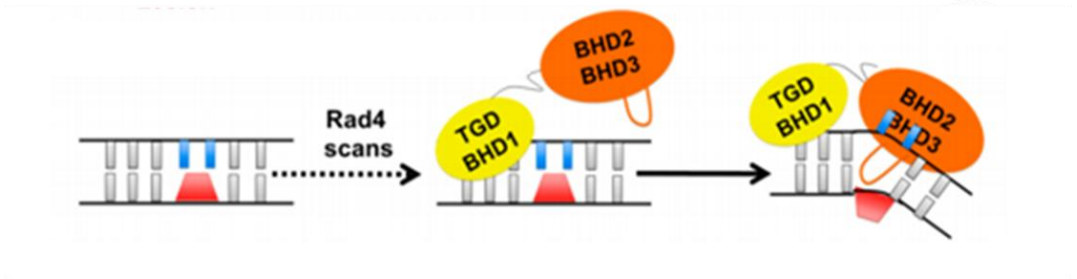

Figure 18: Hypothetical mechanism of lesion recognition by RAD4. Scanning by TGD and BHD1 is accompanied by BHD2 and BHD3 for a locally destabilized region. After region destabilization, BHD3 □-hairpin inserts, the lesion is evicted, and the partner bases flip out to associate with BHD2 and BHD3 domains; the order of these events may be lesion-dependent (Mu, Geacintov et al. 2015).

I.1.a.b) *RAD23A/B* and *CENT-2* regulate XPC activity

The yeast Rad23 protein and the mammalian orthologues RAD23A/RAD23B share N-terminal "ubiquitin-like" (UBL) sequences and two domains associated to ubiquitin activities (UBA, Ubiquitin-Associated domains). Therefore, members of this family have been implicated in the regulation of the proteins degradation in the GGR mechanism.

In vivo, the majority of XPC proteins are complexed with RAD23B (Araki et al. 2001). This could be due to the fact that RAD23B is about 10 times more abundant than RAD23A (van der Spek, Eker et al. 1996). However, the deletion of mHR23B in knockout mice results in developmental defects and intrauterine death of the mice, suggesting a role of RAD23B against XPC degradation (Ng, Vermeulen et al. 2003, Okuda, Nishi et al. 2004). Although the exact functions of RAD23A and RAD23B in NER have not been clearly elucidated yet, it has been suggested that the ubiquitin-like and ubiquitin-associated domains in RAD23 are required for proficient NER. Ubiquitination of XPC could enhance its binding to damaged DNA and subsequent repair (Watkins, Sung et al. 1993, Ng, Vermeulen et al. 2003, Okuda, Nishi et al. 2004, Sugasawa, Okuda et al. 2005, Sugasawa 2006). GGR alteration and survival rate after UV in mouse embryonic fibroblasts (MEF) deficient for both *mRad23A* and *mRad23B* are similar to those observed in XPC-deficient MEF (Ng, Vermeulen et al. 2003, Okuda, Nishi et al. 2004). These data suggest that RAD23 proteins protect XPC against its degradation by the proteasome and regulate quantitatively the activity of the GGR.

Centrin-2 is a calcium binding protein from the calmoduline family. Centrin-2 was initially identified as a component of the centrosome and has been shown to plays an essential role in the duplication of centrioles before mitosis (Nishi, Okuda et al. 2005, Nishi, Sakai et al. 2013). Besides this role, Centrin-2 has been shown to be able to interact with XPC in the cell nuclei (Araki, Masutani et al. 2001). However, *in vitro* studies showed that Centrin-2 is not essential for NER initiation (Araujo, Tirode et al. 2000, Sugasawa, Okamoto et al. 2001), but its interaction with XPC stimulates the binding of XPC-RAD23B complex to DNA bearing 6-4PP lesions and increases the activity of NER *in vitro* (Nishi, Okuda et al. 2005). Alone, Centrin-2 or RAD23B do not present affinity for DNA-bearing-lesions without XPC (figure 16).

I.1.a.c) Role of the DNA damage binding complex (UV-DDB) and ubiquitin ligases in GGR regulation

In vitro experiments showed that XPC has difficulties to recognize CPD lesions in non-transcribed sequences, while in mammalian cells CPD lesions located in the non-transcribed DNA strands are repaired by GGR whose initiation depends on XPC (Mitchell, Brash et al. 1990, Kusumoto, Masutani et al. 2001, Sugasawa, Okamoto et al. 2001). These data suggested that other factors are able to assist XPC in the recognition of CPD in the GGR mechanism.

The UV-Damaged DNA-Binding protein complex (UV-DDB) has been identified as a factor capable to recognize UV-induced photo lesions and to facilitate the recruitment of XPC (Araujo, Tirode et al. 2000, Moser, Volker et al. 2005, Sugasawa 2016). UV-DDB is composed of the heterodimer of DDB1 and DDB2/XPE. DDB2/XPE directly interacts with the lesions present on DNA and presents a particular affinity for CPD and 6-4PP lesions (Sugasawa, Okuda et al. 2005, Scrima, Konickova et al. 2008). It has been shown that mutations in *DDB2* gene in the UV-DDB deficient XP-E cells severely attenuated the repair of CPD lesions, while repair of 6-4PP was less affected. This result suggests that, in the absence of the UV-DDB complex, XPC can preferentially localize to 6-4PP and in a lesser extent to CPDs (Tang and Chu 2002, Wakasugi, Kawashima et al. 2002, Moser, Volker et al. 2005, Yasuda, Nishi et al. 2007). The UV-DDB complex is thus thought to help XPC to recognize CPD lesions. However, it is important to note that the UV-DDB complex cannot substitute for XPC functions. Indeed, XPC is absolutely required to the *in vitro* reconstitution of the GGR machinery, which is not the case for the UV-DDB complex (Araujo, Tirode et al. 2000).

XPC in NER is regulated through the UV-DDB complex by an ubiquitin-ligase complex composed of 2 subunits CuL4A and ROC1 (Shiyanov, Nag et al. 1999, Groisman, Polanowska et al. 2003). This complex associates with the COP9 (CNS) signalosome, a negative regulator of the ubiquitin-ligases activity of cullin type proteins. In the absence of UV, the complex CuL4A/DDB1-2/ROC1 is inactivated by its interaction with COP9. UV exposure leads to: 1) the localization of the CuL4A/DDB1/ROC1 complex at the DNA damages sites and the dissociation of COP9; 2) the activation of the ubiquitination complex. DDB2 polyubiquitination leads to reduce the affinity of the UV-DDB complex to the damage sites and its degradation while XPC

remains unaffected and thus thought to keep the handover of XPC to the lesion (figure 19) (Sugasawa, Okuda et al. 2005, El-Mahdy, Zhu et al. 2006).

I.1.b.The transcription coupled repair (TCR)

DNA damages can disrupt RNA transcription (Khobta and Epe 2012). Prolonged block of transcription can have severe consequences on the cells and may result in induction of apoptosis via the p53 protein. By stopping the progression of RNA polymerase II, the TCR ensures the rapid recovery of transcriptional activity and prevents the induction of apoptosis. Recognition of DNA lesions is directly related to the stalling of the RNA polymerase II (RNAPII) during the transcription process to signal at the transcription machinery to stop (Selby, Drapkin et al. 1997, Svejstrup 2002). The structural characteristics of the blocking lesions, and in particular the distortions of the double DNA helix play a minor role in triggering the TCR. Thus, the CPD and 6-4 PP, two blocking lesions for RNAPII, are recognized and repaired with the same efficiency by the TCR (Fousteri and Mullenders 2008). TCR removes DNA damage from the transcribed active strand. Hence, this pathway is different from GGR and proceeds on specific regions of the active genome (Mellon, Spivak et al. 1987, Tornaletti 2009). Many NER proteins are required in TCR except XPC. TCR needs the active CSA and CSB proteins, proteins that are defective in patients with Cockayne syndrome (CS) (van Gool, van der Horst et al. 1997).

During TCR, the elongating RNA polymerase II detects DNA damages and stalls at the lesions sites. Altogether, transcription complex along with CSA, CSB, XPA, and other proteins, stimulates DNA adducts removal (Saxowsky and Doetsch 2006, Fousteri and Mullenders 2008, Vermeulen and Fousteri 2013). The signal of the repair machinery is dependent on the CSA and CSB proteins. The roles of CSA and CSB are not fully understood.

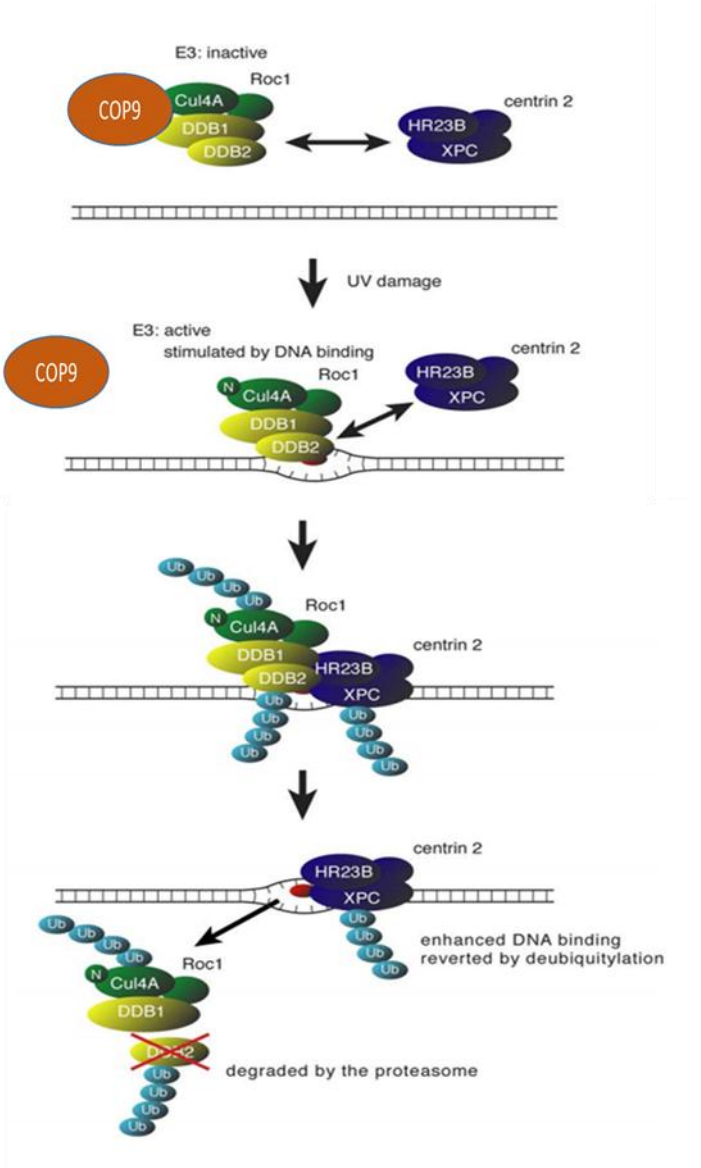

Figure 19 Model for UV-Induced UV-DDB Dependent Ubiquitylation of XPC. In un-irradiated cells, UV-DDB-associated E3 is inactivated by its interaction with the COP9 (CSN) signalosome to protect XPC from ubiquitylation. **After UV irradiation**, UV-DDB translocates onto the damaged site, particularly 6-4PP. UV-DDB dissociates from the COP9 signalosome and the neddylation of cullin 4A (indicated by "N") activates E3. Binding of UV-DDB to the lesion further stimulates E3 activity. The activated UV-DDB-E3 then recruits XPC via protein-protein interaction. XPC, DDB2, and cullin 4A are ubiquitylated at the lesion site. DNA binding activity of UV-DDB to the damaged loses by polyubiquitylated, whereas the DNA binding of XPC is potentiated by its ubiquitylation. The ubiquitylated DDB2 is degraded by the proteasome. The ubiquitylated form of XPC reverts to the unmodified form through deubiquitylation (adapted from (Sugasawa, Okuda et al. 2005)

CSB belongs to the SWI / SNF (Switch / Sucrose Non Fermentable) families. These proteins are involved in the ATP-dependent chromatin remodeling (Citterio, Rademakers et al. 1998, Newman, Bailey et al. 2006). CSB is required for the assembly of the repair complex proteins. CSB also facilitates the accessibility of the damaged DNA sequence by chromatin remodeling protein and recruitment of the histone acetyltransferase p300. CSA contains a WD40 domain, involved in protein-protein interactions. CSA interacts with an ubiquitin ligase activity complex composed among others by the DDB1, Cullin 4 and ROC1/RBX1 proteins (Groisman, Polanowska et al. 2003). UV irradiation provokes the association of the complex with the COP9 and leads to inhibition of the ubiquitin ligase activity. The role of this process in the regulation of the TCR remains to be determined.

I.1.b.a) Opening of DNA around the lesion in NER

The TCR and GGR sub-pathways assemble when the transcription factor TFIIH is recruited (figure 16). TFIIH is involved in genes transcription and NER by unwinding DNA around the lesion (Schaeffer, Roy et al. 1993, Singh, Compe et al. 2015, Compe and Egly 2016). The core of TFIIH consists of 7 subunits: two ATP-dependent helicases XPB/ERCC3 and XPD/ERCC2 together with p62, p52, p44, p34 and p8/TTD-A proteins coupled to the cdk-activating kinase (CAK) complex, which, by itself, consists of 3 subunits: Cdk7, cyclin H and MAT1. During transcription, the CAK complex regulates the phosphorylation of the carboxy-terminal end of the RNAPII (Feaver, Svejstrup et al. 1994, Roy, Adamczewski et al. 1994).

XPB/ERCC3 and XPD/ERCC2 have ATPases/helicase activity. Helicase activity of XPB and XPD unwind the DNA to create a bubble of about 30 nucleotides. XPB unwinds the DNA strand in 3’ to 5’ direction; XPD unwinds the DNA strand in the opposite direction (Schaeffer, Roy et al. 1993, Sung, Bailly et al. 1993).

I.1.b.b) Pre-incision complex assembly

Opening of the DNA double helix stimulates the pre-incision complex assembly (XPA, RPA and XPG). XPA is an essential element of this complex. It contains a "Zinc finger domain” allowing it to bind to DNA with particular affinity for damaged DNA (de Laat, Jaspers et al. 1999, Guthrie 2015). Replication protein A (RPA) is a heterotrimeric

single-stranded DNA-binding protein that is highly conserved in eukaryotes (Iftode, Daniely et al. 1999). RPA is thought to be important in the stabilization of XPA binding to DNA near the 5’ side of the bubble and also to ensure the total opening of dsDNA around lesion (He, Henricksen et al. 1995, Wang, Mahrenholz et al. 2000). RPA is considered as a scaffold protein in replication response to DNA damage. RPA ubiquitination protects it from the proteasomal degradation, and increases upon the replication fork collapse (Scharer 2013, Elia, Wang et al. 2015).

I.1.b.c) Excision of damaged DNA

XPG is a DNA endonuclease. It binds to TFIIH, which is thought to be the earliest process for recruiting its endonuclease activity (Araujo, Nigg et al. 2001, Ito, Kuraoka et al. 2007). Arrival of the XPG protein at the damaged DNA sequence causes the release of the XPC-RAD23B-Centrin-2 complex (Wakasugi and Sancar 1998, Riedl, Hanaoka et al. 2003). The endonuclease activity of XPG is activated when XPA recruits ERCC1-XPF to the 5’ end of the bubble (Fagbemi, Orelli et al. 2011). ERCC1-XPF cuts the DNA at a distance of 16 to 25 nucleotides in 5' of the lesion; and XPG cuts about 2-9 nucleotides in 3' of the lesion (Matsunaga, Mu et al. 1995, Moggs, Yarema et al. 1996, Staresincic, Fagbemi et al. 2009), leading to release of the single strand DNA of about 30 nucleotides which generates a gap in the DNA.

I.1.b.d) Replicative synthesis

After excision, the damaged DNA is segregated to avoid the excised strand to re-bind to the complementary strand (Sugasawa, Akagi et al. 2009). The free DNA fragment serves as a template to fill the gap. Proliferating cell nuclear antigen complex (PCNA) increases the efficacy of DNA polymerase synthesis (Shivji, Kenny et al. 1992), while all other factors of the NER leave the DNA during or after the step of incision. The RPA protein remains bound to the non-damaged strand and participates in the recruitment and setting up of the next proteins necessary for new DNA synthesis (Riedl, Hanaoka et al. 2003). The ligation step occurs after preincision by XRCC1 (X-ray repair cross-complementing protein1) which is considered as a scaffold protein to recruit, stabilize, or stimulate ligase III and other NER enzymes to provide an efficient DNA repair (Scharer 2013).

II. Other roles for XPC in the cell

II.1. XPC and BER

The capacity of XPC is not only restricted to the NER (Gourdin, van Cuijk et al. 2014). XPC also plays a role in base excision repair (BER) mechanism by participating in G/T mismatches. XPC stimulates thymine DNA glycosylase (TDG) to release of TDG from abasic sites and result from the excision of mismatched T bases. In vitro reconstitution experiments, show a new role of XPC in BER. XPC is found to stimulate the activity of OGG1 (a specific DNA glycosylase in BER). OGG1 is important for an efficient cleavage of 8-oxoG caused by the oxidative damage (Fortini, Pascucci et al. 2003, D'Errico, Parlanti et al. 2006). As well XPC has been described to contribute to the regulation of the transcription. XPC seems to play the role of platform for gene transcription in embryonic stem cells (Fong, Inouye et al. 2011).

XPC also seems involved in the MDM2 regulation of the p53 pathway (figure 20) (Krzeszinski, Choe et al. 2014). XPC is involved in MDM2 ubiquitination which mediates the p53 degradation pathway. Furthermore, some pathogenic mutations of XPC perturb MDM2-p53 binding and hence the regulation of the pathway. These observations suggest that XPC participates, together with MDM2, to proteasomal degradation of p53. Mysregulation of p53 stabilization may affect genome integrity, transcription, cell cycle arrest, and apoptosis (Wade, Wang et al. 2010, Brooks and Gu 2011).

Figure 20: A model for XPC mediated p53 degradation (Krzeszinski, Choe et al. 2014).

II.2. Post-translational regulation of XPC

Under physiological conditions, the endogenous XPC protein is quite stable in murine and human fibroblasts, with an estimated half-life between 6 and 9 h (Okuda, Nishi et al. 2004, Yasuda, Nishi et al. 2007). The *de novo* synthesis of XPC is maintained at a low level for limiting its over-expression which appears harmful to the cell (Ng, Vermeulen et al. 2003). RAD23A/B proteins are involved in the stabilization of XPC in part by protecting it from proteasomal degradation (Okuda, Nishi et al. 2004). In *S. cerevisiae*, the RAD4 protein (ortholog of XPC) is also stable under physiological conditions but it is rapidly degraded after UV irradiation (Gillet and Scharer 2006, Gillette, Yu et al. 2006). Similarly, XPC is rapidly degraded by the proteasome in the OSU-2 human fibroblasts cell line after UV irradiation (Wang, Praetorius-Ibba et al. 2007). This phenomenon is accompanied by post-translational modifications of XPC through both ubiquitination and sumoylation (Sugasawa, Okuda et al. 2005, Wang, Zhu et al. 2005). Interestingly, levels of XPC in XP-A cells decreased significantly after UV irradiation. These results suggest that XPA is required for UV-induced modifications of XPC. Transfected XPA cDNA into this XP-A cells restored the expression of XPA and UV-induced XPC modifications to protect it from the degradation. The poly-ubiquitination of XPC is under control of UV-DDB-Cullin4A complex; XPC ubiquitination is reversible. The ubiquitination of XPC increases its affinity for damaged DNA and prevents its degradation by the proteasome pathway (Sugasawa, Okuda et al. 2005). Similarly, it seems that sumoylation of XPC protects it against degradation (Wang, Zhu et al. 2005, Wang, Praetorius-Ibba et al. 2007). After the recognition step, XPC is degraded by the proteasome to allow XPG binding on the site of NER (Riedl, Hanaoka et al. 2003, Wang, Praetorius-Ibba et al. 2007).

Finally, it has been reported that a small proportion of XPC molecules may shuttle between the cytoplasm and the nucleus thanks to nuclear export signal (NES) and nuclear location signal (NLS) sheltered in its sequence (Hoogstraten, Bergink et al. 2008). In the presence of DNA damage, XPC accumulates quickly in the nucleus for several hours to ensure the recognition and repair of lesions such as CPD. Posttranslational modifications of XPC could participate to its nuclear retention (Solimando, Luijsterburg et al. 2009).

III. NER and cell cycle

The ATM (ataxia-telangiectasia mutated) and ATR (ATM- and Rad3-Related) kinases are considered as the central regulators for DNA damage response (DDR) signaling pathways. Both kinases are activated after DNA damage and DNA replication stress, but their specificities and functions are not the same. ATM is activated by double-stranded DNA breaks (DSBs); ATR is activated in the presence of DSBs and a variety of DNA lesions that interfere with replication (figure 21) (Cuadrado, Martinez-Pastor et al. 2006, Marechal and Zou 2013). DDR pathways look like classic signal transduction pathways, composed of sensors, transducers, and effectors. DDR signaling pathways drives the maintenance of genomic stability of organisms (Zhou and Elledge 2000).

The effectors of the DDR signaling pathway are involved in a wide spectrum of processes important in genomic stability, such as the replication and repair of DNA, and cell-cycle control. ATM and ATR have been shown to activate Chk1, Chk2, and MK2 protein kinases (Matsuoka, Huang et al. 1998, Liu, Guntuku et al. 2000, Reinhardt, Aslanian et al. 2007).

Some NER proteins are thought to play a role in the activation of control point through their direct interactions with cell cycle regulatory proteins, notably the cullin ubiquitin ligase 4 (Liu, Lee et al. 2009) and the DDB2 / XPE complex (Stoyanova, Yoon et al. 2008, Stoyanova, Roy et al. 2009); both have been implicated in the control of expression of p21, regulators of cell cycle progression in the G1/S progression (Harper, Adami et al. 1993). The intervention of PCNA in the final step of the NER also plays a major role in controlling cell cycle due to a direct interaction between PCNA and p21 (Waga, Hannon et al. 1994). The activation of ATM after detection of DSBs leads to Brca1, Chk2, and p53 phosphorylation. Brca1 is a tumor suppressor gene whose

product is involved in the maintenance genomic stability, including DNA damage-induced cell cycle checkpoint activation, DNA damage repair, protein ubiquitination, chromatin remodeling, cell-cycle arrest, apoptosis, and other downstream processes which mediates the effects of ATM on DNA repair (figure 22) (Shiloh 2003, Lavin 2008, Maréchal and Zou 2013).

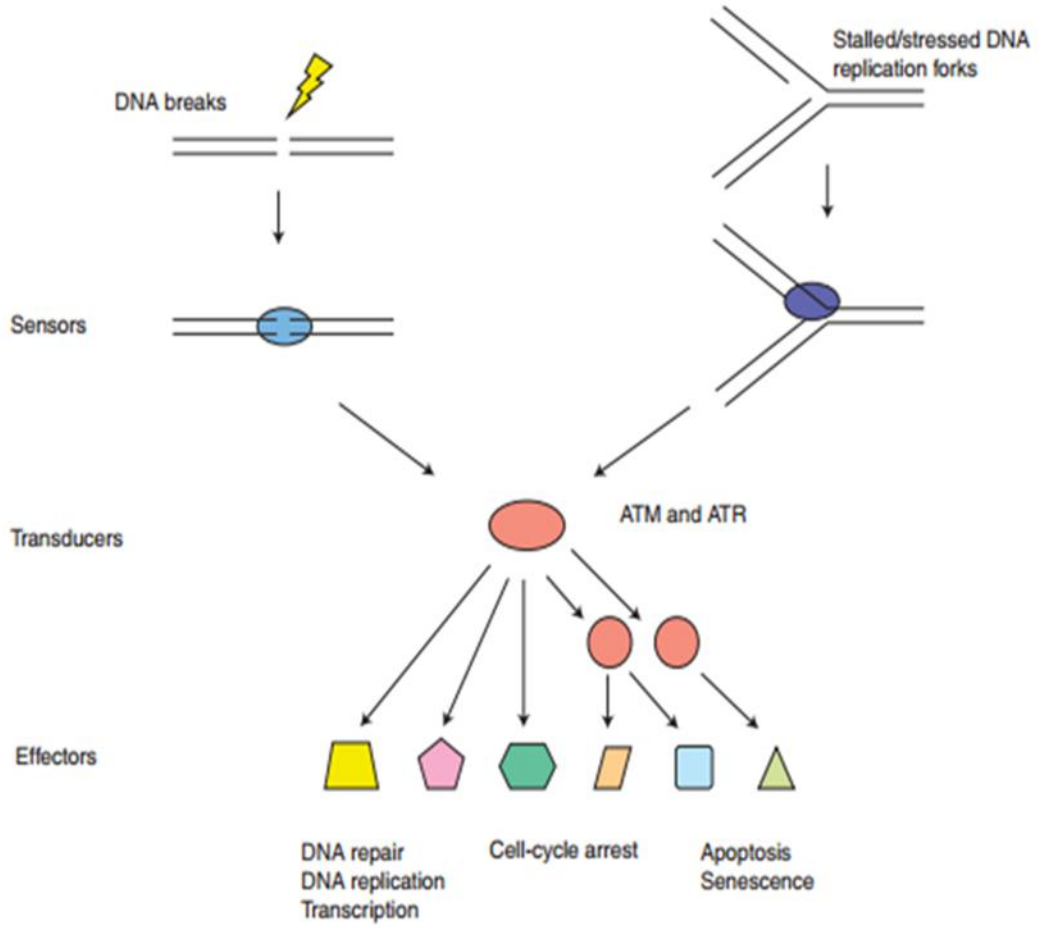

Figure 21: DNA damage response signaling pathway (DDR). The DDR signaling pathway consists of signal sensors, transducers, and effectors. **The sensors** of this pathway are proteins that recognize DNA structures induced by DNA damage and DNA replication stress. **The transducers** of this pathway are kinases, including ATM, ATR, and their downstream kinases. **The effectors** of this pathway are substrates of ATM, ATR, and their downstream kinases. These effectors are involved in a broad spectrum of cellular processes that are important for maintenance of genomic stability of organisms (Maréchal and Zou 2013).

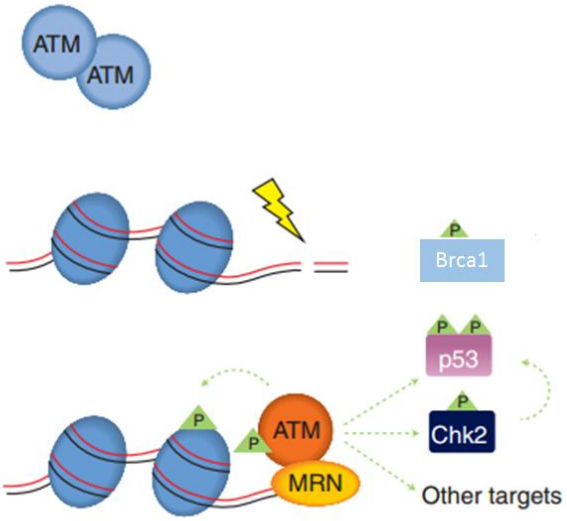

Figure 22 Activation of ATM by DSBs. Recognition of the end of the double strand break (DSB) and chromatin by ATM. The MRN (complex compose of Mre11-Rad50-Nbs1 protein complex functions as a sensor in the initiation of DSB responses through activation of (ATM) (Helmink, Bredemeyer et al. 2009). The activated ATM phosphorylates the substrates such as Chk2 and p53, and the Brca1 (histones in flanking nucleosomes) (Maréchal and Zou 2013).

Chapter six: Syndromes associated with NER defects

I. Xeroderma pigmentosum

Xeroderma pigmentosum (XP) is a genetic disease characterized by hypersensitivity to ultraviolet radiations and hence a high propensity to skin cancer on body area exposed to sun light. This syndrome is due to germinal mutations on either one of the nucleotide excision repair genes. There is no cure for these patients except a full protection from all types of UV radiations (Dupuy and Sarasin 2015).

Xeroderma pigmentosum (XP) was described for the first time by the Hungarian dermatologist Moriz Kaposi in 1870 (Kraemer, Lee et al. 1987, DiGiovanna and Kraemer 2012). This disease was initially called "Xeroderma" (in greek xero: dry and derma: skin). The term "pigmentosum" was added in 1882 in order to highlight the pigmentary anomalies characteristic that make of clinical traits of XP patients. Later on, the German physician Neisser, and then the Italian DeSanctis and Cacchione described the severe neurological abnormalities that may be associated to clinical traits of some (XP-A), but not all XP patients.

Hypersensitivity of XP patients toward UV irradiation has been described as early as in 1964, but the seminal evidence that DNA repair defect by the NER is the cause of this hypersensitivity was made by James Cleaver in 1968 (Cleaver 1968). Cleaver also showed that patient’s cells without neurological disorders have a higher repair capacity than patients with neurological complications. This led Cleaver to hypothesize the presence of a genetic heterogeneity at the origin of the different clinical form. Subsequently, the discovery of a form of a “Xerodermoïde pathology " with normal excision repair capacity but faulty replicative synthesis across DNA lesions allowed the definition by Allan Lehmann of a group called "XP variant" (XP-V) (Lehmann, Kirk-Bell et al. 1975).

The XP disease is composed of seven groups of genetic complementation: XP-A, -B, -C, -D, -E, -F and -G. Corresponding genes, *XPA* to *XPG*, encode proteins involved in NER (table 2). The group XP variant (XP-V) has some clinical symptoms consistent with conventional XP groups, but is not present NER disease as it is associated with mutations encoding for the translational η polymerase (Masutani, Kusumoto et al. 1999).

OMIM	Disease	Inher - tance	Tumor type	Locus	Gene	Protein	Function
	XP	AR	BCC SCC MM				
278700	Complementation group A			9q22.3	*XPA*	XPA	Damaged DNA binding interaction with TFIIH and XPF/XPG endonucleases
133510	Complementation group B			2q21	*XPB/ERCC3*	XPB	3' -> 5' helicase in TFHIII
278720	Complementation group C			3p25.1	*XPC*	XPC	Damaged DNA binding only involved in GGR heterodimer with HHR23B
126340	Complementation group D			19q13.2 -13.3	*XPD/ERCC2*	XPD	5' -> 3' helicase in TFHII
600045	Complementation group E			11q12-13	*DDB1*	XPE P127	Damaged DNA binding only involved in GGR heterodimer with DDB2
600811	Complementation group E			11p11-12	*DDB2*	XPE P48	Damaged DNA binding only involved in GGR heterodimer with DDB1
278760	Complementation group F			16p13.3 -13.13	*XPF/ERCC4*	XPF	5' stucture-specific endonuclease heterodimer with ERCC1
133530	Complementation group G			13q32-33	*XPG/ERCC5*	XPG	3' structure-specific endonuclease stabilization of the open complex
603968	XP variant			6p21.1	*POLh*	POL h	Translesion DNA polymerase

OMIM = Online Mendelian Inheritance in Man; AR = Autosomal Recessive; BCC = Basal Cell Circinoma; SCC = Squamous Cell Circinoma; MM = Malignant Melanoma

Table 2: XP genes, products and their characters (Magnaldo and Sarasin 2004)

II. XP heterogeneity

Although the genes associated with the different complementation groups all encode for proteins involved in the NER, there is a clear clinical heterogeneity features between them (Kondo, Fukuro et al. 1992). Studying the contribution of the different proteins in the NER and also the studies carried on the patients' cells explain the different heterogeneity between the groups (Fassihi, Sethi et al. 2016).

II.1. "Classical" XP groups (XP-A to XP-G)

XP is a rare disease with autosomal and recessive inheritance. Heterozygous parents do not show clinical symptoms. XP incidence in Western Europe is estimated by 2.3 per million livebirths (Kleijer, Laugel et al. 2008), but can reach 1 per to 20,000-100,000 in Japan and 1 per 10,000-30,000 in North Africa. Thus, while the XP-A group accounts for about 16 % Western European XP patients, its frequency in Japan reaches 60 %. Indeed a founder mutation in the *XPA* gene is present in about 1 % Japanese individuals (Hirai, Kodama et al. 2006). In Western Europe, 60 % XP patients belong to the XP-C group (Kleijer, Laugel et al. 2008), other groups are less common.

II.1.a. Life expectancy and tumor development in XP patients

In a study by Kraemer in 1987, only 5 % (among 830) patients were older than 45 years and were presumably XP-V patients. Overall, the life expectancy of XP patients is reduced by about 20 to 30 years compared to the normal population. The mean age of skin cancer onset is 8 years in XP patients and 58 years in the general population (figure 23). The exact cause of death in XP patients is not always determined, but would be due to skin cancers development (Kraemer, Lee et al. 1987). Life expectancy of XP patients depends not only on the severity of symptoms and the complementation group, but also on the patient "lifestyle". A strict protection against UV may improve life's expectancy by limiting onset of sun induced cancers.

The appearance of severe cutaneous symptoms such as freckles (even at birth), photosensitivity and skin dryness are among the first clinical traits of XP patients supporting the hypothesis of the pathology. The median age of onset of first skin abnormalities is between 1 and 2 years. Skin lesions are mostly limited to skin areas exposed to sunlight, the head and the face being mainly affected. XP patients are photosensitive and present in the first months of their lives abnormal reactions to

sunlight exposure. The first symptom observed is usually an intense erythema after minimal exposure to sun. The minimal erythema dose (MED) is different among the complementation groups and would be significantly lower in some of them such as the XP-A group (Kondo, Fukuro et al. 1992). Repeated exposures of XP patients to sunlight mainly induce the formation of AKs the precursor lesions of SCC.

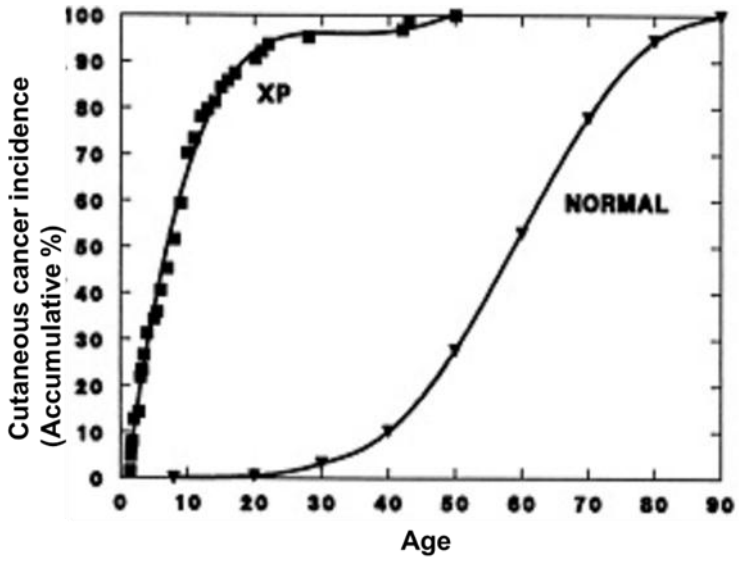

Figure 23: skin cancer incidence as a function of age. The mean age of cancer incidence in 830 XP patients versus in the general population (Normal) is of 8 and 58 years, respectively (according to: (Kraemer, Lee et al. 1987).

XP patients are susceptible to cutaneous cancers SCC, BCC and melanoma (Spatz, Giglia-Mari et al. 2001, Stary and Sarasin 2002), (figure 24). The incidence of SCC and BCC is 4800 times higher in XP patients than in the general population. Beyond the “global” increase of skin cancer incidence, the typology of those cancers is also changed. Increase of skin cancer susceptibility in XP patients is not colinear compared to the general population. Indeed, in the general population the frequency of BCC is about 4 times higher than that of SCC (Alam and Ratner 2001) while this ratio is globally inversed in XP patients with a ratio of about 5 SCCs for 1 BCC. MM develop on average 10 years later than SCC and BCC. The frequency of MM in individuals younger than 20 is about 2000 times higher in XP patients than in the general population. 65 % MM develop on the head, face and neck versus 20 % in the general population. This highlight the major role of UV in the etiology of MM in XP patients. Moreover, about 1.5 to 4 % XP patients develop internal tumors (Kraemer, Lee et al. 1987, Kondo, Fukuro et al. 1992). XP patients under the age of 20 have 10 to 20 times increased risk to develop internal tumors compared to the general population. Those later cancer are often localized in the brain (gliomas) but can also affect the lung, thyroid or digestive system (Giglia, Dumaz et al. 1998).

II.1.b. Ocular damage and neurological disorders

Most XP patients also suffer from ocular lesions affecting the eyelids, the cornea and the conjunctiva, that are among the first targets of UV radiations. Photophobia is one of the first symptom observed in 20% of XP children. XP patients' eyes are often irritated, red and tearful. XP patients can develop ocular neoplasms, mainly SCC and BCC that may appear on the eyelids, the cornea and the conjunctiva. Some cases of ocular melanomas have also been reported. More of half of these tumors develop before the age of 11 and their frequency is 2000 times higher than in the general population.

According to their group of genetic complementation, some XP patients may also suffer from progressive neurodegeneration (notably the XP-A, XP-B, XP-D and XP-G groups (Robbins, Brumback et al. 1991). They express themselves by a decreased of the intellectual capacity, activity and reflexes.

II.2. “Pure” XP groups of genetic complementation

A study in UK from 89 XP patients which included most groups of XP genetic complementation groups recapitulates the clinical heterogeneity of the disease (Fassihi, Sethi et al. 2016). Patients with mutations in DNA damaged recognition proteins DDB2/XPE and XPC, that are both involved in global genome repair (GGR), exhibit almost exclusively skin cancer without neurological disease (Kraemer, Lee et al. 1994, Cleaver, Lam et al. 2009, Bradford, Goldstein et al. 2011). The **XP-E** group, associated with skin disorders, is the less severe form of XP. Mutations in the *DDB2 / XPE* gene lead to reduced levels of NER with approximately 50 % residual activity.

The **XP-A** group of genetic complementation is one of the most severe form of the disease; XP-A patients suffer from extreme susceptibility to cutaneous cancer, severe mental retardation and progressive neurodegeneration. The loss of function of XPA protein abolishes the activity of both GGR and TCR. XP-A cells therefore have extreme sensitivity to UV and very low rates of repair by the NER. The neurodegeneration observed in XP-A patients reflects the importance of TCR in neuron cells.

Surprisingly, although **XPF** is essential for both GGR and TCR, patients from the XP-F group of genetic complementation exhibit average photosensitivity, later onset of skin cancers and rare neurological disorders. XP-F fibroblasts retain very low levels of TCR and GGR (10 %) although patients present with an attenuated phenotype (Matsumura, Nishigori et al. 1998). Presumably, mutations that abolish the function of the XPF protein are lethal, as in the case of mutations affecting the *ERCC1* gene (Tian, Shinkura et al. 2004).

XP-C group is the most common in Western Europe, where it accounts for about 60 % XP patients (Kleijer, Laugel et al. 2008). XP-C patients have a very high photosensitivity to UV and develop numerous skin cancers. XP-C patients develops very rarely neurological disorders. The life expectancy of XP-C patients can be improved according the quality of sun protection and dermatological observation (Slor, Batko et al. 2000). XP-C cells show significant alteration of NER activity with about 10-15 % of total residual activity. This decrease essentially affects the GGR while the TCR is fully functional (Venema, van Hoffen et al. 1990). The fact that TCR is unaffected in XP-C cells may explain the absence of neurological disorders in XP-C patients.

II.3. XP- variant

XP-V patients accounts for about 20 % XP patients, due to defects in the DNA polymerase Polη encoded by the *XPV* gene. Cells from XP-V patients exhibit normal NER but are defective in DNA translesion synthesis after UVR (Lehmann, Kirk-Bell et al. 1975, Johnson, Kondratick et al. 1999, Masutani, Kusumoto et al. 1999). Probably the best interpretation of cancer incidence is life-cumulated UV exposure, reinforcing the necessity to protect XP-Vs from sun exposure (Opletalova, Bourillon et al. 2014).

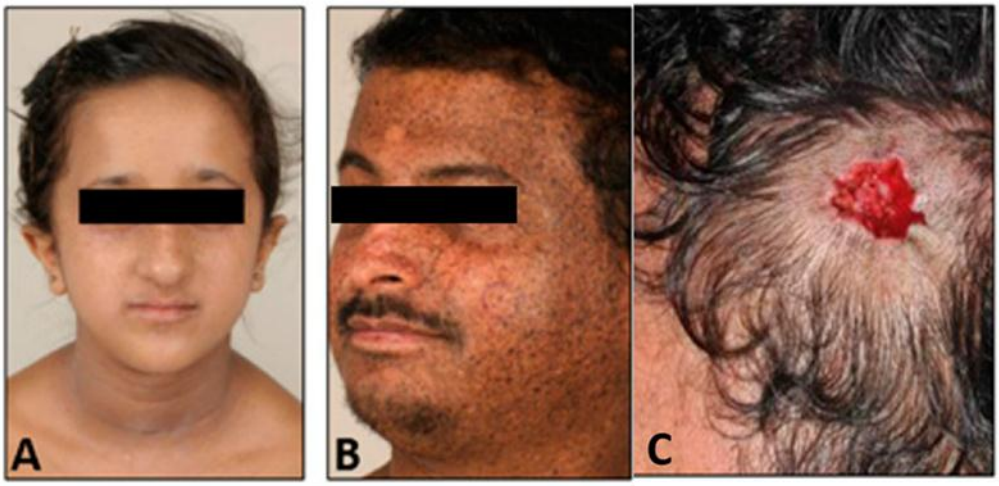

Figure 24 XP-C patients. (A) lentigines on face in patients aged of 12 y; **(B)** extensive pigmentary changes, patients aged of 38 y; **(C)** Site of excised melanoma in the temporal region of the head of a XP-C patient (Fassihi, Sethi et al. 2016).

III. Other syndromes associated with NER defects

Cockayne syndrome (CS) is a rare syndrome resulting from mutations in either *CSA* or *CSB* genes. Most CS patients are photosensitive and develop erythema and severe cutaneous inflammation after UVR. Non-photosensitive CS patients have also been reported (Colella, Nardo et al. 1999). They have a defect in TC-NER and develop neurological anomalies. CS patients have never been reported to develop cutaneous cancer (Nance and Berry 1992, Cleaver, Lam et al. 2009, Brennan-Minnella, Arron et al. 2016). Even if CS patients develop cancer, they are still undetectable due to the short life-span of these patients (12-13 years) compared to XP patients (Brennan-Minnella, Arron et al. 2016).

Another syndrome is called trichothiodystrophy (TTD). TTD cells bear mutations in either *ERCC3 (XPB), ERCC2* (*XPD*), or (P8 or TTD-A) genes coding for proteins playing important roles in both transcription and DNA-repair through the activity of the transcription factor TFIIH; TTD cells are deficient in GGR and TCR (Singh, Compe et al. 2015). Patients present a cutaneous and ocular photosensitivity. As in the case of CS, TTD patients are not predisposed to the development of skin cancers.

Figure 25 illustrates the different genes whose mutations are responsible for the different NER syndromes. XPA, XPC, XPE and XPF are only common between XP; CSA and CSB are only found in CS; XPG is found in pure XP or in a combined XP/CS syndrome; XPB and XPD are common between TTD, XP, or in a combined XP/CS syndrome. TTD-A involve only in TTD.

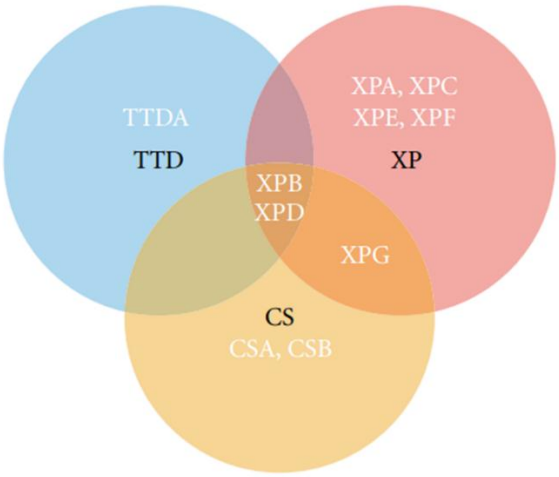

Figure 25: Mutations in the genes involved in NER and the associated syndrome. Mutation in XPA-XPG result in xeroderma pigmentosum **(XP)** syndrome. Mutations in TTDA result in trichothyodystrophy **(TTD)** syndrome. Mutations in CSA and CSB cause the Cockayne syndrome **(CS)**. XPB and XPD are common between all of these syndromes, while XPG is common between XP and CS (Le May, Egly et al. 2010).

Chapter seven: Signaling pathways

I. UV and intracellular transduction signal pathways

Exposure mammalian cells to UV radiation induces agents in the cells as a response to DNA damages, and thus lead to activate many signaling pathways after some minutes of exposure. Devary and colleagues reported that the earliest response to UV-induce damages starts near or at the level of plasma membrane. The response is conveyed by the activation of the Src tyrosine kinase, which in turn activates H-Ras and Raf-1. UV-damage response leads to transcription of genes encoding for transcription factors and proteins involved in the regulation of the major cellular functions as proliferation, metabolism, apoptosis, and repair (Devary, Gottlieb et al. 1992, Devary, Rosette et al. 1993).

II. Receptor tyrosine kinases (RTKs)

The first receptor tyrosine kinases (RTKs) were discovered more than a quarter of a century ago. Many members of this family of cell surface receptors are key regulators for important cellular processes, such as proliferation, differentiation, cell survival, metabolism, cell migration, and cell-cycle control (Ullrich and Schlessinger 1990, Blume-Jensen and Hunter 2001).

There are 58 known human RTKs that fall into 20 subfamilies (figure 26). The molecular architecture of all RTKs is composed of an extracellular region bearing a ligand-binding domain, a single transmembrane helix, and a cytoplasmic region containing a protein tyrosine kinase (TK) domain and carboxy (C-) terminal and juxtamembrane regulatory regions (Lemmon and Schlessinger 2010). Mutations in RTKs or abnormal activation are related with signaling pathways linked to cancers, diabetes, inflammation, bone disorder and angiogenesis.

UV radiations have been shown to result in conformational changes in RTK receptors, membranes distribution, and hence activation to stress or growth factors signaling pathways (Zanke, Boudreau et al. 1996). Production of ROS in cells after UV radiations is also thought to play important roles in the regulation of downstream pathways such as EGFR induction (Paus and Cotsarelis 1999).

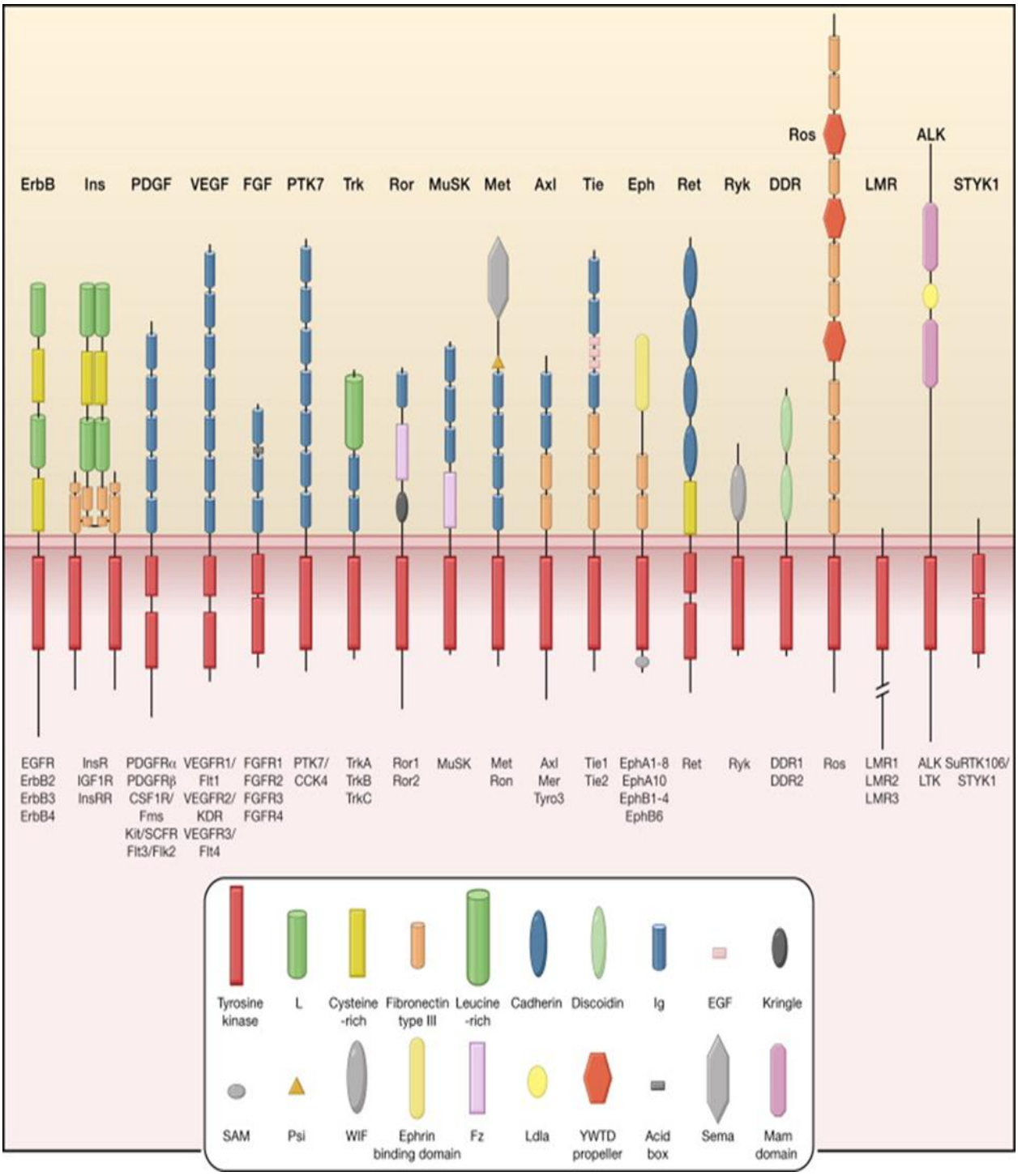

Figure 26 Human receptor Tyrosine Kinase Families. The family of human receptor tyrosine kinases (RTKs) include 20 subfamilies as indicated. Structural domains in the extracellular regions, identified by structure determination or sequence analysis, are marked according to the key. The intracellular domains are shown as red rectangles (Lemmon and Schlessinger 2010).

III. Activation of RTKs

In a general manner, ligands binding activates RTKs by inducing receptor dimerization (Ullrich and Schlessinger 1990). It is important to note that some RTKs can be found as dimers even in the absence of their ligand. For example, the insulin growth factor receptor (IGFR) is present at the plasma membrane as a dimer (Ward, Lawrence et al. 2007). Binding of IGF to its receptor promotes structural conformation changes in the receptor which activates the tyrosine kinase domain. Some studies suggest that EGF bind to pre-existing oligomer of its receptor (Clayton, Walker et al. 2005). Whether monomers or dimers, RTKs require binding of their ligand to be stabilized and activated. Once RTKs are activated, they can phosphorylate tyrosine residues of the intracellular domain, allowing activation of downstream signaling cascades in the cell (figure 27).

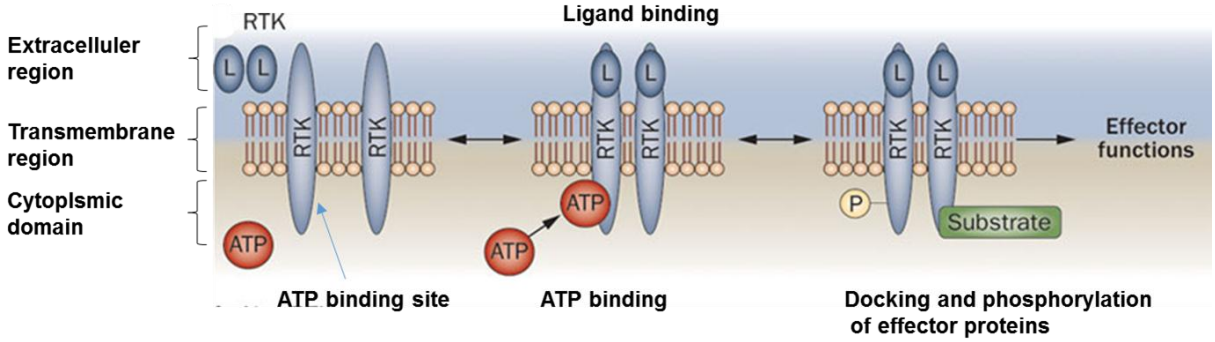

Figure 27 Activation of tyrosine kinase receptor. Ligand binds to extracellular domain of RTK and lead to receptor activation, dimerization and binding of adenosine triphosphate. Phosphorylated tyrosine on the receptor serve as a docking site for effector proteins. Adapted from: (D'Aura Swanson, Paniagua et al. 2009).

IV. Mitogen-activated protein kinases (MAPK) signaling pathways

MAPK signal transduction pathways are among the most widespread mechanisms of eukaryotic cell regulation (Krishna and Narang 2008). MAPKs are Ser/Thr kinases, in mammals, there are more than a dozen MAPK enzymes (Cargnello and Roux 2011), and they convert extracellular stimuli into a wide range of cellular responses as cell growth, differentiation, cell survival, neuronal function and immune response. MAPK pathways fall into three families: 1) The extracellular signal regulated kinases **(ERK1/2)**; 2) The c-Jun N-terminal kinase **(JNK1/2/3)**; 3) The **p38** MAPK (p38 α, β, γ and δ) (Krishna and Narang 2008). Conventional MAPK pathways are shown in figure 28.

Upstream molecules implicated in the cellular MAPK pathways are the Ras and Rho family of GTPases, located near the plasma membranes (Wagner and Nebreda 2009, Kamiyama, Naguro et al. 2015) that drives the switch between the signaling receptors and the downstream pathways. Downstream, MAPK target proteins throughout the cell, from the cytoplasm to the nucleus, regulating, *via* there kinase activity, genes expression, in particular through the regulation of the transcription of downstream effectors. The latter function by modulating "DNA binding, protein stability, cellular localization, and protein-protein interactions, as well as regulating other post-translational modifications" (Yang, Sharrocks et al. 2003). Deregulation of MAPK signaling is implicated in cancer and in many diseases (Wagner and Nebreda 2009).

MAPK pathway components can also function non-enzymatically. It has been found that ERK1 and ERK2 have another role beyond their canonical kinases activity. For example, ERK2 can activate by a direct interaction numbers of proteins such as topoisomerase II and MAPK phosphatase 3 (MKP-3); this does not involve any phosphotransfer activity. Furthermore, ERK2 can acts as a transcriptional regulator by binding to DNA. ERK1 and ERK2 can also regulate cell cycle independently of their kinase functions (Rodriguez and Crespo 2011).

MAPK components associate with many regulatory proteins including protein phosphatases and scaffold proteins. Protein phosphatases remove phosphate group from tyrosine kinase to form feedback loop, while the scaffold proteins interact signals from other pathway to regulate MAPK activity (Morrison and Davis 2003, Owens and Keyse 2007). Table 3, shows the different genes encoded for MAPK proteins (Owens and Keyse 2007).

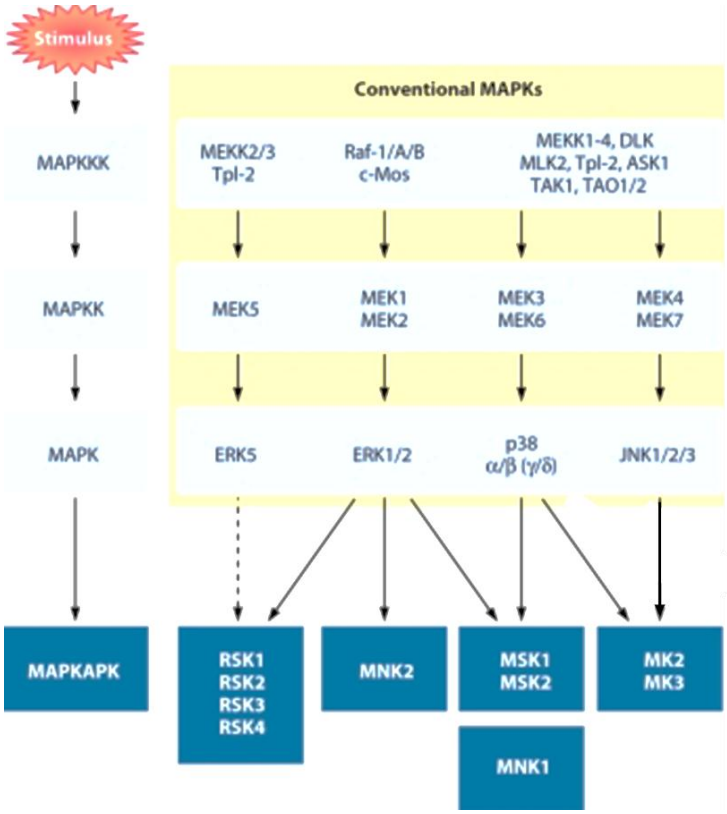

Figure 28: MAPK signaling cascades. MAPK activation eventually leads to activation of the MAPKAPKs. Mitogens, cytokines, and cellular stresses promote the activation of different MAPK pathways, which in turn phosphorylate and activate RSK, MSK, MNK, MK2/3. Dotted lines indicate that, although reported, substrate regulation by the respective kinase remains to be thoroughly demonstrated. The γ and δ isoforms of p38 are in parentheses to indicate that they have not been shown to promote MAPKAPK activation. Adapted from: (Cargnello and Roux 2011).

Gene	MKP	Trivial names	Chromosomal localization	Subcellular localization	Substrate preference	Physiological functions
DUSP1	MKP-1	CL100,erp,3CH134, hVH1	5q34	Nuclear	JNK, p38, ERK	Negative regulator of immune function. Protects mice from lethal endotoxic shock. Plays a key role in metabolic homeostasis and mediates resistance to cellular stress in mouse fibroblasts.
DUSP4	MKP-2	Typ1, Sty8, hVH2	8p12-p11	Nuclear	JNK, p38, ERK	
DUSP2		PAC-1	2q11	Nuclear	ERK, p38	Positive regulator of inflammatory response. Knockout mice are resistant to immune inflammation.
DUSP5		hVH-3,B23	10q25	Nuclear	ERK	
DUSP6	MKP-3	Pyst1, rVH6	12q22-q23	Cytoplasmic	ERK	Negative feedback regulator of ERK2 downstream of FGFR signalling.
DUSP7	MKP-X	Pyst2, B59	3p21	Cytoplasmic	ERK	
DUSP9	MKP-4	Pyst3	Xq28	Cytoplasmic	ERK > p38	Essential for placental development and function (labyrinth formation)
DUSP8		M3/6, hVH5, HB5	11p15.5	Cytoplasmic/nuclear	JNK, p38	
DUSP10	MKP-5		1q41	Cytoplasmic/nuclear	JNK, p38	Functions in innate and adaptive immunity
DUSP16	MKP-7		12p12	Cytoplasmic/nuclear	JNK, p38	

Table 3: MAP kinase and their genes. According to (Owens and Keyse 2007)

IV.1. Activation of ERK1/2 pathway

The activation of RTK specific at the plasma membrane leads to receptor conformation and acts as recognition site for proteins containing Src homology 2 (SH2) or phosphotyrosine the adaptor proteins Shc and Grb2. Then Son of sevenless (SOS) “Guanine nucleotide exchange factor” is recruited to release the guanosine disulphate and activate RAS-GTPase (Gureasko, Galush et al. 2008). Ras in turn leads to activate Raf family members (A-Raf, B-Raf, and c-Raf-1) and subsequently phosphorylates MEK1/2, which in turn activates ERK1/2 (figure 29). After stimulation, phosphorylation of ERK1/2 leads to the induction of ERK1/2 dependent cellular processes, including proliferation, survival, differentiation, and, under some conditions, stress response and apoptosis (Turjanski, Vaque et al. 2007). Deregulation of parts of this cascade may play a major role in several pathologies, including developmental and neurodegenerative diseases, diabetes, and cancer (Dhillon, Hagan et al. 2007, Tanti and Jager 2009, Tidyman and Rauen 2009, Kim and Choi 2010).

Activating mutation in upstream components of ERK1/2 is involved in more than half of carcinogenesis. RAS mutation (∼20 % of all human cancers) and BRAF kinase mutations are involved in ∼7 % of all human cancers (Hagemann and Rapp 1999, Montagut and Settleman 2009).

About 160 substrates have already been discovered for ERKs. Most of these substrates are localized in the nucleus, and seem to participate in the regulation of transcription upon stimulation. Other substrates are found in the cytosol and in other cellular organelles, and those are responsible for processes such as translation, mitosis and apoptosis (Yoon and Seger 2006).

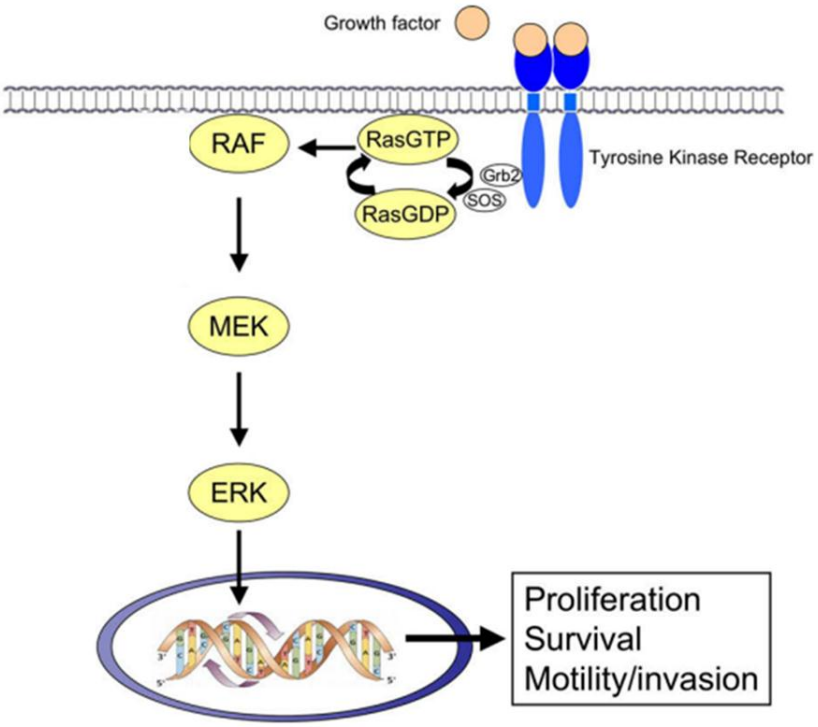

Figure 29 ERK1/2 pathway activation. Adapted from: (Montagut and Settleman 2009).

IV.2. Jun N-terminal kinases (JNKs) pathway

JNKs are also called stress-activated protein kinases (SAPKs). JNKs proteins mediate cellular responses to a variety of intra- and extracellular stresses as cytokines and UV radiation. Stimuli lead to activation of MAPK kinase kinases (MAPKKK) as (MLKs, TAK1, MEKK1/2/3/4, and ASK1), which in turn activate MAPKK (MKK4 and MKK7) which phosphorylate and activate JNKs. Activated JNKs lead to activate c-Jun, AP-1, c-Fos, thus resulting in modulation of gene expression (figure 30).

AP-1 contributes to the control of many cytokine genes and, together with JNKs, is implicated in the inflammatory process. JNKs also associate with inflammation via the promotion of reactive oxygen species production (ROS), as well as calcium release from the endoplasmic reticulum (ER); both ROS and Calcium release activate the transcriptional nuclear factor-κB (NF-κB) during acute-phase response of inflammation (Zhang and Kaufman 2008). Genetic and biochemical studies reported that JNKs also regulate cellular proliferation, apoptosis, and tissue morphogenesis (Hibi, Lin et al. 1993, Ip and Davis 1998, Johnson and Nakamura 2007). In *TP53*-/-

primary mouse embryonic fibroblasts (MEF), the genetic analyses of JNK deficiency show that JNK was a positive regulator of cellular transformation mediating by Ras. Furthermore, JNKs are important in controlling programmed cell death or apoptosis (Tournier, Hess et al. 2000). Dysregulated JNK pathways has been described in neurodegenerative diseases such as (Alzheimer and Parkinson), birth defects and cancer (Hibi, Lin et al. 1993, Derijard, Hibi et al. 1994, Kim and Choi 2010), and diabete (Tanti and Jager 2009).

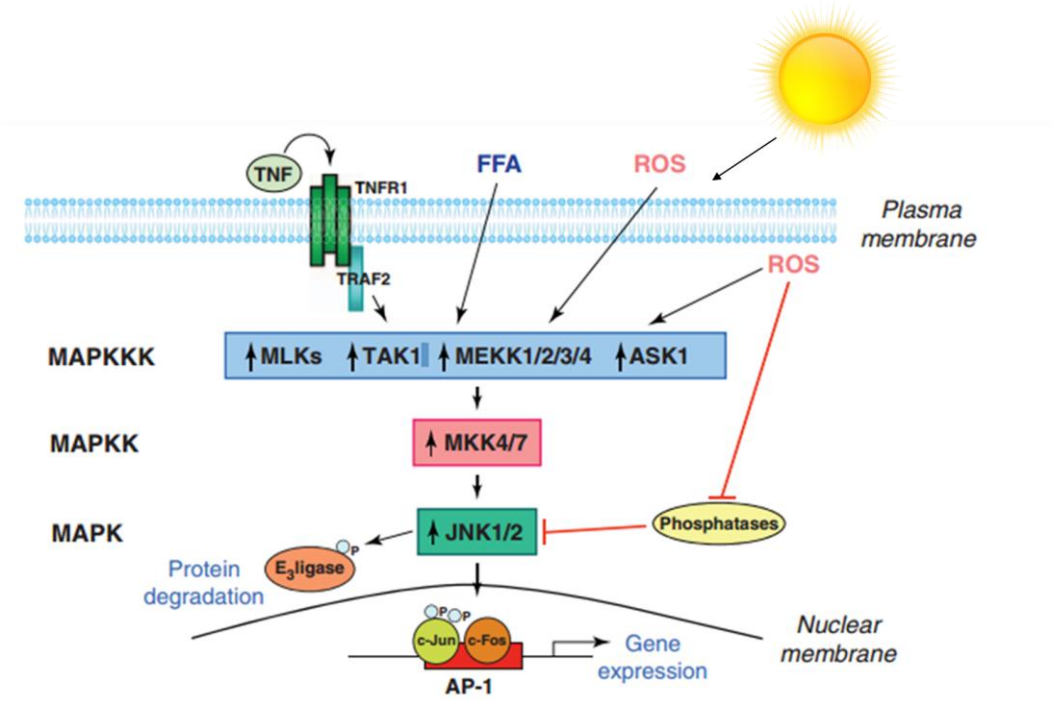

Figure 30 JNK signaling pathway. Several stimuli activate the JNKs signaling cascade such as UVR, Tumor necrosis factor (TNF), free fatty acid (FFA) and extra- or intracellular ROS. Activation of one or more MAPK kinase kinases (MAPKKK) due to stress stimuli lead to activate MAPKK, MKK4 and MKK7, which phosphorylate and activate JNK. The level of JNK activation is negatively regulated by phosphatase action. ROS can activate JNK through the inhibition of phosphatase activity. Activated JNKs phosphorylate Activated protein-1 (AP-1) subunit c-Jun, thus lead to increase its transcriptional activity. As a heterodimer with another subunit such as c-Fos, c-Jun binds to AP-1 promoter regions, increasing gene expression. Alternatively, JNK might also alter protein levels via the phosphorylation and activation of ubiquitin E3 ligase that induce protein degradation. Black lines indicate stimulatory pathways and red lines indicate inhibitory pathways. ASK1, apoptosis signal-stimulating kinase 1; MLK, mixed-lineage protein kinases; TAK-1, TGF-b-activated kinase 1; TNFR1, TNF receptor 1; TRAF2, TNF receptor-associated protein 2 adapted from: (Czaja 2010).

IV.3. P38 pathway

Another stress activated protein kinase (SAPK) is p38, which shows a veritable cross-talk with other MAPK pathways (Kyriakis and Avruch 2001) (Kyriakis and Avruch 2001). p38 is activated due to stress stimuli such as growth factors, hormones, proinflammatory cytokine and UV (Miyazawa, Mori et al. 1998, Rincon, Enslen et al. 1998). Figure 31 shows that upstream p38 are MKK3, MKK6 kinases which are thought to be the major protein kinases responsible for p38 activation (Lin, Minden et al. 1995, Han, Lee et al. 1996).

p38 has important functions in the process of differentiation and cell death. p38 regulates both the G2/M as well as a G1/S cell cycle checkpoint in response to cellular stress such as DNA damage. p38 can also mediate cell survival in specific situations, such as in response to DNA damage (Thornton and Rincon 2009).

Dysregulation of p38 is involved in many pathological manifestation, including inflammation. P38 may therefore be considered as a therapeutic target for inflammation-related diseases (Cuenda and Rousseau 2007, Schieven 2009). It has also been involved in the induction and maintenance of neurodegenerative diseases, diabete, and cancer (Loesch and Chen 2008, Liu 2009, Liu and Cao 2009, Kim and Choi 2010). However, the role of p38 in inflammation is also thought to promote cancer cell proliferation and invasion (Wagner and Nebreda 2009).

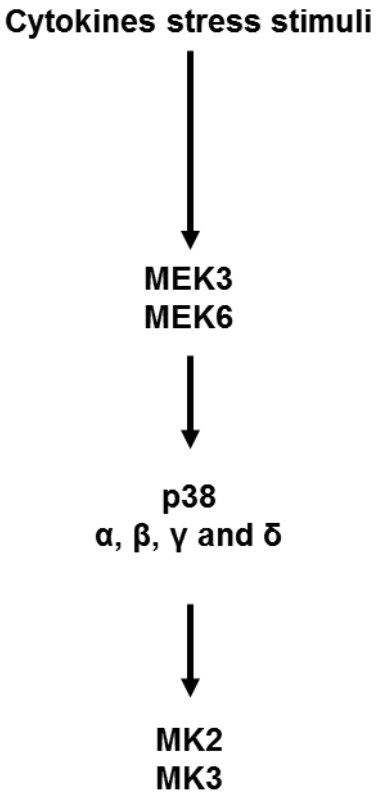

Figure 31 p38 signaling cascades. Stress stimuli lead to activate MEK3/6. Thus in turn activate p38 and as a consequence MK2/MK3. Adapted from: (Cargnello and Roux 2011).

THESIS OBJECTIVES

Thesis objectives:

The aim of this study is to analyze the influence of primary XP-C fibroblasts on the behavior of human squamous cell carcinoma in the absence of UV radiation. We used three dimensions (3D) organotypic cultures mimicking the natural structure of extracellular matrix of the dermis, as well as stratified organization of the epidermal equivalent.

In the context of our studies, it is important to note that the XP-C fibroblasts used were isolated from non photoexposed skin areas. The architecture of the organotypic culture in 3D constitutes an ideal mount to study the interactions between the dermis and the epidermis and allows to directly visualize the influence of each compartment on each other. Furthermore, organotypic skin cultures allow genotoxic and/or pharmacological treatment that would not be possible to carry out in the patients. In addition, organotypic skin cultures allow to assess properties of genetically engineered cells in order to improve approaches of *ex vivo* cutaneous gene therapy *http://ipubli-inserm.inist.fr/bitstream/handle/10608/6476/MS_2008_6-7_607*.

The first organotypic skin cultures from primary XP-C cells were done by Bernerd and colleagues (Bernerd, Asselineau et al. 2001). These studies showed that XP-C keratinocytes exhibited alterations in their proliferation and differentiation features in absence of exposure to sunlight. The histology of reconstructed XP epidermis made from epidermal XP keratinocytes revealed a significant reduction of the thickness of stratum corneum, suggesting an impairment in the differentiation of XP-C keratinocytes, compared with normal controls (Bernerd, Asselineau et al. 2001) (figure 32). Furthermore, *in vitro* reconstructed XP epidermis showed that the stratum corneum contained persisting nuclei, an abnormality called parakeratosis, which accompanies some cutaneous hyperproliferative syndromes. Perhaps, one of most important observation of this study, was that the presence of XP-C fibroblasts promoted the invasion of wild type and XP-C keratinocytes in the dermal compartment (figure 33).

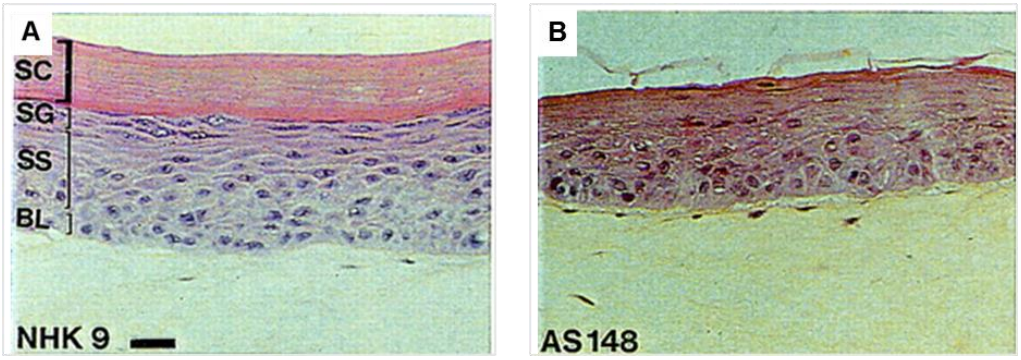

Figure 32: Histology of skin reconstructed in vitro from normal and XP-C keratinocytes on normal dermal equivalent. A) Normal keratinocytes. **B)** XP-C keratinocytes. BL, basal layer; SS, stratum spinosum; SG, stratum granulosum; SC, stratum corneum. Histologies of normal and XP-C epidermis look closely similar, except for a thinner stratum corneum in XP-C epidermis. (Bar = 50 µm) (Bernerd, Asselineau et al. 2001).

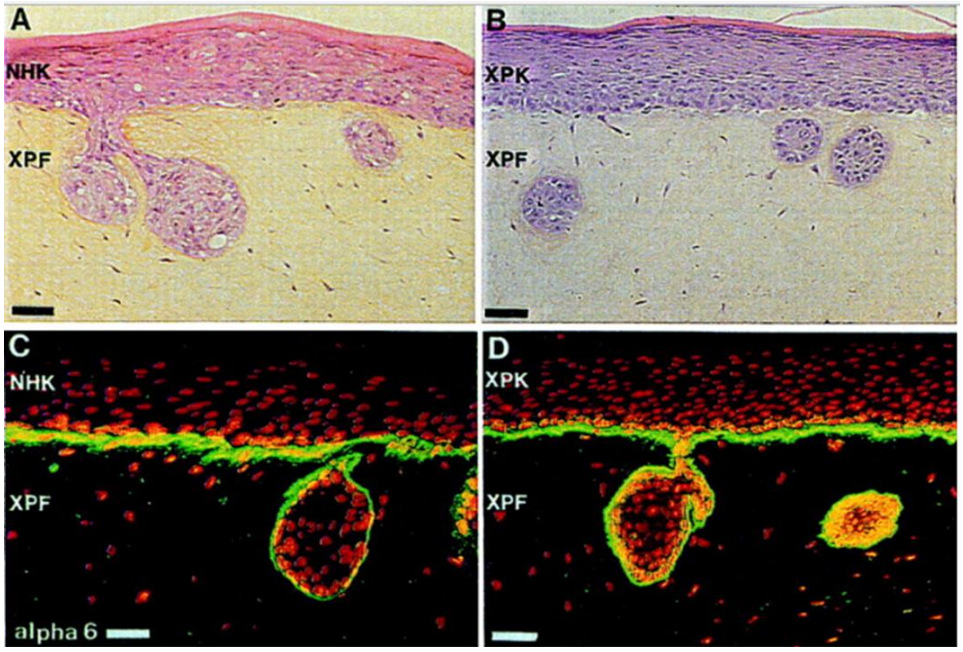

Figure 33 XP-C fibroblasts provoke epidermal invasions in organotypic skin cultures. Wild type (A), and XP-C (B) keratinocytes form epidermal invasions in the dermal equivalent in the presence of XP-C fibroblasts. (C, D) Indirect immmunolabeling of α-6 integrin in organotypic skin culture under the indicated conditions (Bernerd, Asselineau et al. 2001).

Other data from the laboratory also indicated that inclusion of XP-C fibroblasts in the dermal equivalent resulted in higher contractile properties leading to a reduction of the diameter of collagen lattices of about 25 % (Frechet, Warrick et al. 2008). These observations were the first suggesting that cancer susceptibility of epidermal cells towards skin cancer could be elicited by the secretory phenotype of XP-C fibroblasts. In contrary, using an inhibitor of matrix metalloproteinases (MMPs) inhibits the contraction of collagen lattices by fibroblasts (Scott, Wood et al. 1998). Several studies reported on the role of MMPs in tumor progression and invasion (Curran and Murray 1999, Egeblad and Werb 2002) and in skin chronological aging (West 1994) and skin photoaging (Fisher, Datta et al. 1996); other studies have shown an overexpression of MMP1 in XP-C fibroblasts, suggesting that MMP1 could be an actors toward exacerbated tumor susceptibility in XP-C patients. My host laboratory decided to further focus his investigations on factors secreted by XP-C fibroblasts and implicated in the promotion of epidermal invasions. The analysis of secretome of XP-C fibroblasts in culture showed that XP-C fibroblasts secrete hepatocyte growth factor (HGF), a factor known to be implicated in cells morphogenesis, proliferation, malignant transformation and cancer progression (Kalluri and Zeisberg 2006). HGF also has been found to be increased in forestomach in the mice around invasive squamous cell carcinomas (Bhowmick, Chytil et al. 2004). As we mentioned before, XP-C patient present with high propensity to the development of aggressive skin cancers, most notably SCCs and melanoma. Our studies hence focus on the role of XP-C fibroblasts in the promotion of human squamous cell carcinoma with a particular attention to the following points:

- Effects of XP-C fibroblasts culture supernatants on the migration of two SCC cell lines (SCC12, SCC13) *in vitro*, using scratch assay. Taking into account that wound healing could also be due to the proliferation of cells, the analyses were done in the absence or in the presence of mitomycine C to block the proliferation of SCC cells.

- Investigate the role of XP-C fibroblasts on SCC cells invasions in 3D organotypic cultures, using SCC cells in the epidermis and XP-C fibroblasts in the dermis. To determine if HGF overexpressed by XP-C fibroblasts was a major factor of the observed invasions, blocking HGF antibodies were included during the experiments.

- HGF is known to activate MAPK pathways (STAT3, p38, JNKS and ERKs). Each one of these pathways plays an important role in the cell proliferation, apoptosis, differentiation and cell transformation. We then identifying signalisation pathways possibly activated in SCC cells exposed to culture supernatants conditioned by XP-C fibroblasts.

- Find a way to modulate/normalize the pathway activated by HGF using HGF blocking antibodies, and as a result determine which pathway HGF precisely activate and which pathway is implicated in SCC cells invasion.

- As we show that XP-C fibroblasts share high similarity with CAF fibroblasts, we wondered whether XP-C fibroblasts could behave like CAFs by pulling cancer cells and promote invasion. This question was adressed using spheroid assays.

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SCIENTIFIC ARTICLE

Role of XP-C skin fibroblasts in squamous cell carcinoma cell line invasion

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Abstract

Squamous cell carcinoma (SCC) is the most frequent metastatic skin cancer from epithelial origin. In the majority of cases, the etiology of skin SCC cancer is linked to exposure to genotoxic stress, notably ultraviolet radiation (UVR), in the vast of skin cancers. Xeroderma pigmentosum type C (XP-C) is a rare genetic disorder characterized by a severe susceptibility to aggressive SCCs following minimal exposure to UVR, hence compromising the life expectancy of patients. XP-C cells are deficient in the nucleotide excision repair mechanism (NER) of DNA lesions introduced at bipyrimidine sequences upon UVR exposure. Prior results from the laboratory have shown that XP-C dermal fibroblasts constitutively express a phenotype resembling that of stromal fibroblasts associated to cancer cells with accumulation of reactive oxygen species and over expression of matrix metalloproteinase 1 (MMP1).

In this study, we further explored the phenotype of XP-C primary fibroblasts and their effects on the invasion of SCC cells. Our results show that primary XP-C fibroblasts constitutively overexpress hepatocyte growth factor/scatter factor (HGF/SF). In organotypic skin cultures, XP-C fibroblasts promote the invasion of SCC cells. Also, scratch healing of SCC cells was enhanced in the presence of culture supernatants of XP-C fibroblasts through a mitogenic effect connected to increased ratio of SCC cells in the G2-M phase of the cell cycle. Blockage of c-Met activation by blocking HGF antibodies prevented invasiveness of SCC cells within dermal equivalent presumably through the inhibition of p38 or JNK activation by XP-C fibroblasts culture supernatants. Spheroid assay shows that XP-C fibroblasts led SCC invasions. Our data indicated for the first time that absence of XPC in fibroblasts leads to overexpression of cell growth stimulators such as HGF and other factors (MMP1) responsible for the formation of a microenvironment permissive towards SCC cells proliferation and invasion. Moreover, our findings show that XP-C fibroblasts are leaders cells for SCC cells invasion. Therapies targeting XP-C fibroblasts may be considered as a way to control development of aggressive cancer in XP-C patients.

Introduction

Epithelial cancers are by far the most frequent neoplasia (about 80 %) in the human, among which skin carcinoma occupy the first place (about 30 %) (DePinho 2000). Since about three decades, evidences accumulated showing that development and dissemination of the tumor mass are highly dependent on the interactions between carcinoma cells and their surrounding stroma. In normal skin, keratinocytes form the major part of the surface stratified squamous epithelium (epidermis); epidermal cells from the basal layer are thought to be those from which most cancers of the integument develop (i.e. basal cell carcinomas, BCCs ; squamous cell carcinomas, SCCs). Underneath the epidermal compartment, a well separated supporting complex tissue, the dermis, constitutes a stroma including dermal fibroblasts, immune cells and adipocytes, embedded in an extracellular matrix (ECM) schematically composed of a network of collagen and elastin fibers and various proteoglycans (Sorrell and Caplan 2004). During ontogenesis, interactions between the mesodermis and the ectodermis orchestrate development of the epidermis and of ectodermal annexes such as hair, nails and teeth (Fuchs 2007).

Besides their role in development, the phenotypes and inductive activities of stromal cells, notably of fibroblasts, may be profoundly perturbed in the presence of cancer cells but the exact nature of the interactions between fibroblasts and cancer cells still disserve much investigations. *Ex vivo* experiments, however, established that stromal (dermal) fibroblasts in the vicinity of cancer cells, acquire traits enabling them to promote carcinoma cell invasion. The influence of cancer cells may change their program of gene expression through transcriptional and / or epigenetic modifications (Kalluri 2016) and signaling pathway regulations (Albrengues, Bertero et al. 2015), hence impacting expression of growth factors (Albrengues, Bourget et al. 2014), cytokines, and matrix remodeling proteases such as MMPs (Kaplan, Riba et al. 2005, Gaggioli, Hooper et al. 2007, Kalluri 2016). Fibroblasts associated with cancer cells (Cancer Associated Fibroblasts, CAFs) produce a biological context resembling that observed in inflammatory tissue with massive production of reactive oxygen species (ROS), growth factors (e.g. TGF□, FGF-7), and MMPs, as well. CAFs may then act on both the proliferation (Kopelovich 1982) and the motility of cancer cells (Schor, Haggie et al. 1986), as well as on the components and organization of the ECM. The vital prognostic associated with epithelial cancers decreases in the presence of CAFs, most notably due to their proinvasive, promutagenic and prometastatic effects (Olaso, Santisteban et al. 1997, Orimo, Gupta et al. 2005). Prior work from the laboratory has revealed that, under some genetic conditions, dermal fibroblasts may also spontaneously behave like fibroblasts associated with cancer cells but in the absence of cancer cells, indicating that changes in

regulatory networks of gene expression not only depend on cell interactions but may also occur in a cell autonomous manner (Valin, Barnay-Verdier et al. 2009).

Xeroderma pigmentosum (XP) is a very rare (about 1.5 10^{5} newborns) recessively inherited genetic disease due to severe alteration(s) in the mechanism of nucleotide excision repair (NER) which removes DNA lesions introduced following sunlight exposure, notably UVB wavelengths (You, Lee et al. 2001). While individuals from the general population are equipped to efficiently eliminate UV-induced DNA lesions, the congenital incapacity of XP patient cells to remove these lesions and hence the persistence of these DNA lesions result in high mutagenic rates and a very high susceptibility toward skin cancers. NER defects are due to various levels of NER alteration, as a function of the mutation(s) in one of the 7 genes (*XPA* to *XPG*) implicated in one of the seven groups of XP genetic complementation in the human (XP-A to XP-G) (Masutani, Kusumoto et al. 1999). Surprisingly enough, not only the rate of skin cancers is increased in XP patients, but also their aggressiveness, i.e. their typology. BCCs are generally non-metastatic while SCCs present with a significant potential of distal invasion and metastasis. Indeed, in the general population, about 5 to 8 basal cell carcinoma (BCC) develop for 1 squamous cell carcinoma (SCC) in photoexposed skin. In XP patients, the rate of BCCs versus SCC is at average 1 for 8 (Kraemer, Lee et al. 1987, Kraemer 1997). Together with a high proneness toward highly metastatic skin melanoma, the life expectancy of XP patients may be severely altered. These clinical observations suggested to us that primary XP fibroblasts could express phenotypic resemblances with sporadic CAFs and, possibly, influence the typology of skin carcinoma. Previously, we reported overexpresssion of MMP1 in primary fibroblasts isolated from XP patients belonging to the XP-C group of genetic complementation, which is mostly represented in Europe, Africa, and USA (Frechet, Warrick et al. 2008).

The aim of this study was to better document the reasons why XP (XP-C) patients not only develop several thousand times more skin cancer than individual from the general population, but also why the typology of skin cancers is preferentially aggressive and metastatic. Our data indicate that primary XP-C fibroblasts over express in a cell autonomous manner the mitogenic and motogenic factor HGF/SF. Inhibition of HGF-dependent pathway activation was accompanied by total inhibition of carcinoma cell invasion. Our data provide further evidence that XPC mutations result in pejorative deregulation of gene expression leading to cancer cell invasion and suggest a potential for better-targeted therapeutic approaches.

Results

XP-C primary fibroblasts provoke invasiveness of the SCC 13 squamous carcinoma cells

Our previous results indicated that primary fibroblasts isolated from non photoexposed skin biopsies taken on XP-C patients elicited invasion of epidermal keratinocytes in the dermal compartment from organotypic skin cultures (Bernerd, Asselineau et al. 2001). Here, we measured the impact of XP-C primary fibroblasts on the behavior of cells derived from human squamous cell carcinoma (SCC13). Histological stainings in figure 1A show that neither WT nor SCC13 keratinocytes exhibited invasive capacities in organotypic skin cultures comprising a dermal equivalent populated with control WT fibroblasts. In contrast, inclusion of XP-C primary fibroblasts in dermal equivalents resulted in epidermal invasions with both WT keratinocytes and SCC13 cancer cells. Epidermal invasions formed by WT cells most often displayed a round and quite regular shape; in contrast, cell invasions formed by SCC13 cancer cells appeared collective, as observed in the presence of carcinoma associated fibroblasts (Gaggioli, Hooper et al. 2007) (Figure 1A). Also, it was worth noting frequent dermo-epidermal clefts in organotypic skin cultures composed of both SCC13 cells and XP-C fibroblasts. The epithelial origin of invading cells was verified by indirect immunolabeling of the K14 keratin (Figure 1B).

XP-C primary fibroblasts elicit proliferation of the SCC13 squamous carcinoma cells

The proinvasive activity of primary XP-C fibroblasts observed in organotypic skin cultures (Figure 1A) could be due to either mitogenic or motogenic effects, or both. To address these issues, we performed scratch healing assays in either the absence or the presence of mitomycin C (MMC), a chemotherapeutic drug which blocks cell proliferation by inducing irreversible DNA damages. In the absence of MMC, scratch closure of SCC13 cells incubated in culture supernatants from WT fibroblasts was complete about 7h30 following injury. When SCC13 cells were incubated in culture supernatants conditioned by XP-C fibroblasts, time to complete scratch closure were shortened to about 3 to 4 hours, then confirming the influence of XP-C fibroblasts on either epithelial cell proliferation or migration. After pretreatment of SCC13 cells using MMC, extent of scratch closure 7 to 8 hours after injury was found even less efficient than that of WT keratinocytes with only 50 to 60 % (see S1). Together, these observations suggested that primary dermal fibroblasts isolated from XP-C patients were principally endowed with mitogenic stimuli towards SCC cells, but poorly promoted cell migration (Figure 2 B). Based on these observations, we then further analyzed the impact of

culture supernatants conditioned by WT versus XP-C fibroblasts on cell cycle characteristics of SCC cells. Table in Figure 2C highlights the impact of culture supernatants conditioned by XP-C fibroblasts. SCC13 in XP-C fibroblasts' culture supernatants exhibited about twice as much numbers of G2-M cells, such as SCC13 cells conditioned by WT fibroblasts.

Since CYCLIN B1 is a master protein of the G2-M cell cycle progression, we then measured its levels of expression in SCC13 cells conditioned by culture supernatants conditioned by either WT or XP-C fibroblasts. Figure 2D shows a substantial increase of CYCLIN B1 accumulation in SCC13 cells exposed to XP-C fibroblasts culture supernatants, thus confirming at the molecular level the promitotic influence of XP-C fibroblasts.

XP-C primary fibroblasts lead cancer cell invasion in spheroid assay

Whether XP-C fibroblasts could behave as CAFs, notably by making tracks to pull cancer cells within the ECM (Gaggioli, Hooper et al. 2007), was then analyzed using a spheroid assay. This assay enables real time monitoring of epithelial cell invasion in the presence of fibroblasts from various origins (e.g. WT, XP, CAF). Figure 3A shows that SCC13 cells spread out of the spheroid and behave as podocytes in the presence of XP-C primary fibroblasts compared to spheroid composed of SCC13 cells and WT fibroblasts. These observations suggested that XP-C primary fibroblasts were able to lead tracks of cancer keratinocytes as efficiently as *bona fide* CAFS (data not shown; Gaggioli et al., 2007). To further document these observations, we performed live microscopy. SCC13 cells were labelled using a retroviral construct allowing expression of the green fluorescent protein. Control (data not shown) or XP-C primary dermal fibroblasts were stained in red using the cell tracker fluorescent dye, which is compatible with live cells. Under these circumstances, XP-C fibroblasts tend to rapidly accumulate at the periphery of spheroids to occupy a leader position (Figure 3B).

Hepatocyte growth factor / scatter factor, a molecular determinant of the proinvasive behavior elicited by XP-C primary fibroblasts

We aimed at identifying factors possibly implicated in the expression of the proinvasive phenotype led by XP-C primary fibroblasts. Since Hepatocyte Growth Factor / Scatter Factor (HGF/SF) is a major growth factor secreted by CAFs, we measured its expression in primary XP-C fibroblasts cultured in both conventional culture conditions and in dermal equivalents. Under both circumstances, *HGF* mRNA and HGF secretion were found significantly increased (about 10 to 15 times, and 3 times, respectively) in XP-C compared to WT fibroblasts (Figure 4A).

The HGF receptor, c-Met, is activated by auto phosphorylation upon binding of its ligand HGF, leading to phosphorylation of its targets including p38, JNK1/2, ERK, as well as the transcription factor STAT3 (Trusolino, Bertotti et al. 2010). On these bases, we assessed the effects of culture supernatant from primary XP-C fibroblasts on the activation of the c-Met/HGF pathway. Western blot analysis in figure 4C show that incubation for 1h30 of SCC13 cells in culture supernatants from any of the 4 independent XP-C fibroblasts strains resulted in phosphorylation of c-Met with an average increase about 7 folds compared to control compared to SCC13 cells cultured in supernatants conditioned by WT primary fibroblasts.

Upon binding of HGF, the c-Met receptor results in pleiotropic effects, among which triggering of the mitogen-activated protein kinase cascades whose final major effectors are ERK1, 2, JNK1, 2, 3 and p38 $\alpha, \beta, \gamma, \delta$, and the signal transducer and activator of transcription STAT3. These pathways regulate various cellular processes including proliferation, transformation, differentiation, apoptosis and tubulogenesis. We then measured the phosphorylated forms of those HGF effectors in SCC13 cells exposed to culture supernatants conditioned by primary XP-C fibroblasts (4 independent strains). The amounts of P-p38 and P-JNK1/2 forms were significantly increased in the presence of XP-C supernatants (Figure 4D, E). In contrast, neither the phosphorylated form of ERK1, 2 nor STAT3 appeared affected in the presence of XP-C fibroblasts culture supernatants (Figure 4F, G), (see S1).

Inhibition of c-Met activation counteracts the pro-invasive influence of XP-C fibroblasts

In light of the pro-invasive influence of XP-C primary fibroblasts on SCC13 cells together with our observations indicating the accumulation of HGF in XP-C fibroblasts, we then aimed at analyzing the impact of HGF binding inhibition on c-Met activation and SCC13 cancer cells invasion. SCC13 cells were then cultured in media conditioned by XP-C primary fibroblasts in the absence or the presence of a monoclonal antibody able to block c-Met activation by HGF. Western blot analysis in Figure 5A shows that P-c-Met was abundant in SCC13 cells cultured in medium conditioned by primary XP-C fibroblasts (2 independent strains). Accumulation of P-c-Met was abrogated in SCC13 cells cultured in XP-C fibroblasts culture supernatants complemented with the inhibitory anti-HGF antibody. In good accordance, under the same circumstances, P-p38 accumulations was abrogated (Figure 5B).

We then assessed the functional impact of HGF inhibition with the HGF blocking antibodies on SCC13 invasion in organotypic skin cultures. First, we found that diameters of dermal equivalents were smaller when populated with XP-C fibroblasts compared to WT fibroblasts.

The presence of the anti-HGF antibody in the culture medium of dermal equivalents counteracted the increased contractile properties in the presence of XP-C fibroblasts (Figure 5C, D). Finally, in organotypic skin cultures, epidermal invasions observed in the presence of XP-C fibroblasts were no longer observed when the HGF blocking antibody was added to the culture medium (Figure 5E). These results suggest a paramount effect of XP-C primary fibroblasts on extracellular matrix remodeling and contraction, as well as in the promotion of cancer cells invasion.

Materials and methods

Skin cells isolation and culture

Human primary fibroblasts and keratinocytes were isolated from healthy, non photo-exposed skin biopsies from either control (WT) individuals or XPC patients (Table S2). SCC-13 cells were a generous gift from Dr. James Rheinwald (Rheinwald and Beckett 1981). The study was approved by a local French ethic committee and was conducted after informed written consent of XP-C patients or their representatives.

Primary dermal fibroblasts were cultured in DMEM supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine, 1 % penicillin/streptomycin, 1 mM sodium pyruvate, 1 % non-essential amino acids, and 0.2 % fungizone, at 37 °C, 5 % CO_2. Fibroblasts (passages P6 to P10) were seeded at 10^4 cells/cm², and cultured up to 80-90 % confluency before processing. Primary keratinocytes, SCC-13, and SCC13-GFP (Bergoglio, Larcher et al. 2007) cells were cultured on feeder layers of lethally irradiated 3T3-J2 Swiss mouse fibroblasts in cFAD keratinocyte medium (Rheinwald and Beckett 1981).

Conditioned fibroblast media for keratinocyte cultures

For scratch assays, fibroblasts were seeded at 6600 cells/cm^2, cultured for 72 hours in standard cFAD and then in FAD medium containing 0.5 % FCS for 48 hours. Prior scratch assay, primary keratinocytes and SCC-13 cells were first cultured in medium previously conditioned for 48 hours by feeder 3T3-J2 Swiss mouse fibroblasts.

For RNA and protein analyses, keratinocytes (WT or SCC cells) were seeded at 4000 cells/cm² on lethally irradiated 3T3-J2 Swiss mouse fibroblasts in cFAD for 48 hours to reach about 50 % confluency. Feeder cells were then removed after incubation in PBS-EDTA 0,02 % and keratinocytes were refed for 48 hours in FAD containing 0.5 % FCS; cells were then cultured for 1h30 in FAD 0.5 % FCS, conditioned for 48 hours by either WT or XP-C human primary fibroblasts. Before treating the keratinocytes with the fibroblasts supernatants, the conditioned supernatants media were supplemented with 0.66 ng/ml of the anti-HGF blocking monoclonal antibody (MAB294, Monoclonal mouse IgG& clone#24612, MAB294 R&D systems), and incubated for 1h hours at 37 °C and the directly used to conditioned the Keratinocytes. Keratinocytes lysate were used for RNA and proteins preparations.

Scratch assay

Keratinocytes were seeded in 6 wells plates (x cells /cm^2) in culture supernatants previously conditioned by irradiated 3T3-J2 Swiss mouse fibroblasts for 24h at 37°C, 10 %

CO2. Once confluent, when indicated, in order to block proliferation, cells were pretreated with 10 µg/ml mitomycin C for 2 hours. Cells were then scratched using 200 µL « pipetman » tips, washed in PBS, and incubated in culture supernatants conditioned by either WT or XP-C fibroblasts as described above. Scratch closure was assessed High – throughput Live Epi. Fluorescence microscope (Zeiss) during about 7h30. % of scratch closure was calculated using ImageJ program.

Contractile properties and invasion assays in organotypic skin cultures

For gel contraction assay, 25 × 10^3 fibroblasts were embedded in 100 µl of a collagen/matrigel mix (collagen concentration of approximately 4.6 mg ml^{-1} and a final Matrigel concentration of approximately 2.2 mg/ml^{-1}), and seeded in triplicate into 96-wells plate. After 1 h at 37 °C, matrix gels were overlaid with 100 µl of cFAD 0.5 % medium in either presence or absence of 0.66 ng/ml of HGF blocking monoclonal antibody (MAB294, Monoclonal mouse IgG& clone#24612, MAB294 R&D systems). After 24 hours, the mean diameter of the gels was measured using the ImageJ software. The percentage of gel contraction was calculated using the formula 100 × (well diameter−gel diameter)/well diameter.

Organotypic cultures were set as described by Gaggioli (Nystrom, Thomas et al. 2005, Gaggioli, Hooper et al. 2007). Briefly, 5.10^5 fibroblasts were embedded in a 2:1 mixture of collagen I (#354249; BD Biosciences, Oxford, UK) and Matrigel (#354234; BD Biosciences). After 1 hour at 37°C, 5.10^5 keratinocytes were plated on top of the gels, and incubated overnight at 37°C, 10 % CO2. Gels were then mounted at the air-liquid interface on stainless steel grids and fed through capillary diffusion of the culture medium. After 7 days, the cultures were harvested and fixed in 3.7 % paraformaldehyde, 0.25 % glutaraldehyde, before paraffin embedding. When indicated, organotypic cultures were processed in the presence of 0.66 ng/ml of the anti-HGF blocking monoclonal antibody (MAB294, Monoclonal mouse IgG& clone#24612, MAB294 R&D systems) which incorporated in the collagen/matrigel/fibroblasts mixture. Invasion index was calculated using the following formula: [1 – (non-invading keratinocytes area/invading and non-invading cells area)] values represent the average of results of the analyses of 3 total sections from two independent organotypic cultures.

Histology and immunostaining

Hematoxylin-Eosin (H&E) and immuno labellings on paraffin sections were performed as described (Brellier, Bergoglio et al. 2008). Immunostaining of Keratin 14 was performed

using anti-K14 mouse mAb (1/20; NCL-LL002; ab7800, abcam) and Alexa Fluor 594 rabbit anti mouse secondary antibody (1/200; alexa flour ® day 594, Thermo Fisher scientific) (Bernerd and Asselineau 1997).

Protein extraction and western blotting

For western blot experiments, proteins were solubilized in 150 µl TLB buffer {Tris 20mM pH 7.5, NACL 137mM, EDTA 2mM pH7.5, Triton x100 1%, Na PPi 2mM, Glycerol 10%) with complete protease inhibitor cocktail (Roche, Germany) and phosphatase inhibitor cocktail (Phosstop, Roche, Germany). Proteins (30 µg) were separated on 8 % SDS-PAGE, transferred onto polyvinyl difluoride (PVDF) membrane (Millipore, Corporation, Billerica, MA, USA), and revealed using anti- human Met, (D1C2 XP® rabbit mAb, 1/1000, # 8198, Cell signaling technologies), anti-human p-Met, (NL8 rabbit mAb, 1/5000, # 04-9000 Millipore), anti-human, p ERK and pSAPK/JNK (56G8) (rabbit mAb, 1/1000, # 9102 and #9258 respectively, Cell signaling technologies), p38 (polyAb, 1/1000, # 9212 Cell signaling technologies), anti-human pSTAT3 (ab76315 rabbit mAb, 1/5000, abcam). Membranes were re-probed using the anti-GAPDH mouse mAb (1/5000; Abcam # 9484, Cambridge, UK) or the anti-Tubulin TUB2.1 mouse mAb (1/5000, Sigma Aldrich, St Louis, MO) for loading/transfer controls. Membranes were then analysed using the Luminata crescendo western HRP substrate (Millipore Corporation, Billerica, MA, USA). Results of western blot were then quantified using the Fusion-Capt software (Vilber Lourmat, Eberhardzell, Germany). Antibodies used are listed in S3 table.

RNA extraction and quantitative RT-PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed from 1 µg RNA using the Superscript II Reverse Transcriptase (Roche Applied Science, Basel, Switzerland) and hexameric random primers. Real Time-PCR was carried out on cDNAs using Fast SYBR Green Master Mix (#18064-014; Applied Biosystems, Foster City, CA) and the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers used are listed in S4 Table, expression of each gene was normalized to the respective *GAPDH and SB34* expression (Brellier, Marionnet et al. 2004).

Spheroid invasion assay

For spheroid invasion assays, cultured SSC13-GFP cells and fibroblasts were trypsinized and resuspended at 10^6 cells/ml in cFAD medium and fibroblasts medium,

respectively. Prior to experiments, fibroblasts were pre-stained for 30 min with a nuclear cell tracker (# R37106, NucBlue^{R}Live ReadyProbes^{R} reagent, molecular probes, Life Technologies), spined, and resuspended. An equal ratio (25 X 10^3 each) of SCC13-GFP cells and nuclear stained fibroblasts were suspend in 50 µl drops in the center of inverted 60 mm dishes, incubated for 72 hours at 37°C, 10 % C02 (Gaggioli 2008). Aggregated cells were then carefully harvested and deposited on a thin layer prepared mixture of collagen I (#354249; BD Biosciences, Oxford, UK) and matrigel (#354234; BD Biosciences) as described earlier. Gels were previously prepared and incubated for 10-30 min before adding the sphere of cells) on 14 mm glass bottom cell culture dishes (P35GC- 1.5-14-C, Mattek corporation, ASHLAND, USA). Aggregated cells were then maintained for spheroid development and analysed 24 hours later using confocal microscopy (Nikon A1R, Leica AM-TIRF).

Cell cycle

SCC cells were seeded at 2500 cells/cm² on lethally irradiated 3T3-J2 mouse fibroblasts in cFAD. At 50 % confluence, 3T3 gamma were removed. Cells were then depleted by adding cFAD 0.5 % SVF. After, 48h, culture supernatants were removed and SCC cells were washed with PBS and conditioned with the supernatants of XP-C fibroblasts for 6h at 37 °C. SCC cells were harvest and centrifuged at 4 °C for 5 min at 100 rcf. Cells were washed with 10 ml of PBS, centrifuged at 4 °C for 10 min at 300 rcf, fixed with 20 % ethanol in PBS, and kept on ice for 30 min. Cells were then washed twice with PBS and spin at 4 °C, 300 rcf for 5 min. Cells then were incubated 30 min at 37°C with 0,1 mg/ml Propidium iodide and 20 ng/ml of RNase I in PBS. Cytometric analysis were performed on FacsCanto Becton Dickinson.

Discussion

The relation between SCC invasion and stromal skin fibroblasts in XP-C patients is poorly documented. To learn more about this process, we screened for the effect of XP-C fibroblasts on SCC invasion and known regulators of SCC invasion. This work shows that overexpression of HGF by XP-C fibroblasts is critical for SCC cells invasion.

In this study, we showed that culture media conditioned by XP-C fibroblasts activate c-Met and downstream p38 and JNK in SCC treated cells. We also showed that XP-C fibroblasts in culture overexpress HGF. The HGF c-Met receptor has been implicated in many cellular processes, including cell motility, survival, proliferation, and cancers. (Liu, Newton et al. 2010, Lee, Kim et al. 2011). In the context of malignancies, activation of c-Met leads to different signalling kinases cascades that initiate an invasive and/or metastatic program (Trusolino, Bertotti et al. 2010). Our study clearly showed that activation of the c-Met pathway in SCC cells conditioned by XP-C media is due to HGF secreted by XP-C fibroblasts. Indeed, although many proteins can affect the activation of c-Met including cell surface, membrane-spanning molecules or receptors, (Christensen, Burrows et al. 2005, Xu and Yu 2007, Furlan, Kherrouche et al. 2014), blockage of HGF activity with HGF blocking antibodies in culture supernatants suppress the activation c-Met, p38 and JNK in treated SCC cells. The importance of HGF secreted by XP-C fibrobasts in SCC cell invasion was also hylighted in our 3D organotypic cultures. Indeed, we showed that primary XP-C fibroblasts promote invasion of human SCC cells and that blocking HGF antibodies in 3D cultures suppress the invasion of SCC cells.

Scratch assays using SCC13 cells showed that wound closure was significantly more rapid in the presence of XP-C fibroblasts culture supernatants. Whether XP-C fibroblasts affected either cell migration or proliferation was assessed after treatment of SCC13 cells with the antimitotic drug mitomycin C, and showed that the major effect of XP-C primary fibroblasts was mitogenic. In good accordance, we found that accumulation of the Cyclin B1 protein was significantly increased in SCC13 cell cultures conditionned by XP-C cell culture supernatants. Our observations showing the effects of HGF accumulation in XP-C primary fibroblasts are in good agreement with the seminal description of HGF secretion by mesenchymal cells and its effects as a mitogenic protein for hepatocytes and subsequent identification as the ligand of the proto-oncogene c-Met tyrosine kinase receptor (Nakamura, Nawa et al. 1984, Naldini, Vigna et al. 1991). HGF is secreted by mesenchymal cells and acts as a multi-functional cytokine on cells from epithelial origin. HGF regulates cell growth, cell motility and morphogenesis in numerous cells and tissue types and plays a role in angiogenesis and tissue regeneration. HGF is then considered as a mitogen and a motogen factor. Our scratch healing

assays using XP-C primary fibroblasts from skin (i.e. from mesenchymal origin) in presence or absence of mitomycin C, showed that HGF played a major role in the multiplication rather than in the migration of cancer SCC cells. In good accordance, our data indicated that SCC13 cells treated with culture supernatants of XP-C fibroblasts presented with increased expression of the cyclin B1 protein; as a consequence, the ratio in G2-M phase of cell cycle was increased by about 100 %, (i.e. x 2), also suggesting that the secretome of XP-C primary fibroblasts had a mitogenic effect on SCC13 cells.

Our observations also revealed increased contraction of collagen dermal equivalents and collagen matrigel spheroid mounts in the presence of XP-C compared to WT fibroblasts. Interestingly, increased gel contraction has also been described in the presence of CAFs *ex vivo*, as well as *in vivo in* human cancer mass, which represents a criterion of pejorative prognostic (Daniel, Robinson et al. 2001, Pollard 2001, Gaggioli, Hooper et al. 2007). Yet, the role of the HGF secreted by XP-C fibroblasts disserves further investigations, it appeared that its specific inhibition using an anti-HGF antibody counteracted both (XP-C) fibroblast-dependent contractile and invasive properties. These properties seemed to result from activation of the SAPK kinase p38 and JNK but not the ERK MAPK pathway as described in the presence of human CAFs. Neither, in contrasts to similar experiment using human CAFs we failed to measure any substantial activation of the STAT3 transcriptional factor in SCC cells cultured in the presence of XP-C fibroblasts culture supernatants (Sanz-Moreno, Gaggioli et al. 2011). Despite these differences between CAFs and XP-C fibroblasts, spheroid assay clearly indicated that patients behaved as leader cells by “pulling” SCC cells *ex vivo*.

In conclusion, organotypic cultures have identified a key role for XP-C fibroblasts in proliferation and invasion of SCC cells. Furthermore, we have revealed that the promotion of SCC cells invasion is connected to the production of HGF by XP-C fibroblasts and the activation of the c-Met signalling pathways in carcinoma cells. In absence of specific treatment dedicated to XP-C patients, targeting of the HGF/c-Met pathway could prevent the development of aggressive carcinoma as already applied in individuals from the general population suffering from aggressive skin carcinomas (Grugan, Miller et al. 2010).

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Legende of figures

Figure 1: XPC fibroblasts promote SCC13 cells invasion in organotypic skin cultures

(A) Representative images of H&E staining of sections from organotpic cultures generated from either WT (KTM1) or SCC13 cells cultured in an organotypic system in the presence of either A, upper panels WT (FH29), or, A, lower panel H&E staining from organotypic skin cultures with a dermal equivalent composed of XP-C primary fibroblasts (AS433 or AS798). Scale bar: 50 µm. (B) Quantification of SSC13 cells invasions. Values are presented as means ± SD of three independent areas. Asterisks $p < 0.05$ (0.0001). (C) Immunofluorescence stainings of the K14 keratin (green) and nuclei (blue) show that invading cells are from epidermal origin. Scale bar: 20 µm.

Figure 2: XP-C fibroblasts exert a pro-mitotic effect on SCC13 cells

Scratch healing assay on SCC13 cells culture supernatants conditioned from either WT or XP-C fibroblasts in absence (A) or in presence (B) of mytomycine C. Representative images from the time lapse sequence of SCC13 cells 0 and 7h30 after scratching (upper panels). Lower panels: kinetics of wounding were quantified and expressed as the percentage of cell coverage over the initial cell-free zone, in surface units. (C) Quantification of the impact of XP-C fibroblasts culture supernatants on the distribution of SCC13 cells within the cell cycle in either the presence of WT or XP-C fibroblast; note the significant. (D) Immunoblot analysis of cyclin B1 in lysates of SCC13 cells cultured in medium conditioned by either WT (FH29, FTM1) or XP-C (AS433, AS875, AS798) fibroblasts. Beta tubulin was used as loading control. Right panel: Quantification of cyclin B1 expression in the different conditions. Values are presented as means ± SD. Note the significant increase of Cyclin B1 accumulation in SCC13 cell cultures in the presence of culture supernatants of WT versus XP-C fibroblasts $P<0.05$.

Figure 3: XP-C fibroblasts lead cancer cells

(A) Spheroid assays. SCC13 cells (labelled in green following GFP retraoviral transduction) in presence of either WT (A, left) or XP-C (A, right) fibroblasts invade the surrounding matrix (right panel) compared to spheroid composed of WT fibroblasts (left panel). Scale bar 250 µm. (B) Live confocal microscopy images of spheroids showing GFP SCC13 cells (Green) and XP-C fibroblasts (red) after different times of cultures following generation of spheroids. Lower

panels represent higher magnification of insets delineated in the upper panel of the figure. Scale bar: 40 µm. Arrowheads point to XP-C fibroblasts stained in red prior the experiment.

Figure 4: XP-C fibroblasts express high levels of HGF and activate the c-Met pathway in SCC13 cells in a p38 and JNK dependent manner.

(A) Accumulation of HGF mRNAs measure by RT-Q-PCR in WT (N = 3) and XP-C fibroblasts (n = 4). Values are presented as means ± SD. P<0.05. (B) Accumulation of HGF levels in culture media conditioned by WT (N = 3) and XP-C (N = 3) fibroblasts as measured by Elisa analyses. Values are presented as means ± SD. (C) Immunoblot analysis of Met and P-Met in lysates of SCC13 cells treated with culture media conditioned by WT (FH29) or XP-C (AS148, AS433, AS875, AS798) fibroblasts. GAPDH was used as loading/transfer control. Right panel: quantification of P-Met expression under the different conditions. Values are presented as means ± SD of two independent experiments. Asterisk $p < 0.05$. (D, E, F and G) Immunoblot analysis of phosphorylated froms of p38, JNK1,2,3, ERK1,2 and STAT3 in lysates of SCC13 cells treated with culture media conditioned by WT (FTM1, FH29) or XP-C (AS148, AS433, AS875, AS798) fibroblasts. Beta tubulin or GAPDH were used as controls of loading and transfer. For each western blot representative illustration, right panels show quantitative analyses of the amount of phosphorylated proteins under the indicated conditions. Values are presented as means ± SD of two independent experiments. Asterisk, $p < 0.05$.

Figure 5: Overexpression of HGF by XP-C fibroblasts contributes to the invasive phenotype of SCC13 cells

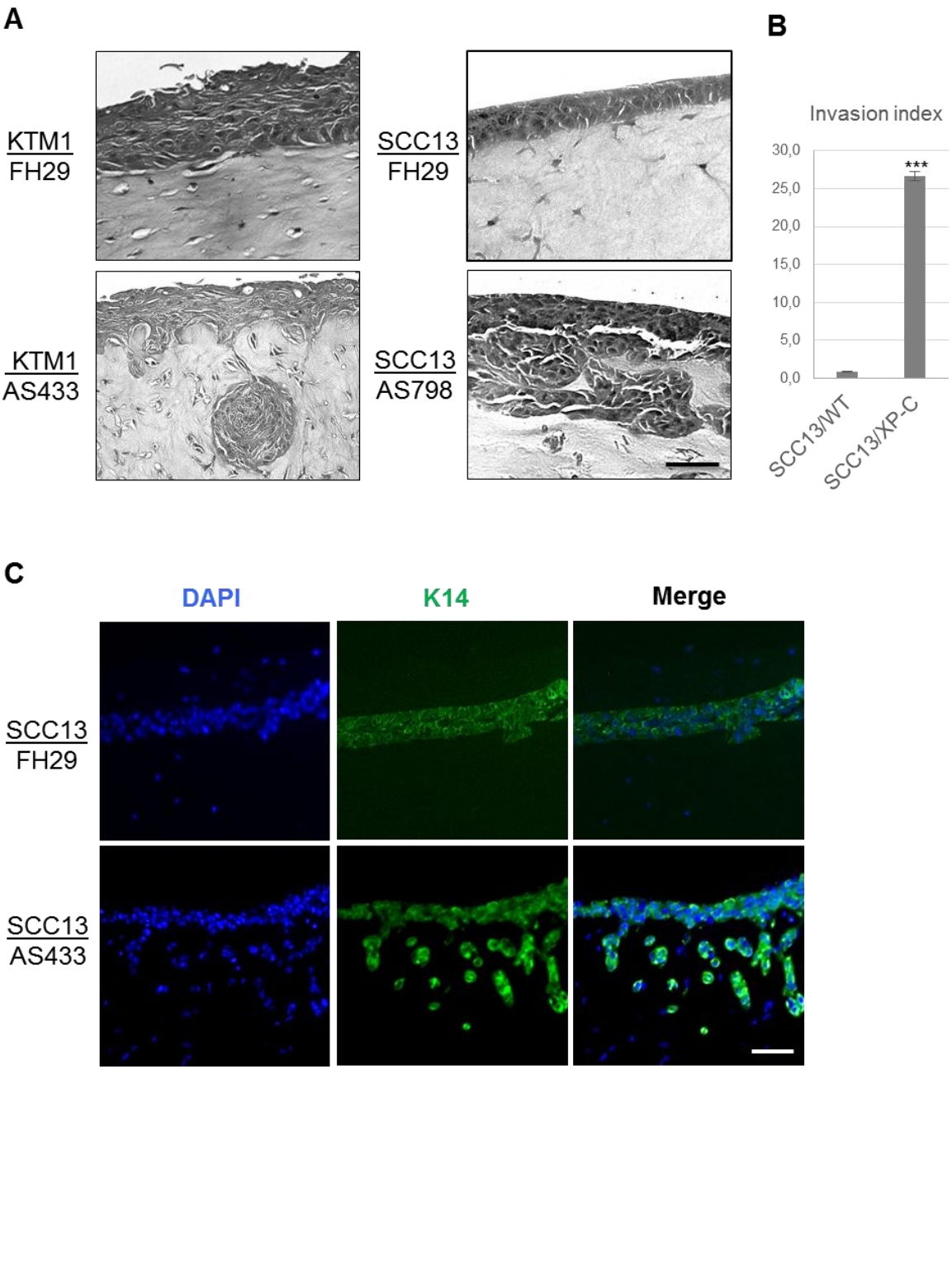
(A) Immunoblot analysis of total Met and P-Met in lysates of SCC13 cells cultured in media conditioned by XP-C (AS875, AS798) fibroblasts in absence (-) or in presence (+) of a HGF blocking antibody (HGFAb). GAPDH was used as loading control. Right panel: quantification of P-Met accumulation in the absence (-HGF Ab or the presence (+HGF Ab). Values are presented as means ± SD. Asterix $p < 0.05$ (0,01).

(B) Immunoblot analysis of P-p38 in lysates of SCC13 cells cultured in the presence of media conditioned by XP-C (AS798) fibroblasts in absence or presence of 1 and 1.2 ng/ml of HGF blocking antibody. Tubulin was used as loading/transfer control. Right panel: quantification of P-p38 expression in the different conditions. Values are presented as means ± SD. Asterix $p < 0.05$ (0,026). (C) Representative images of gel contraction assays of matrigel/collagen lattices populated with WT (FH29) or XP-C (AS433, AS798) in absence or presence of 0.66

ng/ml HGF blocking antibody. Scale bar, 0.3 cm. (D) Quantification of gel diameters under the different conditions as indicated. Values are presented as means ± SD. Asterix $p < 0.05$ (0,0001). (E) Representative images of stained sections of organotypic cultures composed SCC13 cells and XP-C (AS798) fibroblasts in absence or presence of 0.66 ng/ml HGF blocking antibody. Sections were stained with H&E or with K14 antibody (red) and nuclei (blue). Scale bar 50 µm.

Note the total inhibition of SCC13 cells invasiveness in the presence of XP-C fibroblasts and the anti-HGF/SF blocking antibody

Figure 1


A
KTM1
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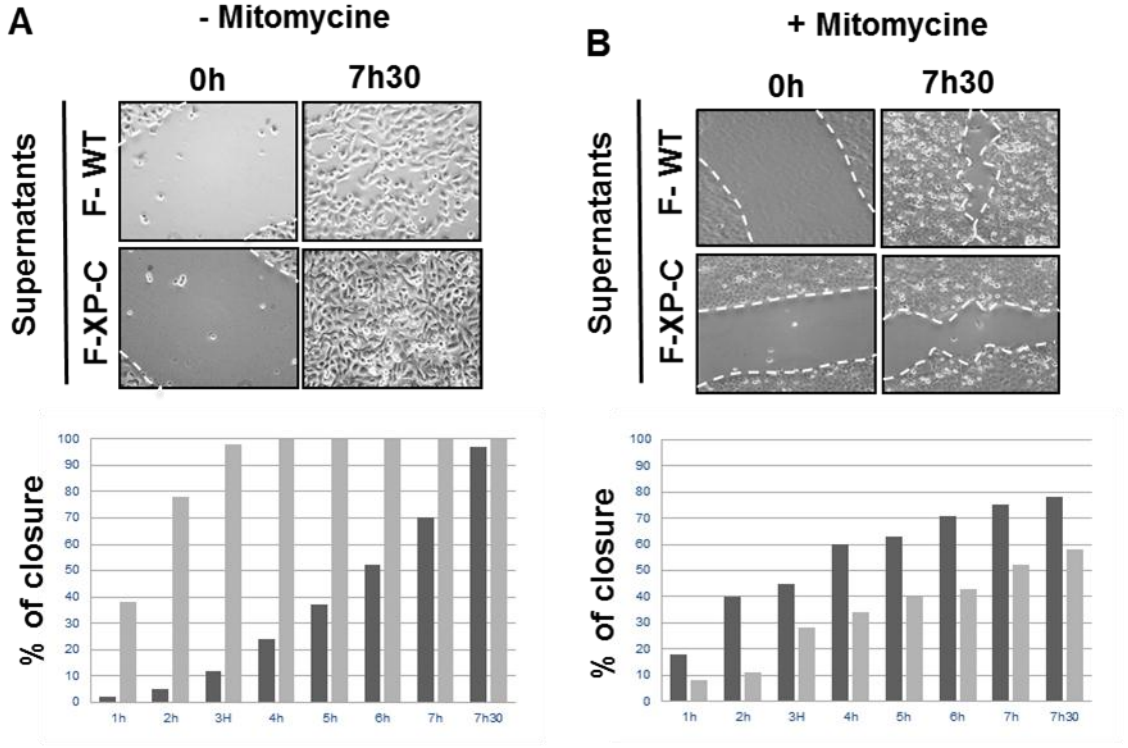
30,0
25,0
20,0
15,0
10,0
5,0
0,0
***
SCC13/WT
SCC13/XP-C
C
DAPI
K14
Merge
SCC13
FH29
SCC13
AS433

Figure 2



C

	SCC13 + Sn F-WT	SCC13 + Sn F-XP-C
G0-G1 (%)	73,4	61,7
S (%)	12	13,4
G2-M (%)	12,9	23,8

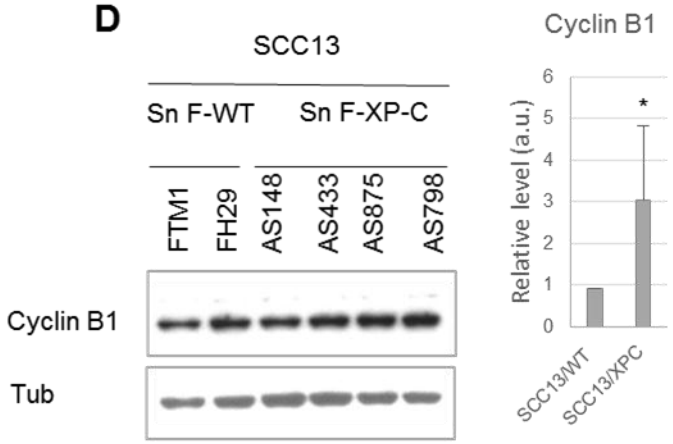


Figure 3

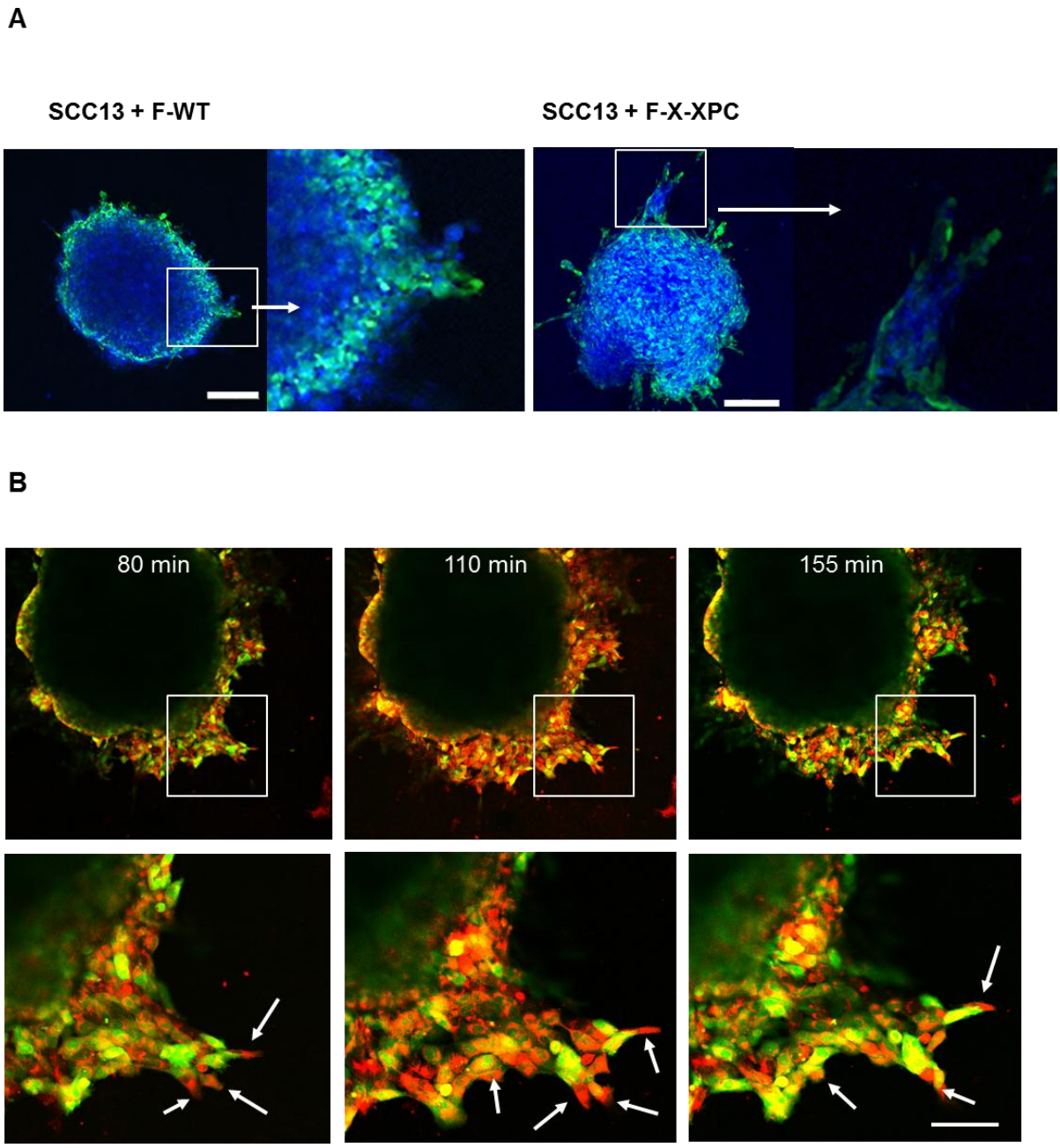


Figure 4

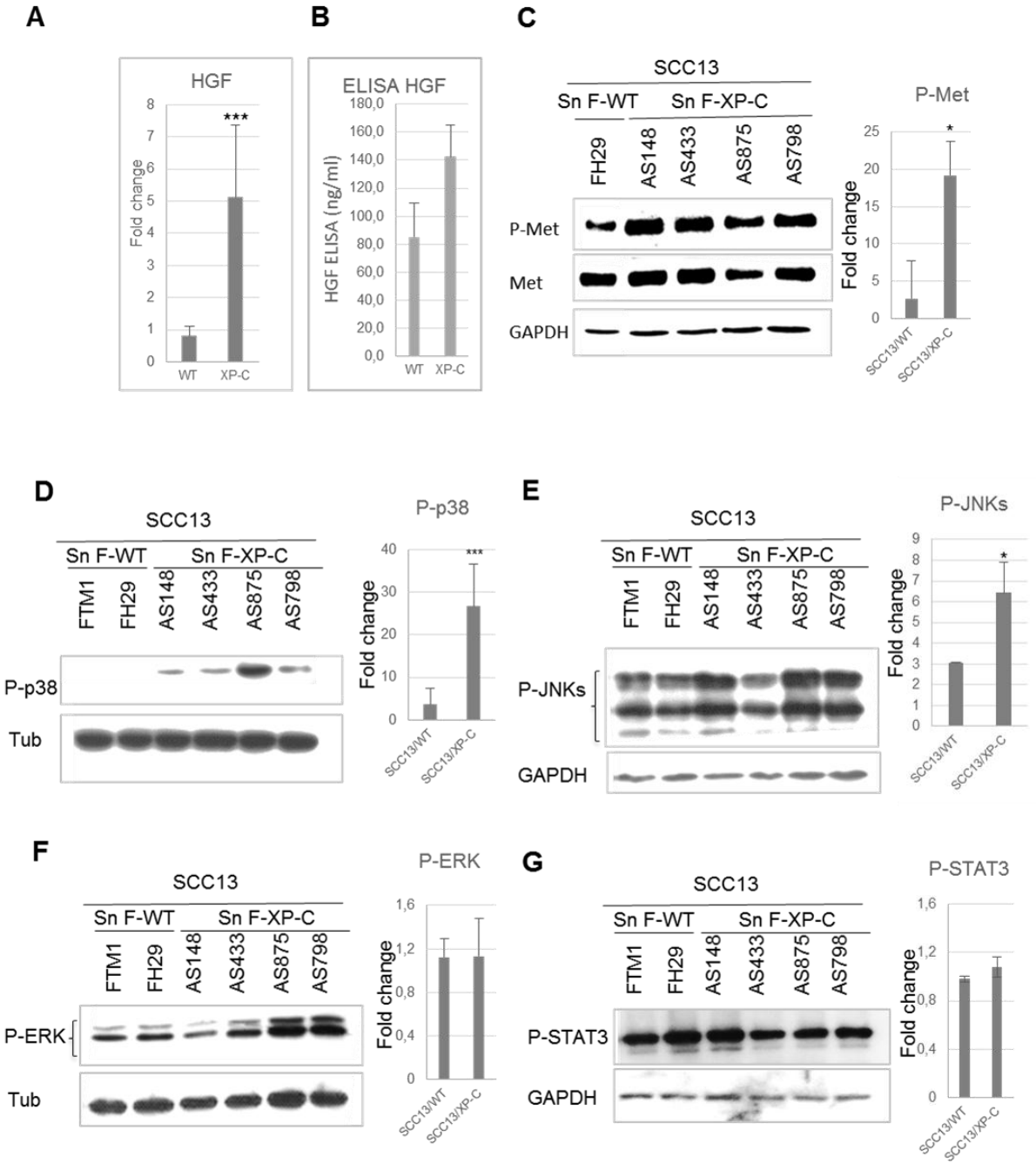
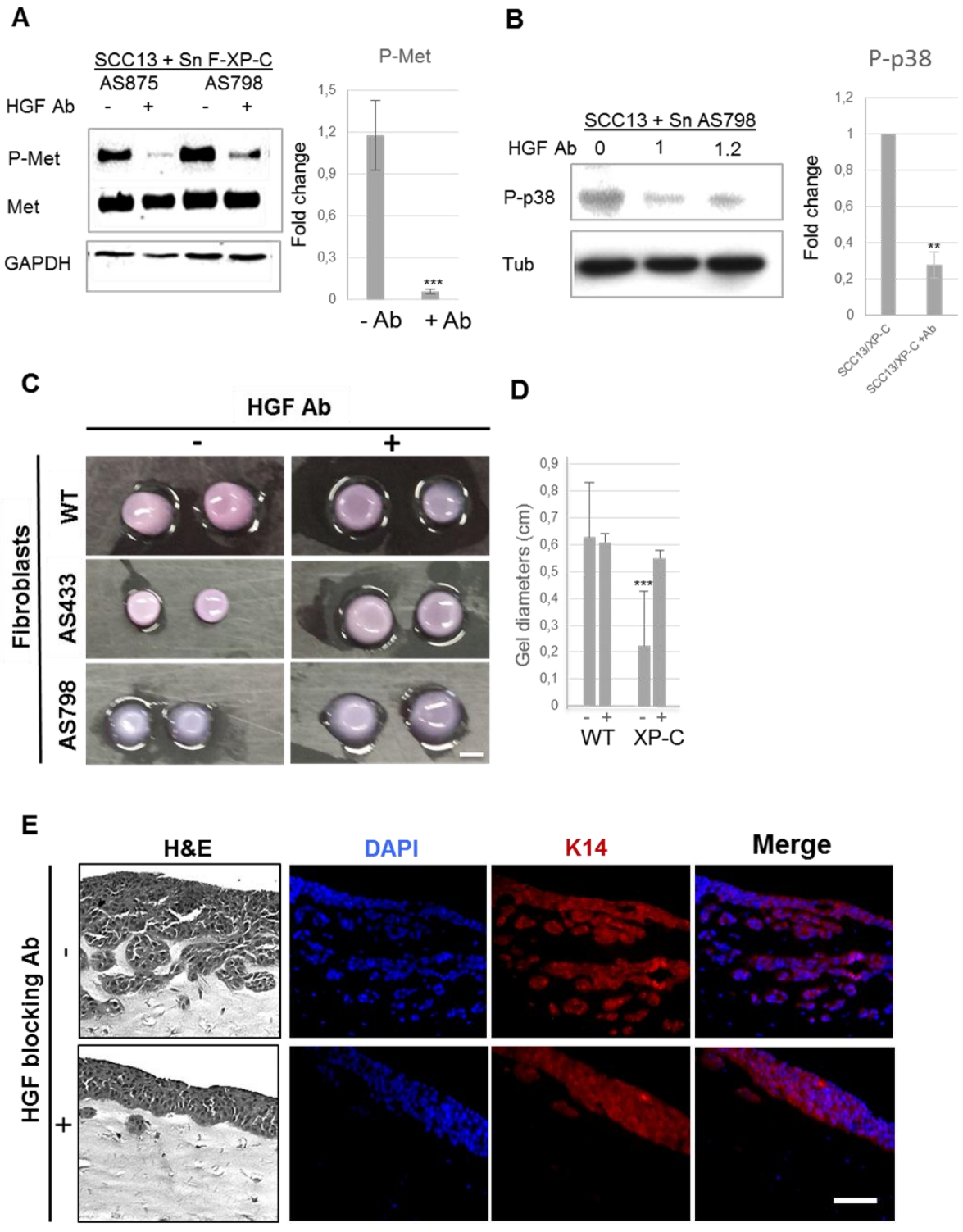

A
HGF
Fold change
***
WT
XP-C
B
ELISA HGF
HGF ELISA (ng/ml)
WT
XP-C
C
SCC13
Sn F-WT
Sn F-XP-C
FH29
AS148
AS433
AS875
AS798
P-Met
Met
GAPDH
P-Met
Fold change
*
SCC13/WT
SCC13/XP-C
D
SCC13
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-p38
Tub
P-p38
Fold change
***
SCC13/WT
SCC13/XP-C
E
SCC13
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-JNKs
GAPDH
P-JNKs
Fold change
*
SCC13/WT
SCC13/XP-C
F
SCC13
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-ERK
Tub
P-ERK
Fold change
SCC13/WT
SCC13/XP-C
G
SCC13
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-STAT3
GAPDH
P-STAT3
Fold change
SCC13/WT
SCC13/XP-C

Figure 5


A
SCC13 + Sn F-XP-C
AS875
AS798
HGF Ab - + - +
P-Met
Met
GAPDH
P-Met
Fold change
1,5
1,2
0,9
0,6
0,3
0
***
- Ab
+ Ab
B
SCC13 + Sn AS798
HGF Ab 0 1 1.2
P-p38
Tub
P-p38
Fold change
1,2
1
0,8
0,6
0,4
0,2
0
**
SCC13/XP-C
SCC13/XP-C +Ab
C
HGF Ab
-
+
Fibroblasts
WT
AS433
AS798
D
Gel diameters (cm)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
***
- + - +
WT
XP-C
E
H&E
DAPI
K14
Merge
HGF blocking Ab
-
+

Supplementary Data

S1. Table 1: Patients and cell characteristics.

S2. Table 2: Antibodies used for western blot.

S3. Table 3: Oligonucleotides used in quantitative reverse-transcription PCR experiments Gene.

Figure 1: Immunoblot analysis of phosphorylated and non-phosphorylated forms of (A) ERK1,2 (B) STAT3 and (C) p38 in lysates of SCC13 cells treated with culture media conditioned by WT (FTM1, FH29) or XP-C (AS148, AS433, AS875, AS798) fibroblasts.

Figure 2: Scratch assay movies. 1) SCC13/FH29 –MMC. 2) SCC13/FH29 +MMC. 3) SCC13/AS798 –MMC. 4) SCC13/AS798 +MMC*. *(MMC = Mitomycin).

Figure 3: Immunofluorescence staining of α-SMA in different types of fibroblasts. α-SMA was stimulated in FTM1 as well as in XP-C fibroblasts in the presence of TGF-β. CAF was used as control.

Figure 4: XP-C fibroblasts express high levels of LIF. Accumulation of LIF mRNAs measure by RT-q-PCR in WT (FTM1, FTM5, FH84) and XP-C fibroblasts (AS148, AS433, AS798).

Figure 5: A) Immunoblot analysis of phosphorylated of JUN in lysates of SCC13 cells treated with culture media conditioned by WT (FTM1, FH29) or XP-C (AS148, AS433, AS875, AS798) fibroblasts. **B)**) Immunoblot analysis of P-JNK in lysates of SCC13 cells cultured in the presence of media conditioned by XP-C (AS798) fibroblasts in absence or presence of 1 and 1.2 ng/ml of HGF blocking antibody. Tubulin was used as loading/transfer control.

Figure 6: HGF expresses by XP-C fibroblasts and activates the c-Met pathway in SCC12 cells in a p38 dependent manner. Immunoblot analysis of phosphorylated p38 (A), JNK1,2,3 (B), ERK1,2 (C) and STAT3 (D) in lysates of SCC13 cells treated with culture media conditioned by WT (FTM1, FH29) or XP-C (AS148, AS433, AS875, AS798) fibroblasts. Beta tubulin or GAPDH were used as of loading and transfer controls.

Supplementary data 1

Patients and cell characteristics

Fibroblasts	Age at biopsy	Race	Phenotype	Clinical characteristics	Onset of tumors
FTM1	years	40 years	NA	NA	NA
FH29	30 years	Caucasian	NA	NA	NA
XP148	12 years	Caucasian	XP-C	Multiple BCCs and SCCs, face and exposed areas	9 years
XP433	2 months	Caucasian	XP-C	NA	NA
AS875	20 years	Caucasian	XPC		
AS798	2 ans	Caucasian	XP-C	Photovieillissement (mains). Kératose Actinique. Ephélides	

Supplementary data 2

Antibodies used for western blot.

Nom	Type	Clone	dilution	Fournisseur	Reference	Taille
Phospho-p44/42 MAP Kinase (Thr202/Tyr204)	Rabbit	X	1:1000 (WB)	Cell Signaling Technology	9104	42, 44 kDa
Phospho-p38 MAP Kinase (Thr180/Tyr182)	Rabbit	X	1:1000 (WB)	Cell Signaling Technology	9211	43 kDa
Phospho-SAPK/JNK1 Thr183/Tyr185)	Rabbit	X	1:1000 (WB)	Cell Signaling Technology	9251	46 kDa (Phospho-JNK1) 54 kDa (Phospho-JNK2/3)
Anti-rabbit IgG HRP-linked antibody	Goat	X	1:1000 (WB)	Cell Signaling Tecnhology	7074	X
c-Jun (N-term)	Rabbit monoclonal IgG	X	1:2000 (WB)	Epitomics	1254-5	39 kDa
Cyclin B1	Mouse monoclonal IgG	V152	1:1000 (WB)	Abcam	ab72	48 kDa
GAPDH	Mouse monoclonal IgG	X	1:5000 (WB)	Abcam	ab9484	40.2 kDa
p44/42 MAP Kinase	Rabbit polyclonal IgG	X	1:1000 (WB)	Cell Signaling Technology	9102	42, 44 kDa
p38 MAP kinase	Rabbit polyclonal IgG	X	1:1000 (WB)	Cell Signaling Tecnhology	9212	43 kDa
SAPK/JNK (56G8)	Rabbit monoclonal IgG	56G8	1:1000 (WB)	Cell Signaling Technology	9258	46, 54
Phospho-Met (Tyr1234/1235)	Rabbit monoclonal IgG	NL8	1:10000 (WB)	Millipore	04-9000	140 kDa
Met (D1C2) XP	Rabbit monoclonal IgG	D1C2	1:1000 (WB)	Cell Signaling Technology	8198	140, 170 kDa
STAT3 (phospho Y705)	Rabbit monoclonal IgG	X	1:10000 (WB)	Abcam	ab76315	88 kDa
Inhibitor p38				Santa Cruz	sc-204168	
HGF	Mouse monoclonal IgG1	24612		R&D	MAB294	
Cytokératine 14	Mouse monoclonal	LL002		Novocastra	NCL-LL002	
β-Tubulin	Mouse monoclonal	TUB 2.1		Sigma-Aldrich	T4026	

Table 3: Oligonucleotides used in quantitative reverse-transcription PCR experiments

Gene		
GAPDH	**F- CAGTCCATGCCATCACTGCCACCCAG**	**R- CAGTGTAGCCCAGGATGCCCTTGAG**
SB34	**F- GCATCAGTACCCCATTCTATCAT**	**R- AGGTGTAATCCGTCTCCACAGA**
HGF	**F- TCACGAGCATGACATGACTCC**	**R- AGCTTACTTGCATCTGGTTCC**
Met	**F- CATGCCGACAAGTGCAGTA**	**R- TCTTGCCATCATTGTCCAAC**

Figure 1

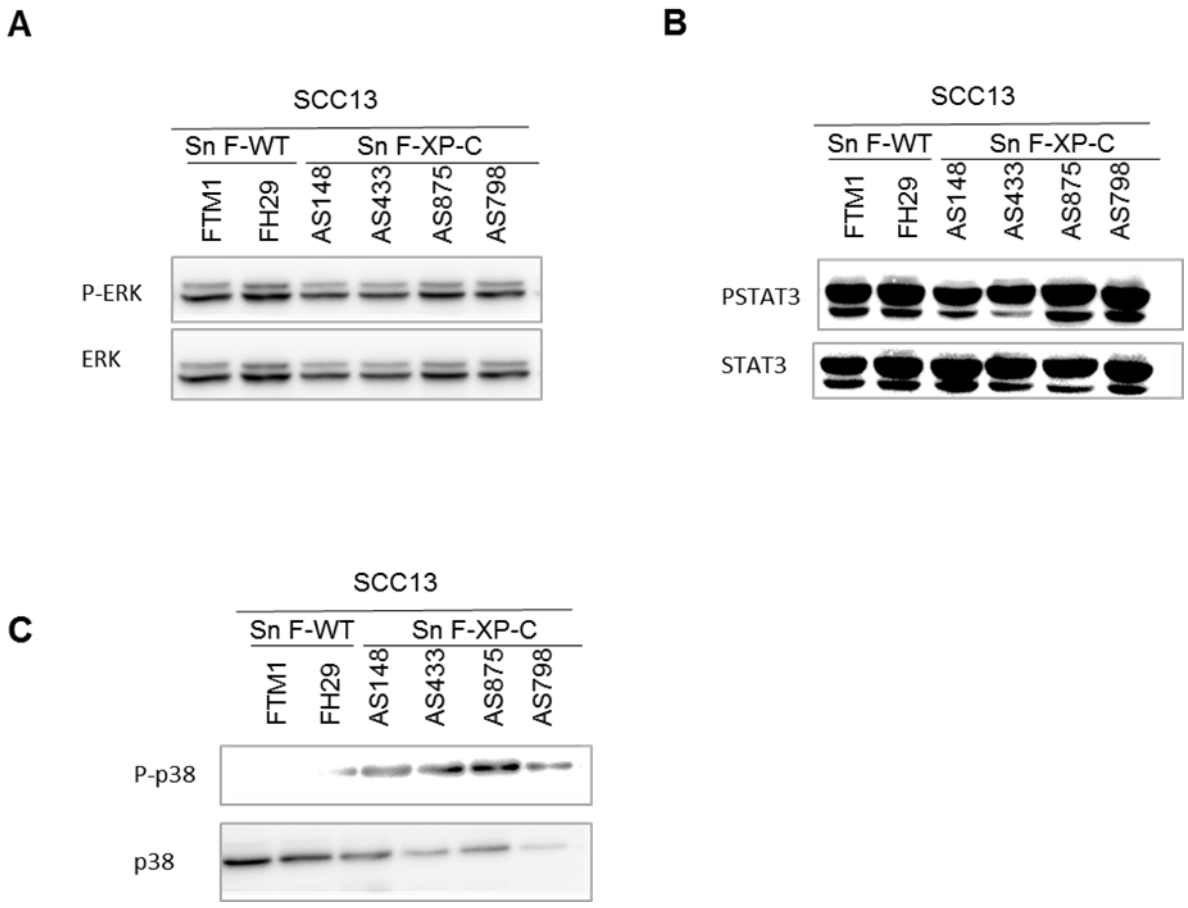
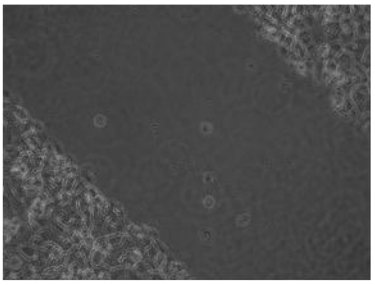
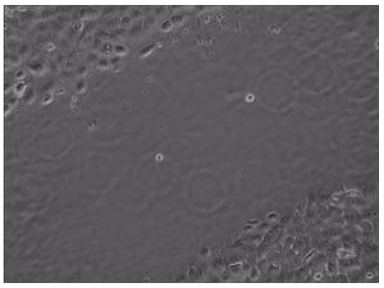

A
SCC13
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-ERK
ERK
B
SCC13
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
PSTAT3
STAT3
C
SCC13
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-p38
p38

Figure 2

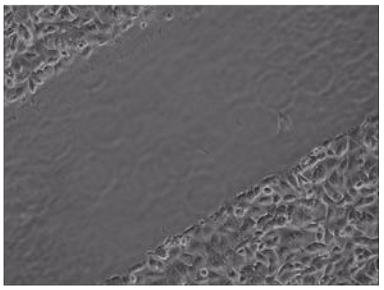
1) SCC13/WT - MMC



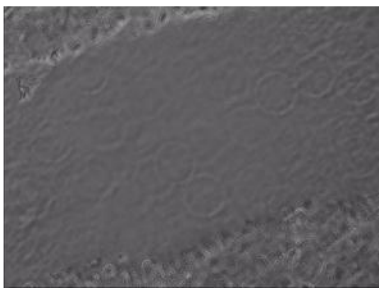
2) SCC13/WT + MMC



3) SCC13/XP-C - MMC



4) SCC13/XP-C + MMC



Média1 SCC13WT+MMC.avi

Média1 SCC13XP-C-MMC.avi

Média1 SCC13XP-C+MMC.avi

Média1 SCC13WT-MMC.avi

Figure 3

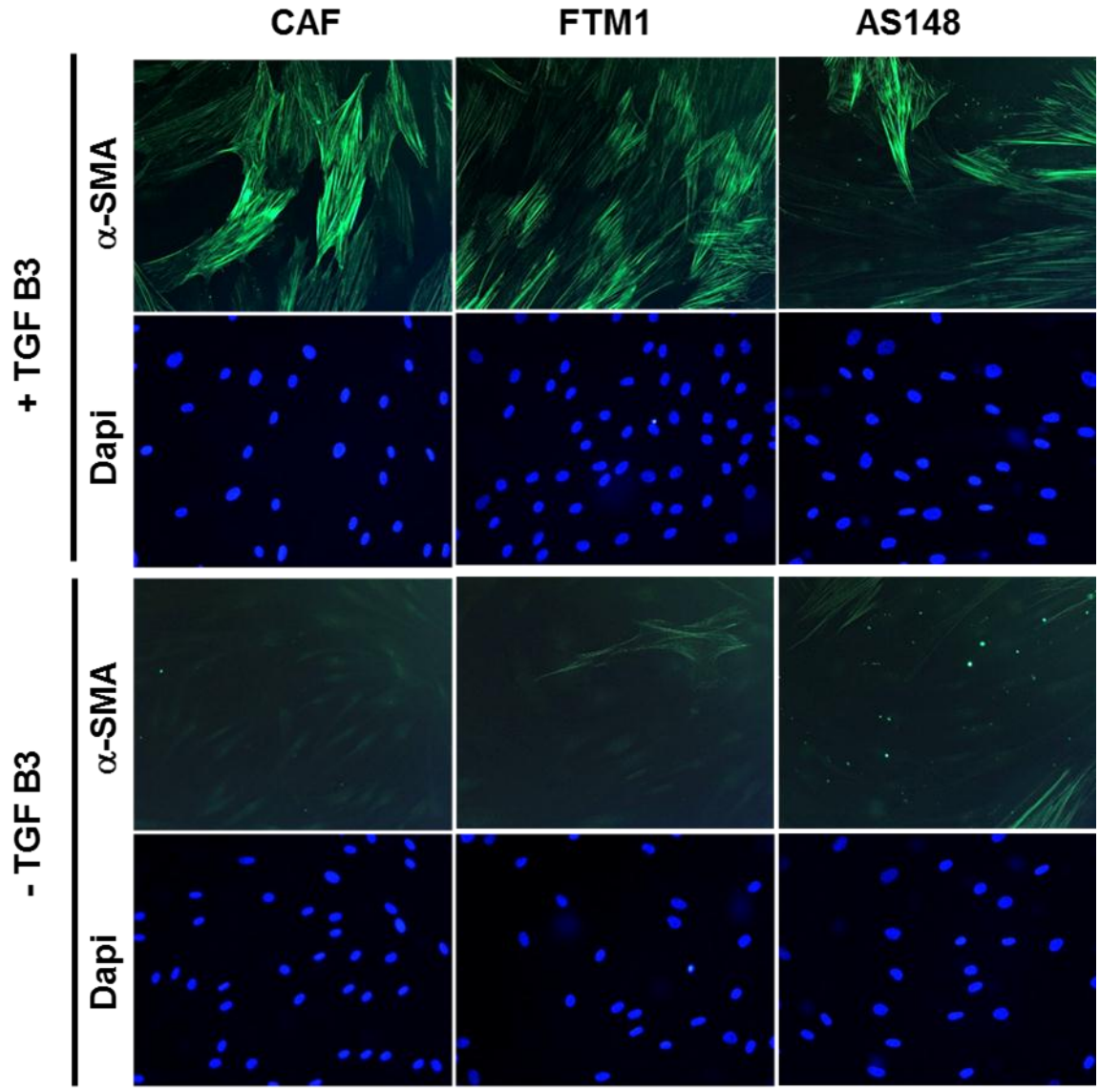


Figure 4

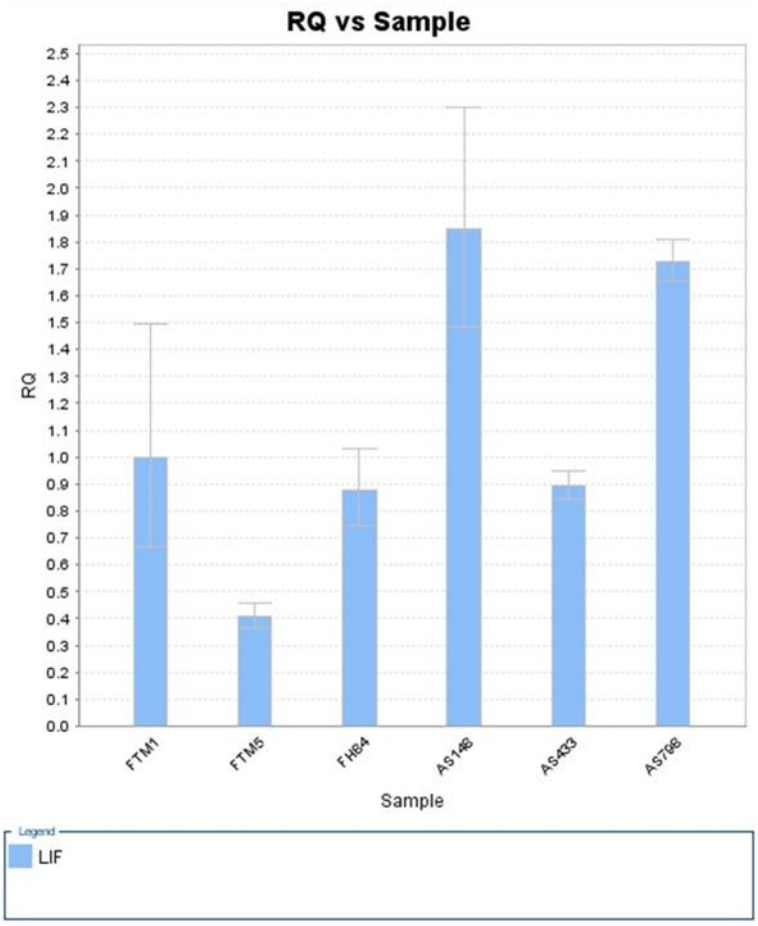

RQ vs Sample
RQ
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
FTM1
FTM5
FH84
AS148
AS433
AS796
Sample
Legend
LIF

Figure 5

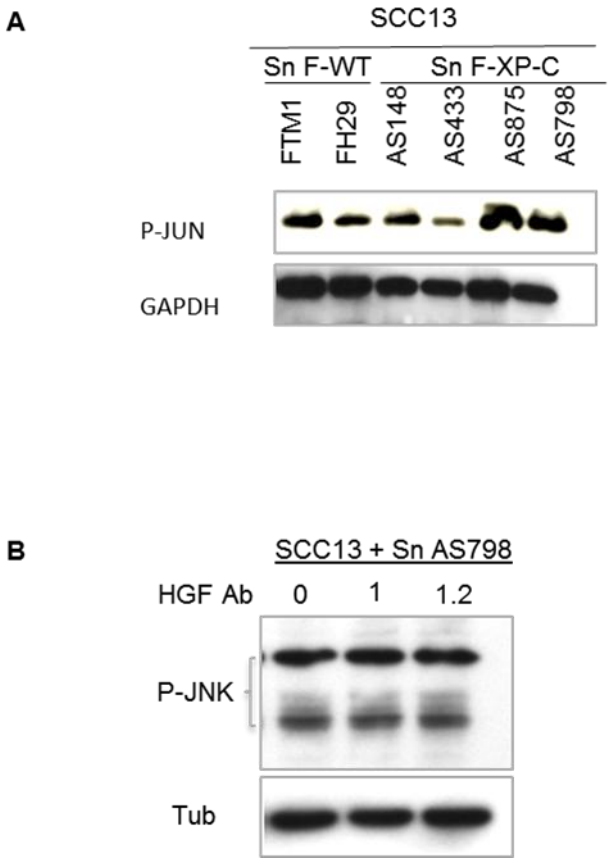
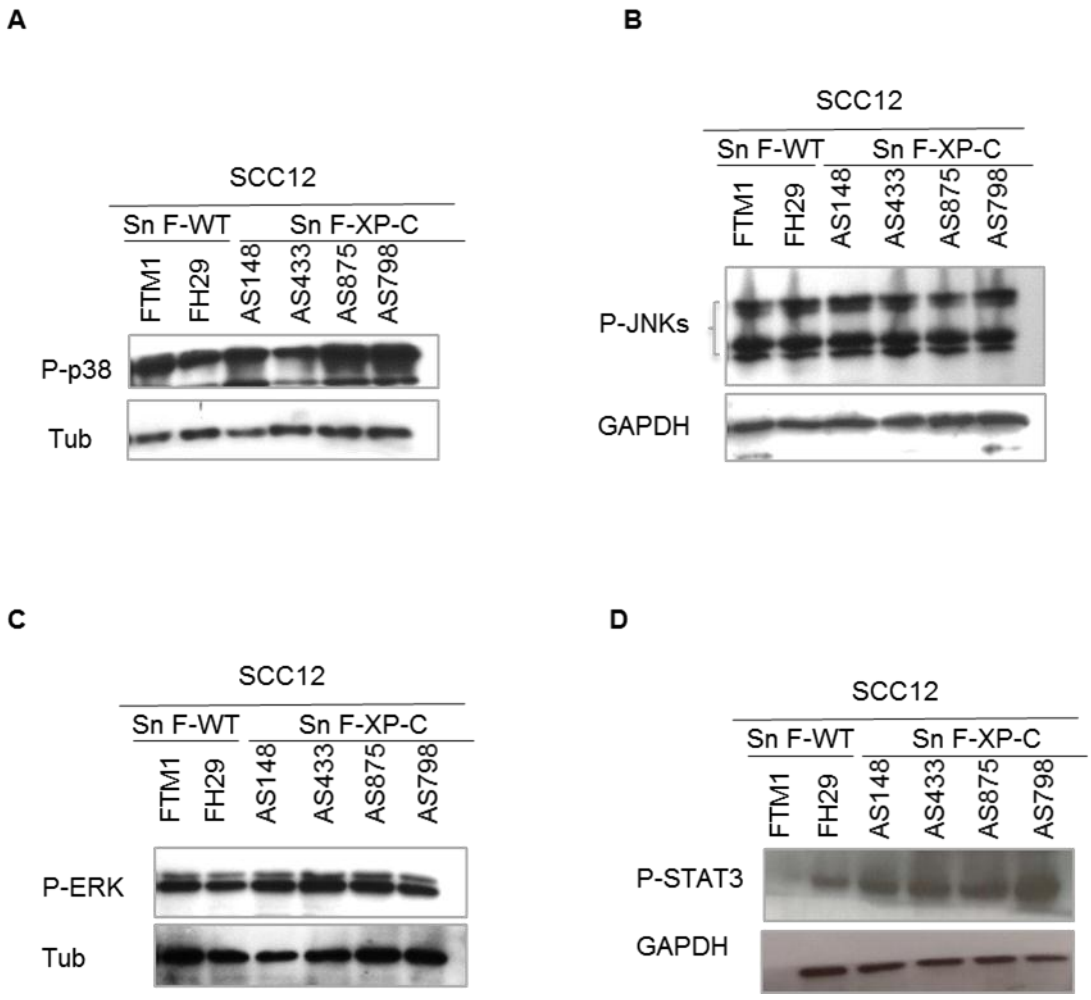

A
SCC13
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-JUN
GAPDH
B
SCC13 + Sn AS798
HGF Ab
0
1
1.2
P-JNK
Tub

Figure 6


A
SCC12
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-p38
Tub
B
SCC12
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-JNKs
GAPDH
C
SCC12
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-ERK
Tub
D
SCC12
Sn F-WT
Sn F-XP-C
FTM1
FH29
AS148
AS433
AS875
AS798
P-STAT3
GAPDH

DISCUSSION AND PERSPECTIVES

Discussion and perspectives

I. Characteristics of XP-C fibroblasts

I. a. XP-C fibroblasts present an aged-like phenotype

Previous studies of the laboratory have shown that dermal fibroblasts isolated from non photoexposed skin of XP-C patients have an aged-like phenotype. In collagen I dermal equvalents, they present elongated and dendritic shape compared to wild type fibroblasts. These features are reminiscent of chronically and photoaged skin in individuals from the general population (Fligiel, Varani et al. 2003, Varani, Schuger et al. 2004, Frechet, Warrick et al. 2008).

In the general population, MMP1, a zinc-containing endopeptidases enzyme, and ROS, accumulate after skin exposition to UVR (Stadtman 1992, Fisher, Quan et al. 2009). In the general population, MMP1 is increased with age; this enzyme degrades collagen I of the extracellular matrix (ECM) and contributes to skin aging and carcinogenesis (Scharffetter, Wlaschek et al. 1991, Fisher, Datta et al. 1996, Pittayapruek, Meephansan et al. 2016). MMP1 accumulates in AKs and cSCC, suggesting its role in tumor dissemination and metastasis (Tsukifuji, Tagawa et al. 1999, Overall and Kleifeld 2006). Previous *in vitro* studies from the laboratory have shown the accumulation of MMP1 in XP-C fibroblasts isolated from non photoexposed skin areas as well as in skin sections of XP-C patients (Frechet, Warrick et al. 2008). Other observations have shown that XP-C keratinocytes are prone to invade dermal equivalent populated with XP-C primary fibroblasts (Bernerd, Asselineau et al. 2001). Altogether, these data strongly suggested that XP-C fibroblasts could play an active role in ECM remodeling and promotion of cancer cells invasion.

In their study, Frechet and colleagues reported that the amount of ROS increased 1.5 fold in XP-C fibroblasts compared to wild type fibroblasts (Frechet, Warrick et al. 2008). In good agreement, the activity of catalase, an enzyme protecting the cell from oxidative damages through the conversion of the H202 into oxygen and water, was found decreased in all XP cells tested. Accumulation of ROS presumably contributes to the aged-like phenotype of XP-C fibroblasts and, as one consequence, the propensity of XP-C patients to SCC development (Liotti, Bodo et al. 1987, Vuillaume, Daya-Grosjean et al. 1992). We thus aimed at further characterizing the proinvasive phenotype expressed in XP-C fibroblasts in absence of UV exposure.

I. b. XP-C fibroblasts express high levels of hepatocyte growth factor (HGF), a growth factor implicated in carcinogenesis

Our data indicated that SCC13 cells treated with culture supernatants of XP-C fibroblasts have increased expression of cyclin B1 protein; in accordance, the ratio in G2-M phase of cell cycle is increased by about 100 %, suggesting that XP-C secretome has a mitogenic effect on SCC13 cells. We also found that XP-C fibroblasts expressed and secreted high amounts of the Hepatocyte growth factor/scatter factor (HGF/SF) compared to controls fibroblasts. We hypothesized that XP-C fibroblasts act in a paracrine manner. Indeed, culture supernatants conditioned by primary XP-C fibroblasts activate the c-Met pathways in SCC13 cells. This activation of c-Met was counteracted in presence of HGF blocking antibodies. Moreover SCC13 scratch closure was accelerated in the presence of XP-C fibroblasts culture supernatants while pretreatment of SCC13 cells with mitomycin C counteracted this effect. This suggested a mitogenic effect of HGF secreted by XP-C fibroblasts on SCC13 cells.

HGF was discovered in 1984 and was described as a mitogenic protein for hepatocyte cells and then was identified as the ligand for the proto-oncogene c-Met receptor (Nakamura, Nawa et al. 1984, Naldini, Vigna et al. 1991). HGF is secreted by mesenchymal cells and acts as a multi-functional cytokine on cells from epithelial origin. HGF regulates cell growth, cell motility and morphogenesis in numerous cells and tissue types and plays a role in angiogenesis and tissue regeneration. It is considered as a mitogen (stimulation of cell growth), a motogen (stimulation of cell motility), and a morphogen (induction of multicellular tissue-like structure) factor (Nakamura 1994).

Studies by Daniels and their colleagues have reported that HGF is implicated in the cancerogenesis in human corneal epithelial cells (HCEC). These studies showed that exogeneous HGF, in dose dependant manner, promotes HCEC migration and increases MMP 2 and 9 secretion (Daniels, Limb et al. 2003).

Other studies reported a cross-talk between HGF/Met and EGF. In human retinal pigment epithelium cells (RPE), HGF transactivates EGFR, leading to activation of downstream pathways such as AKT and JNK and to DNA synthesis and cell proliferation. In contrast, EGFR activation induces c-Met ectodomain shedding and leads to the downregulation of the HGF signaling in RPE. Inhibitors of the EGFR kinase

blocked wound healing and EGFR transactivation in these cells, suggesting that c-Met and EGFR play a key role in regulating RPE cell migration, proliferation, and wound healing (Christensen, Burrows et al. 2005, Xu and Yu 2007, Furlan, Kherrouche et al. 2014).

In the light of these studies, several questions arose: is EGFR induced in SCC cells in the presence of XP-C fibroblasts culture supernatants? What happens if EGFR is blocked using kinase inhibitor? Did the blocking of EGFR inhibit scratch healing of SCC cells even in the presence of HGF? And what about EGFR downstream activation (Akt, JNK) and cell cycle?

HGF promotes the migration and invasion of uval melanoma (UM) cells by activating its receptor c-Met and the downstream PI3K/AKT pathways. Blocking HGF with antibodies inhibited the migration and the invasion of cells. In xenograft models, inhibited HGF significantly decreased tumor growth in nude mice by suppressing the phosphorylation of c-Met and PI3K/AKT (Wang, He et al. 2017). Other studies showed that using combination of anti-cMet antibodies may oppose UM intrinsic resistance to MEK inhibitors (Cheng, Chua et al. 2017).

HGF causes disruption of cell junctions and causes a rapid elevation of unbound β-catenin. In turn, unbound β-catenin translocates in the nucleus. Activation of its transcriptional targets, in a WNT independent manner, induces local motility and cells scattering (Stoker, Gherardi et al. 1987, Weidner, Behrens et al. 1990, Murai, Shen et al. 2004).

Other studies have reported that HGF blocking antibodies inhibit HGF-mediated c-Met receptor phosphorylation and, subsequently, the proliferation and survival of the glioblastoma U-87 MG cells and the invasion of the MDA-MB-435 melanoma cell line in matrigel. Importantly, *in vivo,* these antibodies impaired HGF-dependent tumor growth and caused significant regression of tumors development following xenografts of U-87 MG glioblastoma cells in nude mice (Burgess, Coxon et al. 2006, Kim, Wang et al. 2006).

c-Met is a transmembrane receptor tyrosine kinase (RTKs). HGF and c-Met have essential functional roles in embryonic development and organogenesis and are strongly implicated in carcinogenesis (Liu, Newton et al. 2010, Lee, Kim et al. 2011).

Studies have shown that in c-Met mutant mice, epidermal keratinocytes were unable to reepithelialize skin wounds, suggesting that c-Met is not only essential for cell growth and migration during embryogenesis but also to stimulate epithelial proliferation during (skin) wound healing (Chmielowiec, Borowiak et al. 2007).

c-Met is found to be involved in melanocyte growth and melanoma development. In human melanocytes and mouse melanoma cells, c-Met is overexpressed by the activation of cyclic adenosine monophosphate (cAMP) (used in cellular signal transduction). Up-regulation of c-Met expression by cAMP leads to the activation of HGF signaling and allows HGF to protect melanocytes and melanoma cells from apoptosis and promote their survival (Beuret, Flori et al. 2007).

It has been shown that the c-Met proto-oncogene was activated under hypoxia conditions, leading to amplification of the HGF signaling and promoting branching morphogenesis and invasion of cancer cells. Conversely, inhibition of c-Met expression prevents hypoxia-induced invasive growth. These data show that hypoxia promotes tumor invasion by sensitizing cells to HGF stimulation, providing a molecular basis to explain c-Met over-expression in human cancers (Pennacchietti, Michieli et al. 2003). In the light of these studies, study by Frechet, showed that using the antioxidant, Epigallocatechin gallate (EGCG) led to decrease both ROS and the secreted MMP1 in XP-C cells (Frechet, Warrick et al. 2008).

Can ROS be implicated in HGF overexpression in XP-C fibroblasts? It will be interesting to treat XP-C fibroblasts with antioxidants and look at the level of HGF and as consequences, at the migration and invasion of cancer cells. Such an approach could have a therapeutic impact in the future.

Studies on two cell lines derived from the same cervical carcinoma have reported that HGF induced collagen gel contraction (Wong, Leung et al. 2000, Okazaki, Yoshimura et al. 2002). Gel contraction was also accelerated in the presence of oral fibroblasts. Compared to skin fibroblasts, oral fibroblasts produce higher level of HGF than the dermal fibroblasts (Okazaki, Yoshimura et al. 2002, Shannon, McKeown et al. 2006). Our results using a collagen/matrigel matrix showed that XP-C fibroblasts exhibit a higher ability to contract the lattice compared to wild type fibroblasts. Interestingly, incoporation of HGF blocking antibodies in the gel blocked lattices contraction.

The process of collagen gel contractions has been shown to be regulated by cytokines such as TGF-β, IL-1 and HGF. This regulation appeared complex, and could involved synthesis and degradation of matrix components, as well as the tension generated in the cytoskeleton of the resident cells.

In 1997, Ingber using the model of tensegrity (model proposed that cells are as hard wired and can transmitted the mechanical stresses signals between the cells and ECM through for example integrins and with other cells through cadherins, selectins, and cellular adhesion molecules). Many signal transducing molecules that are activated by cell binding to growth factors and extracellular matrix associate with cytoskeletal scaffolds within focal adhesion complexes and that lead to change in scaffold geometry or molecular mechanics (Ingber 1997).

TGF-β is known to enhance wound healing (Hebda 1988, Beck, Chen et al. 1990). The mode of action of TGF-β depends on the nature of cell-cell contacts and cell shape, the presence or absence of specific molecules found in ECM, as well as the presence or absence of other cells, which may amplify or modify its actions (Sporn and Roberts 1990).

In human osteoblast-like MG-63 cells cultured within collagen gels, TGF-β stimulates matrix accumulation through increased expression of ECM molecules as collagen and enhanced MMP-1, MMP-3, and α2 integrin mRNA levels (Parreno and Hart 2009). In MG-63 cells collagen gel contraction occurs through two mechanisms, one involving the endogenous cytoskeleton and the other, cell motility (Katoh, Kano et al. 2001).

In a general manner, tumor microenvironment consists of tumor, immune, stromal, and inflammatory cells. This microenvironment produces cytokines, growth factors, and adhesion molecules that promote tumor progression and metastasis. Of particular interest in this setting is interleukin-1 (IL-1), a pleiotropic cytokine with numerous roles in both physiological and pathological states. IL1 is known to be up regulated in many tumor types and has been implicated as an important factor in tumor progression via the expression of metastatic and angiogenic genes and growth factor (Lewis, Varghese et al. 2006).

Interleukin-1 (IL-1) includes a family of closely related genes; the two major agonistic proteins, IL-1α and IL-1β, affect mainly inflammation, immunity and hemopoiesis. IL-1 is abundant at tumor sites, where it may affect the process of carcinogenesis, tumor growth and invasiveness and also the patterns of tumor–host interactions (Apte, Dotan et al. 2006). Interestingly, recent studies have also shown IL-1 to be involved in the dynamics of the actin cytoskeleton and cell junctions. For example, treatment of different epithelia with IL-1*α* often results in activation of RhoA kinase signaling pathway and the restructuring of actin network and cell junctions, thereby leading to junction disassembly (Lie, Cheng et al. 2012). It has been found that, Rac1 (subfamily of GTPase proteins) participates in IL-1 activity for the formation of lamellipodia in SH-SY5Y neuroblastoma cells that were grown on collagen and treated with IL-1*β* (Barth, Stewart-Smeets et al. 2009).

Other factor can also be implicated in the cytoskeleton organization is HGF. In Caco-2 colon cancer cells, adhesion molecules, and structural proteins such as E-cadherin, villin, and F-actin were upregulated by HGF and actin fibers were reorganized (Kermorgant, Dessirier et al. 2001).

In small cell lung cancer (SCLC), it was reported that HGF/c-Met activation pathway contributes to ROS increase (Jagadeeswaran, Jagadeeswaran et al. 2007). Hypoxia was shown to induce a reorganization of F-actin in primary cultures of human umbilical vein endothelial cells (HUVECs). Thus was characterized by the accumulation of stress fibers, the recruitment of vinculin to focal adhesions between the integrin and the actin, and loss the ruffles of cell membrane. Moreover, hypoxia-induced p38 activation results in activation MAPK kinase 2/3, and phosphorylation of the F-actin polymerization modulator and heat shock protein 27 (HSP27) (Huot, Houle et al. 1997).

In the light of this finding we can hypothesize that an interaction between ROS and HGF in XP-C fibroblasts cells could exist. HGF can induces carcinoma cells migration and invasion through 1) alteration of carcinoma cells cytoskeleton organization via the interaction between HGF and ROS. 2) HGF activate p38 pathways which implicate in the reorganization of carcinoma structural proteins. We can summarized these data by: Molecules targeting HGF/c-Met, ROS or p38 can be used

to prevent carcinoma cells structural transformations. Thus provide novel therapeutic intervention for XP-C patients and as well as for general population.

Study by Cheng and their colleagues have been reported that there is a complex functional roles for HGF and TGF-β signaling in mediating tumor-stromal interactions during mammary tumor cell scattering and invasion, with important implications in the metastatic process (Cheng, Chytil et al. 2008). Knocked out the TGF-β type II receptor gene in mouse mammary fibroblasts (Tgfbr2fspKO) was enhanced HGF expression at RNA level as well as protein secretion level (Cheng, Bhowmick et al. 2005, Cheng, Chytil et al. 2007).

A number of studies have shown an important role for transforming growth factor-b (TGF-β) signaling in regulating stromal-tumor interactions. TGF-β signaling exerts multiple effects in both stromal and epithelial cells, including inhibiting cell proliferation, promoting motility, and regulating differentiation. All of these cellular functions are achieved by binding of the TGF-β ligand to its cell surface type II receptor, which leads to recruitment and activation of its type I receptor and subsequent downstream signaling in the cytoplasm of multiple pathways, including SMAD, mitogen-activated protein kinase (MAPK), and Rho pathways (Daniel, Robinson et al. 2001, Pollard 2001).

By higlighting this study we can hypothesize that: TGF-□ can have a crucial role in the secretion of HGF in XP-C fibroblasts. It will be very interesting to study the level of TGF-□ in XP-C fibroblasts and also looking to the effect of inhibiting TGF-□ on the secertion and expression of HGF and to the downstream activation of TGF-□ as SMAD2 and ATF. As activating transcription factor (ATF) is implicated in the activation of stress pathway, and for sure as results on cancer cells promotion and invasion.

II. XP-C fibroblasts and carcinogenesis

II. a. Role of Stromal cells in carcinogenesis

Epithelial/mesenchymal interactions regulate the proliferation and differentiation of epithelial cells (Kalluri and Zeisberg 2006). The stroma has been considered for a long time as a passive element upon carcinogenesis. However, many evidences now suggest an active role of cancer associated fibroblasts (CAF) in evolution of cancer

cells and their dissemination. Carcinomas cells induce changes in the stroma due to the expression of growth factors, chemokines, and ECM proteases. Fibroblasts in the tumor stroma become "activated" (CAF) (Bhowmick, Chytil et al. 2004, Kalluri and Zeisberg 2006) and express markers such as alpha-smooth-muscle-actin (α-SMA), specific to the myofibroblasts that are involved in the healing process, Tenascin C, and Extradomain A-fibronectin (EDA-FN) (figure 34 A).

The ECM, together with cellular components of the tumor microenvironment (TME), are actively remodelled and reprogrammed by CAFs (figure 34 B). Metabolic reprogramming of dermal fibroblasts that become CAFs may also fuel the TME and other cells in the stroma enhance the growth of cancer cells (table 4).

Molecules	Functions
Intrcellular adhesion molecule 1 (ICAM1)	Serve as a docking site for activation or repression of immune cells
Programmed cell death protein 1 ligand 2, (PDL1) and (PDL2)	Mediate immunosuppressive functions of innate immune surveillance by Natural killer cells (NK), Prostaglandin E2 (PGE2)
Collagens, Fibronectin, Matrix metalloproteinases (MMPs), Tissue inhibitor of MMPs (TIMPs)	Alter the ECM and trigger the expression of Tenascin-C
Vascular endothelial growth factor (VEGF-A), Transforming growth factor-β (TGF-β), Platelet-derived growth factor- α (PDGF-α), Interleukin-6 (IL-6)	Increase vascular permeability, generate vascular leaks, leading to reactive perivascular areas containing fibrin and platelets, angiogenesis
Chemoattractant protein 1 (MCP1), Chemokines as CXCL9, CXCL10, Stroma derived factor-1 (SDF-1)	Immune cells activation and recruitment
Epidermal growth factor (EGF), Insulin growth factor (IGF), Interleukin-6 (IL-6)	Communicate with cancer cells, provide potential oncogenic signals to promote cancer cells survival, proliferation, invasion and metastasis

Table 4: Communication between CAF and other cells in the stroma. According to: (Kalluri and Zeisberg 2006, Kalluri 2016)

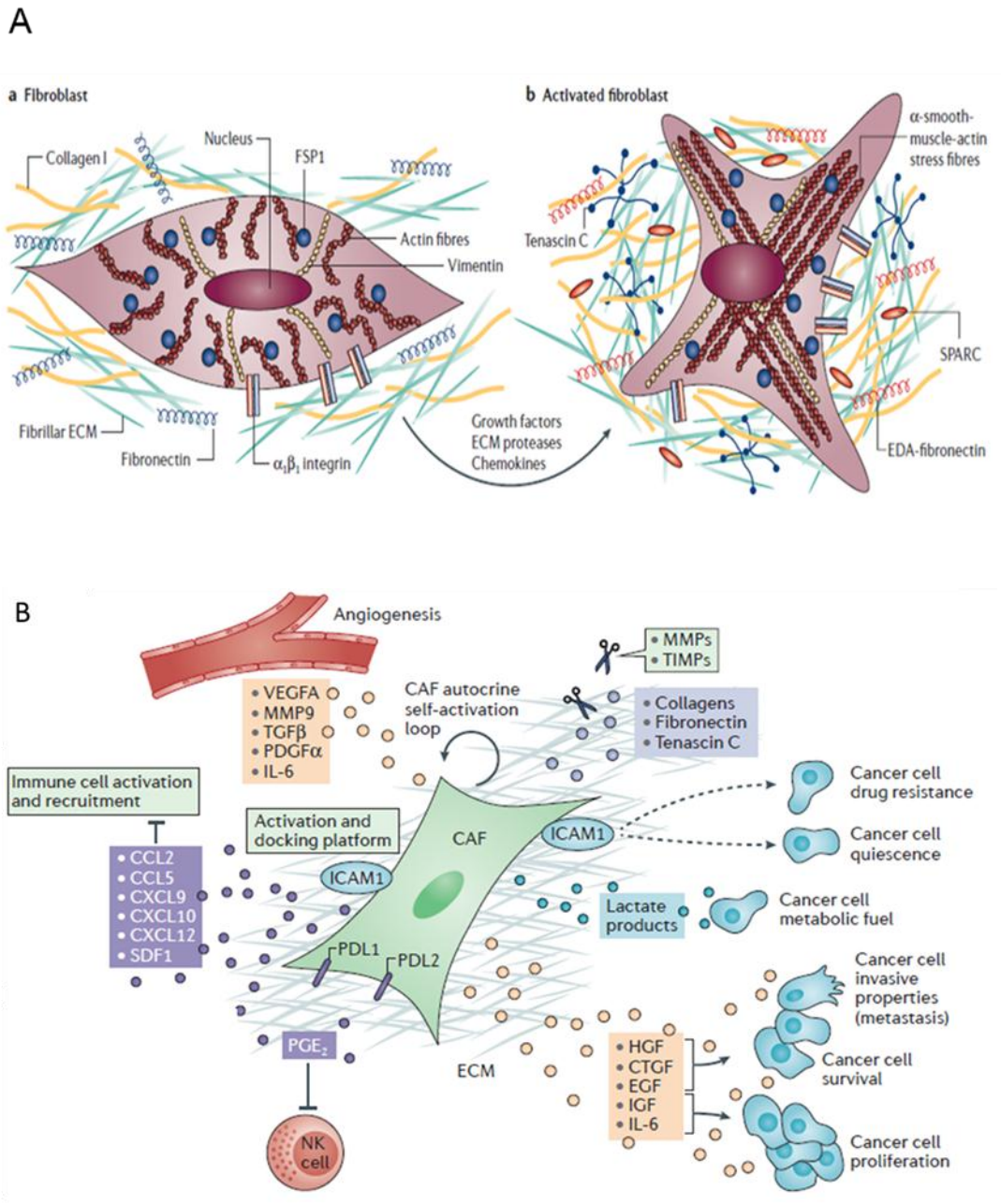


Figure 34: The fibroblasts and their microenvironment. (A) Normal fibroblasts (a) have a fusiform shape with prominent actin cytoskeleton and vimentin intermediate filaments. They are embedded within an extracellular matrix composed of type I collagen and fibronectin. They interact with the surrounding microenvironment through integrins such as the α1β1 integrin. Activated fibroblasts (b) have a dendritic shape. They express the alpha smooth muscle actin and secrete tenascin C and the EDA-fibronectin. **(B)** CAF communicate with surrounding cells by their secretome and promote cancer cells development (Kalluri and Zeisberg 2006, Kalluri 2016).

Provided the highest proportion of aggressive versus non aggressive cancers in XP-C patients compared to the general population, and their early onset, we aimed at comparing the phenotype of XP-C fibroblasts to that of CAF.

Our studies indicate that XP-C fibroblasts share some properties with CAF, notably the overexpression of MMP1 and HGF, and accumulation of ROS. However, primary XP-C fibroblasts were negative for alpha-SMA (a main activation marker of CAFs) and for markers described for senescent fibroblasts with the «Senescent Associated Secretory Phenotype» as p16 and β-galactosidase.

Studies conducted in our laboratory showed that XP-C fibroblasts culture supernatants activate melanoma cells (Mel501), and lead to tumor formation in mice, suggesting that the secretome of HGF by XP-C fibroblasts is probably implicated in SCC and MM in XP-C patients (Gache et al., unpublished data). Our organotypic assays showed that XP-C fibroblasts promote the invasion of the SCC12 and SCC13 in the dermal equivalents. Using HGF blocking antibodies counteract cancer cells invasion. A more detailed study will therefore have to be carried out in order to better characterize and understand the impact of XP-C fibroblasts and the stroma in early stages of cancerogenesis and aging in XP-C patients.

II. b. SCC13 invasions in XP-C dermal equivalent: is there a leader cell ?

Depending on the cell type and tissue environment, cancer cells possess a broad spectrum of migration and invasion mechanisms including both individual and collective cells migration strategies. The tumor microenvironment, and in particular the stromal organization, influences the mode and dynamics of invasion (Clark and Vignjevic 2015). Individual cell migration is characterized by a lack of cell-cell interactions (figure 35 a). Increased actomyosin contractility, under the control of the Rho-Rho kinase (ROCK) signaling, favors a round amoeboid migration. Single migrating cells can also use a mesenchymal phenotype in which cells display Rac-driven actin-rich protrusions (Sahai and Marshall 2003, Wolf, Mazo et al. 2003, Sanz-Moreno, Gadea et al. 2008, Bergert, Chandradoss et al. 2012, Clark and Vignjevic 2015).

Besides, carcinoma cells may also invade in a collective manner. Collective invasion occurs in many physiological and pathological processes, including

morphogenesis, tissue repair and cancer. In human epithelial cancers such as SCC, invasive cells are typically observed to migrate collectively (Friedl and Gilmour 2009). In collective invasion, carcinoma cells retain their cell-cell junctions that mediate cell-cell cohesion, cell polarity, and cell-cell signaling. Upon collective invasion, one or several leader cells with mesenchymal phenotypes form multicellular strands and generate tracks and pericellular tissue proteolysis (Gaggioli, Hooper et al. 2007, Khalil and Friedl 2010). Under these circumstances, carcinomas cells use MRCK (myotonic dystrophy kinase-related CDC42-binding protein kinases) which belongs to the family of Rho GTPases and regulates cytoskeletal organization. MRCK phosphorylates myosins light chains (MYL) to promote actin fiber contractility and cancer cells migration. Aberrant activation of Cdc42 results in tumorigenesis and tumor progression (Sinha and Yang 2008). In turn, stromal fibroblasts generate tracks for cancer cells within the ECM by using both protease (MMP) and force-mediated matrix remodeling. Force-matrix remodeling is mediated by integrins alpha3 and alpha5, and by Rho dependent regulation of myosin light chain (MLC) activity in fibroblasts (figure 35 b).

As we mentioned above, XP-C primary fibroblasts present important similarities with CAFs. We then assessed the possibility that XP-C fibroblasts may also behave as leader cells to drive the invasion of carcinoma cells. This was indeed the case since 3D spheroid assays showed that XP-C fibroblasts take the lead of SCC13 cells invasion.

Further studies are needed to characterize the pathways activated in XP-C fibroblasts to generate tracks for SCC cells invasion. Identification of specific inhibitors of these pathway could have clinical outcomes.

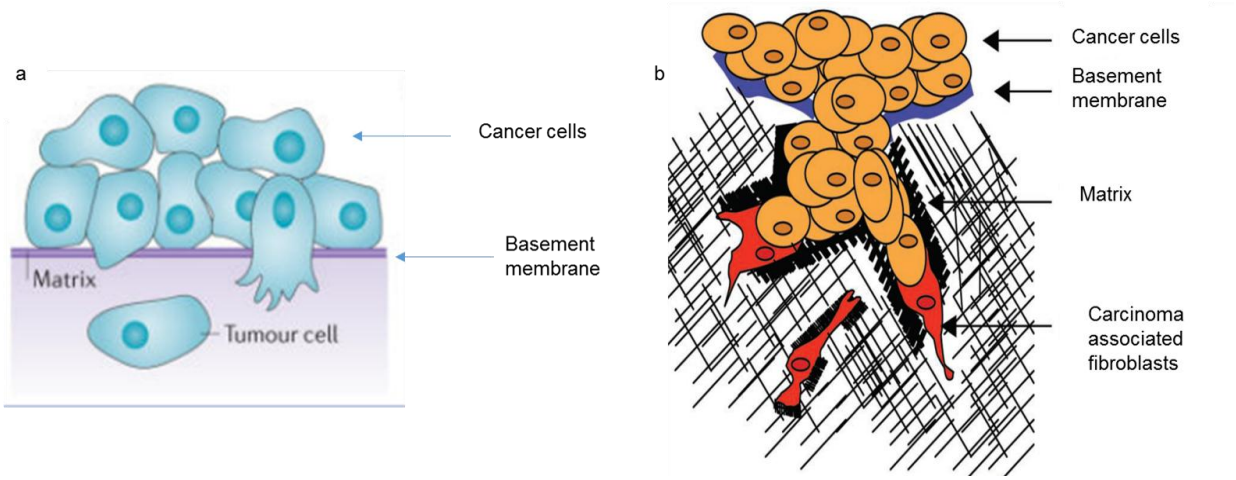


Figure 35: Schematic representation of models of carcinoma invasions. a) Single cell carcinoma invasion, cell-cell interaction is decreased with increasing actomysin contractility to promot individual cell invasions **b)** Collective invasion of Carcinoma cells, CAFs (red) lead of a collective invading chain of SCC cells (brown) (Gaggioli 2008, Slattum and Rosenblatt 2014).

III. **HGF secreted by XP-C fibroblasts activates major pathways in cSCC in vitro.**

III. a. HGF/c-Met structure

We chose to talk in the discussion about the structure of HGF and c-Met because it was found that HGF is overexpessed in the XP-C fibroblasts. These aspects are not presented in the introduction. HGF and its receptor c-Met are composed of several important domains for activation or downregulation of their downstream pathways. HGF consists of two chains: α chain compose of an amino-terminal hairpin loop (HL), four kringle domains (K1–K4; each defined by three conserved disulphide bonds) and of β–chain is a serine protease homology (SPH) domain that lacks proteolytic activity (figure 36a).

The HGF receptor c-Met is composed of extracellular α-subunit bond to a transmembrane β-subunit through disulphide which contains the intracellular catalytic activity (figure 36b). The extracellular region of c-Met includes three functional domains: the sema domain (which is also found in the semaphorins and plexins); the plexin, semaphorins and integrin domain (PSI) is connected to the transmembrane helix through four IPT (immunoglobulin-like, plexins and transcriptional factors)

domains. The intracellular segment is composed of a juxtamembrane sequence that downregulates kinase activity; a catalytic region that positively modulates kinase activity following trans-phosphorylation of Tyr1234 and Tyr1235; and a carboxy-terminal multifunctional docking site that contains two docking tyrosines (Tyr1349 and Tyr1356). These tyrosines are involved in the recruitment of several transducers and adaptors (Trusolino, Bertotti et al. 2010)**.**

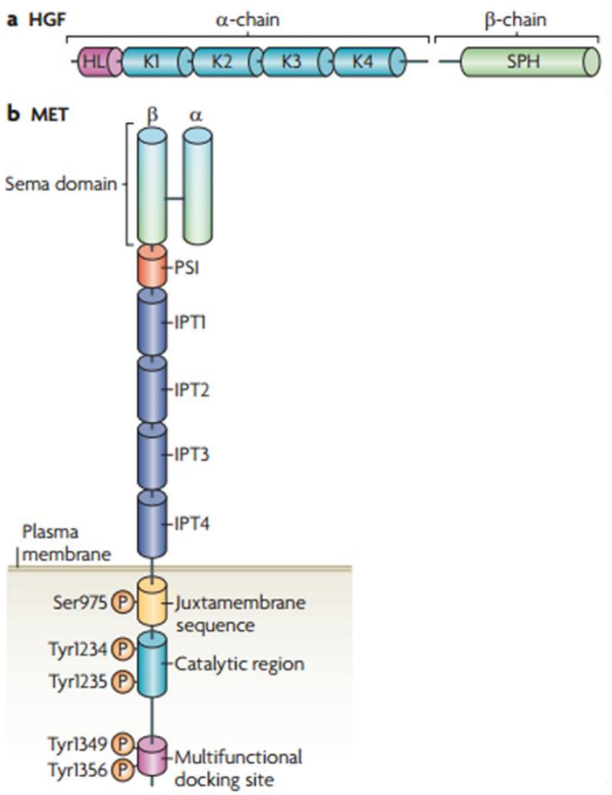


Figure 36: HGF and their receptor c-Met. a) Hepatocyte growth factor/scatter factor (HGF/SF) is composed of α and β chain. α–chain from amino-terminal hairpin loop (HL), four kringle domains (K1–K4), and β-chain from a serine protease homology (SPH)b) c-Met-HGF receptor compose of extracellular region of c-Met contains: the sema domain from plexin, semaphorins and integrin domain (PSI). Transmembrane helix from IPT (immunoglobulin-like, plexins and transcriptional factors) domains. The intracellular segment is composed of a juxtamembrane from a catalytic region following by Tyr1234 and Tyr1235; and a carboxy-terminal multifunctional docking site that contains two docking tyrosines (Tyr1349 and Tyr1356) (Trusolino, Bertotti et al. 2010).

III . b. HGF secreted by XP-C fibroblasts activates major pathways in cSCC *in vitro*.

Aberrant activation of c-Met has been shown to be implicated in several cancer cells, including cervix, hepatoma and osteosarcoma (Pennacchietti, Michieli et al. 2003, Benvenuti and Comoglio 2007). The activation of Met led to activate many downstream effectors. Met activates extracellular signal-regulator kinase (ERKs). Met activates RAS directly through the Grb2–SOS complex activation or indirectly through SHC adaptor protein (Ponzetto, Bardelli et al. 1994, Pelicci, Giordano et al. 1995). ERKs activation are by stimulating RAS following by phosphorylation of Raf and MEK1, 2 (figure 37 a). After activation, ERKs translocate in the nucleus where they phosphorylate and stabilize several transcription factors involved in cell proliferation and transformation. Another way lead to ERKs activation by involving the tyrosine phosphatase SHP2, which dephosphorylates the P120-RAS-GAP binding site on GAb1; this prevents the recruitment of P120-RAS-GAP, which usually deactivates RAS, to promote RAS activation (Maroun, Naujokas et al. 2000, Montagner, Yart et al. 2005).

Signal transducer and activator of transcription 3 (STAT3) monomers bind to c-MET through their SH2 domain and become trans-phosphorylated. Each STAT3 uses its SH2 domain to recognize the phosphotyrosine of its partner, and translocates to the nucleus to function as a transcription factor (figure 37b). This results STAT3 to dissociation from the receptors, its homodimerization (through their SH2 domains), and subsequent translocation to the nucleus where it regulates the expression of several genes implicated in cell proliferation or differentiation (Boccaccio, Ando et al. 1998, Zhang, Wang et al. 2002).

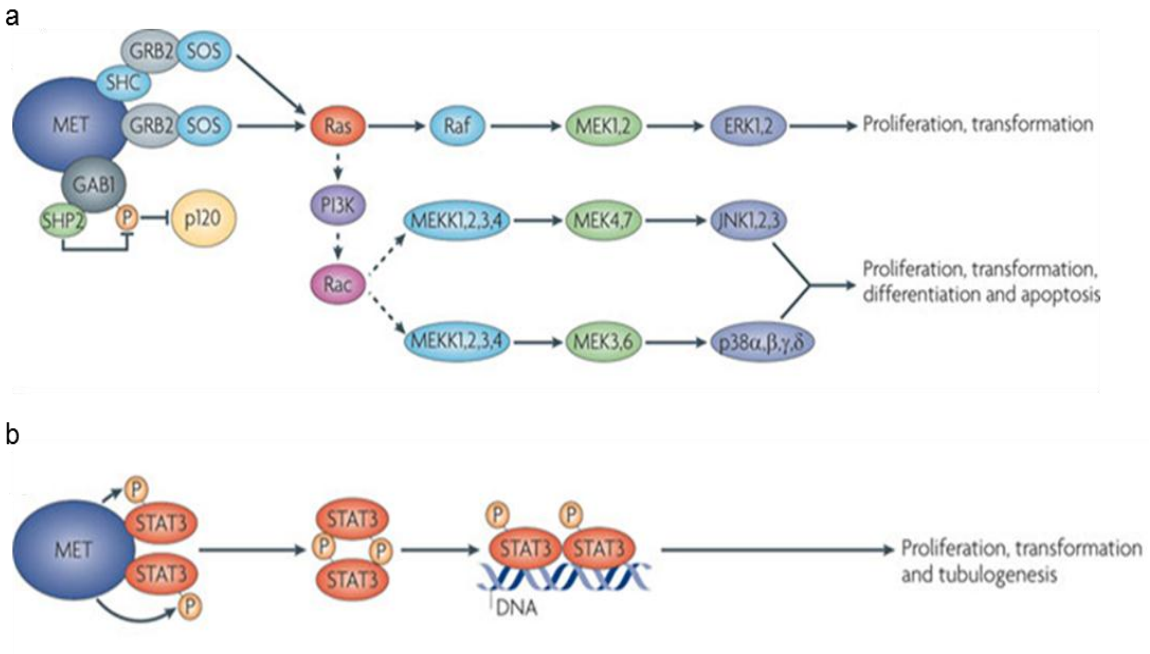


Figure 37: Major MET-regulated signaling pathways. a) MET activates Ras which can interact directly through Grb2–SOS or indirectly through SHC adaptor protein. Ras activation leads to activate Rac, Raf, MEK1, 2 and ERK1, 2. If the activation of Ras is followed by the activation of PI3K and Rac as result leads to p38 and JNKs activation through the MEKK1, 2, 3, 4, followed by MEK4, 7 activation to activate JNK, or MEK3, 6 to activate p38. **b)** Met activates STAT3 leads to disassociate from their receptors and homodimerize through their SH2 domains (Trusolino, Bertotti et al. 2010).

Met is also able to activate the JNK and p38 MAPKs. For both of them, first RAS-PI3K is activated, leading to activation of Rac (a small GTPase); this is followed by cascades of activations of MEK kinases (MEKK1–MEKK4). MEK4 and MEK7 phosphorylation leads to JNK1, JNK2 and JNK3 activation, whereas phosphorylation of MEK3 and MEK6 leads to activations of p38α, p38β, p38γ and p38δ (figure 37a). Both JNKs and p38 control many cellular processes such as cell proliferation, differentiation, transformation and apoptosis (Rodrigues, Park et al. 1997, Coltella, Rasola et al. 2006).

Our results showed that in SCC13 cells, both of JNKs and p38 are activated in the presence of XP-C fibroblasts culture supernatants. Using antibodies blocking HGF

prevent the phosphorylation of p38 but JNK is still not affected, suggesting that under these circumstances, the phosphorylation of p38 is HGF-dependent. Due to the importance of p38 in controlling the cell cycle, presumably, SCC13 proliferation is depending on p38 activation.

In H441 pulmonary epithelial cell, addition HGF in the cell culture lead to increased thymidine incorporation about 1.6 fold, while using inhibitors of p38 was found to reduce tymidine uptake in the DNA (Awasthi and King 2000). Interestingly, p38 is implicates in the G2–M checkpoint in response to DNA damages to arrest the cell cycle and initiate DNA repair. Furthermore, p38 regulates p53 by phosphorylating the Ser33 and Ser46 residues. In contrast, p53-defective tumor cells, p38 recover their checkpoint response and become dependent on the p38/MK2 pathway for survival after DNA damage despite the functional ATR-Chk1 (Bulavin, Saito et al. 1999, Reinhardt, Aslanian et al. 2007).

For more investigation of the role of p38 in SCC cells, study the p38 blocking on cancer cells proliferation, apoptosis and senescence after the treatment with XP-C fibroblasts culture supernatants must be done.

A global study of the gene expression profile (transcriptome) and / or proteome of XP-C fibroblasts in dermis equivalents, in the presence or absence of keratinocytes, will allow characterization of other intrinsic defects of XP-C fibroblasts. New candidates likely to be targeted by a pharmacological approach could then be identified.

ANNEXES

ANNEX i

RESEARCH ARTICLE

Basal Cell Carcinoma in Gorlin's Patients: a Matter of Fibroblasts-Led Protumoral Microenvironment?

Yannick Gache[1,2], Florence Brellier[3], Sophie Rouanet[1,2], Sahar Al-Qaraghuli[1,2], Maria Goncalves-Maia[1,2], Elodie Burty-Valin[3], Stéphanie Barnay[7], Sabine Scarzello[1,2], Martial Ruat[4], Nicolas Sevenet[5], Marie-Françoise Avril[6], Thierry Magnaldo[1,2]*

The ultraviolet (UV) content of the sunlight is by far the most frequent etiological factor in cutaneous carcinogenesis; in this context, exposure to sunlight is responsible for basal- (BCC), and squamous- cell carcinoma (SCC) as well as malignant melanoma (MM). Although my thesis essentially focused on the role of XP-C fibroblasts in SCC development, our laboratory investigated also the role of stromal fibroblasts in the onset, growth, and fate of BCC. BCCs is the commonest skin cancer in human. They exceptionally metastasize but may present with a high potential of local invasiveness. About 70% sporadic BCCs bear somatic mutations in the *PATCHED1* tumor suppressor gene which encodes the receptor for the Sonic Hedgehog morphogen (SHH). *PATCHED1* germinal mutations are associated with the dominant Nevoid Basal Cell Carcinoma Syndrome (NBCCS or Gorlin-Goltz syndrome). A major trait of NBCCS patients is their susceptibility to develop numerous BCCs, including in non photoexposed skin are. Furthermore, NBCCS primary cells exhibited normal NER levels and UV cell survival suggesting that susceptibility toward BCCs is not linked to inappropriate responses to DNA damages such as observed in the xeroderma pigmentosum syndrome. In contrast, NBCCS fibroblasts expressed a transcriptional signature composed of 18 genes resembling the signature described in CAFs from sporadic BCC.

In the present study, we aimed at better understanding the fact that NBCCS patients develop BCCs in skin areas preserved from apparent genotoxic insults. As the SHH/PATCHED pathway is known to be involved in intercellular control of proliferation, we hypothesized that deregulation of the SHH/PATCHED pathway in mesenchymal

cells may affect proper control of epithelial cell growth and contributes to the expression of microenvironmental factors leading to the development of BCCs. The functional impact of NBCCS fibroblasts was assessed in organotypic skin cultures with control keratinocytes. Our results indicate that the presence of *PATCHED1* +/- NBCCS fibroblasts in the dermal compartment of organotypic skin cultures is sufficient for severely perturb epidermal differentiation, proliferation, and cell cycle control in the overlaying WT epidermis. Extinction of P53 in WT keratinocytes resulted in an invasive phenotype in the presence of NBCCS fibroblasts. Unexpectedly, keratinocyte proliferation was severely reduced and showed high levels of nuclear P53 in both organotypic skin cultures and in fibroblast-led conditioning experiments. Although β–galactosidase, a senescence marker, was found overexpressed in keratinocytes in the presence of NBCCS fibroblasts, other markers of senescence as p16 and p21 were not activated. We also found that secretion of SHH was dependent on the presence of both fibroblasts and keratinocytes and is further increased in the presence of NBCCS fibroblasts. In conclusion, **o**ur observations further document the role of the SHH/PATCHED pathway in cutaneous homeostasis in the human. Altogether, these data suggest that the repertoire of diffusible factors (including SHH) expressed by primary NBCCS fibroblasts generate a stress affecting keratinocytes behavior and epidermal homeostasis. Our findings suggest that the dermal microenvironment led by NBCCS fibroblasts may play a functional role in high levels of BCCs in NBCCS patients in absence of external genotoxic stress.

PLOS | ONE

RESEARCH ARTICLE

Basal Cell Carcinoma in Gorlin's Patients: a Matter of Fibroblasts-Led Protumoral Microenvironment?

Yannick Gache[1,2], Florence Brellier[3], Sophie Rouanet[1,2], Sahar Al-Qaraghuli[1,2], Maria Goncalves-Maia[1,2], Elodie Burty-Valin[3], Stéphanie Barnay[7], Sabine Scarzello[1,2], Martial Ruat[4], Nicolas Sevenet[5], Marie-Françoise Avril[6], Thierry Magnaldo[1,2]*

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Abstract

Basal cell carcinoma (BCC) is the commonest tumor in human. About 70% sporadic BCCs bear somatic mutations in the *PATCHED1* tumor suppressor gene which encodes the receptor for the Sonic Hedgehog morphogen (SHH). *PATCHED1* germinal mutations are associated with the dominant Nevoid Basal Cell Carcinoma Syndrome (NBCCS), a major hallmark of which is a high susceptibility to BCCs. Although the vast majority of sporadic BCCs arises exclusively in sun exposed skin areas, 40 to 50% BCCs from NBCCS patients develop in non photo-exposed skin. Since overwhelming evidences indicate that microenvironment may both be modified by- and influence the- epithelial tumor, we hypothesized that NBCCS fibroblasts could contribute to BCCs in NBCCS patients, notably those developing in non photo-exposed skin areas. The functional impact of NBCCS fibroblasts was then assessed in organotypic skin cultures with control keratinocytes. Onset of epidermal differentiation was delayed in the presence of primary NBCCS fibroblasts. Unexpectedly, keratinocyte proliferation was severely reduced and showed high levels of nuclear P53 in both organotypic skin cultures and in fibroblast-led conditioning experiments. However, in spite of increased levels of senescence associated β-galactosidase activity in keratinocytes cultured in the presence of medium conditioned by NBCCS fibroblasts, we failed to observe activation of P16 and P21 and then of bona fide features of senescence. Constitutive extinction of P53 in WT keratinocytes resulted in an invasive phenotype in the presence of NBCCS fibroblasts. Finally, we found that expression of SHH was limited to fibroblasts but was dependent on the presence of keratinocytes. Inhibition of SHH binding resulted in improved epidermal morphogenesis. Altogether, these data suggest that the repertoire of diffusible factors (including SHH) expressed by primary NBCCS fibroblasts generate a stress affecting keratinocytes behavior and epidermal homeostasis. Our findings suggest that defects in dermo/epidermal interactions could contribute to BCC susceptibility in NBCCS patients.

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Introduction

In the human, most sporadic cancers are from epithelial origin and are thought to arise following sequences of genotoxic injuries [1]. The vast majority of cancers affecting the general population (about 30%) derive from skin, notably from the epidermal compartment. The ultraviolet (UV) content of the sunlight is by far the most frequent etiological factor in cutaneous carcinogenesis; in this context, exposure to sunlight is responsible for basal- (BCC), and squamous-cell carcinoma (SCC) as well as malignant melanoma (MM) [2]. Patients suffering from the dominantly inherited disease Nevoid Basal Cell Carcinoma/Gorlin-Goltz Syndrome (NBCCS) [3] express a variety of traits including developmental failures such as polysyndactily, bifid ribs, odontogenic keratocysts, as well as proneness toward medulloblastoma and ovarian cancers. However, the most severe genetic affliction in NBCCS patients is by far their constitutive susceptibility toward BCCs [3, 4, 5]. For yet unexplained reasons, 40 to 50% of those NBCCS BCCs, develop in non photo-exposed skin areas, [6] suggesting that susceptibility toward BCCs is not linked to inappropriate responses to DNA damages such as observed in the xeroderma pigmentosum nucleotide excision repair genetic syndrome [7].

Germinal mutations responsible for the NBCCS encompass the *PATCHED1* (*PTCH1*) tumor suppressor gene, most of which are non-sense and are thought to be deleterious. No evidence of a clear genotype-phenotype relationship has yet been put forward. PATCHED is a putative 12-pass trans-membrane receptor that binds the diffusible morphogen molecule SONIC HEDGEHOG (SHH) [8–10]. In brief, control of the pathway depends on the absence or the presence of SHH. In the absence of SHH, PATCHED acts as a repressor leading to inactivation of the GLI transcriptional activators. Upon receipt of the SHH signal, active forms of GLI transcriptional factors are produced to modulate the expression of SHH target genes [11].

In more than 50% sporadic BCCs, somatic mutations in the *PATCHED1* gene have also been identified, with frequent loss of heterozygosity including the *PATCHED1* locus [12]. However, since virtually 100% sporadic BCCs exhibited accumulation of *GLI* mRNAs, [13] it has been proposed that constitutive de-repression of the SHH/PATCHED pathway could result in inappropriate control of the cell proliferation and differentiation balance, developmental failures and cancer development, most notably BCCs [11].

In sporadic cancers, overwhelming evidences have accumulated indicating that the microenvironment of epithelial cells could play a decisive role in the onset, growth, and fate of neoplastic cells. Cancer cells can activate their microenvironment, leading to remodeling of components of the extracellular matrix and tumor cell growth through *de novo* secretion of various cytokines and growth factors [14]. In animal models, the tumor microenvironment has also been shown to generate oxidative damages and genetic instability, hence promoting invasive capacity of epithelial cells [15].

Based on the fact that sun exposure is not an essential etiological factor of NBCCS BCCs, [16] we investigated whether altered dermo-epidermal interactions may play a role in BCCs predisposition of NBCCS patients. More specifically, as the SHH/PATCHED pathway is known to be involved in intercellular control of proliferation, we hypothesized that deregulation of the SHH/PATCHED pathway in mesenchymal cells may affect proper control of epithelial cell growth and contributes to the expression of microenvironmental factors leading to the development of BCCs. In this respect, our previous transcriptome analyses of primary NBCCS fibroblasts cultured in 3D organotypic systems, revealed their significant inclination to express a phenotype closely resembling that of fibroblasts associated to sporadic BCCs [17, 18]. The most significant differential expression between wild type (WT) and NBCCS fibroblasts concerns proteins involved in the composition of the extracellular matrix and its remodeling, cellular proliferation, invasion, angiogenesis, and control of the production of reactive oxygen species (ROS).

In the present study, we aimed at better understanding the fact that NBCCS patients develop BCCs in skin areas preserved from apparent genotoxic insults. We took into account that mesodermal-ectodermal interactions play a major role upon development, maintenance and repair of adult organs as well as cancer development [19]. Our results indicate that the presence of *PATCHED1* +/- NBCCS fibroblasts in the dermal compartment of organotypic skin cultures (OSC) is sufficient for severely perturb epidermal differentiation, proliferation, and cell cycle control in the overlaying WT epidermis. These perturbations, however, did not correlate with expression of *bona fide* markers of apoptosis or senescence. Altogether, the present data strongly suggest for the first time that the dermal microenvironment led by NBCCS fibroblasts may play a functional role in high levels of BCCs in NBCCS patients in absence of external genotoxic stress.

Materials and Methods

This study was approved by a local French ethic committee (Commité consultatif pour la protection des personnes en recherche biomédicale, CCPPRB: CSET935) and was conducted after informed written consent of NBCCS patients.

Skin biopsies and primary cell cultures

Human primary fibroblasts and keratinocytes were isolated from healthy, non photo-exposed skin biopsies of either control individuals or NBCCS patients bearing either nonsense or missense mutations in the *PATCHED1* gene (S1 Table).

Primary dermal fibroblasts were grown in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 1% non essential amino acids, and 0.2% fungizone, at 37°C, 5% CO_2. Fibroblasts strains (passages P6 to P9) were seeded at 10^4 cells/cm², and cultured up to 80–90% confluence before processing. Under standard conditions, primary keratinocytes, E6-E7-immortalized keratinocytes, and SCC13 cell line were cultured on feeder layers of lethally irradiated 3T3-J2 Swiss mouse fibroblasts in cFAD keratinocyte medium [20]. When indicated, cFAD was replaced by a FAD medium containing 0.5% FCS. Immortalization of keratinocytes was performed using the pLXSN16E6-E7 retroviral vector [21] and Phoenix helper cells (http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html).

Culture of keratinocytes in culture medium conditioned by fibroblasts

Fibroblasts were seeded at a density of 10^4 cells/cm² and cultured for 72 hours. Fibroblasts were then cultured in FAD containing 0.5% FCS and conditioned supernatants were collected after 48 hours. Keratinocytes were seeded at 11 000 cells/cm² on lethally irradiated 3T3-J2 Swiss mouse fibroblasts in cFAD for 24 hours. Cells were then cultured in FAD containing 0.5% FCS for 24 hours before being incubated in the fibroblasts conditioned media for 72 hours. Keratinocytes were then lysed for RNA and proteins preparations.

Expression of SHH in culture supernatants from either fibroblasts or fibroblasts and keratinocytes co-cultures

For analyses of SHH secretion by fibroblasts, cells were seeded at 10^4 cells/cm² and cultured in fibroblasts medium for 48 hours. Cells were then incubated in fibroblasts medium without FCS for 48 hours. For analyses of SHH secretion in fibroblasts-keratinocytes co-cultures, fibroblasts were first seeded at 10^4 cells/cm² in cFAD for 24 hours. Keratinocytes were then added to the fibroblasts cultures at a density of 7 500 cells/cm². Co-cultures of fibroblasts and keratinocytes

were incubated in keratinocyte-SFM medium (Gibco, Life technologies, NY, USA) for 48 hours. Conditioned media were then collected, concentrated on Vivaspin columns (Sartorius Stedim Biotech GmbH, Goettingen, Germany), and protein extracts were analysed by western blot. Three independent conditioning experiments were performed.

Organotypic skin cultures

OSC were prepared as described [22]. Briefly, human dermal fibroblasts (passages P5 to P8) were embedded into a type I bovine collagen gel at a concentration of 2.10^6 cells/ml. After 4 days of contraction of the dermis equivalent, 5.10^4 keratinocytes were seeded onto the dermis equivalent and cultured in immersion for 7 days before being raised at the air-liquid interface for 7 additional days. When indicated, OSC were processed in the presence of 5 μg/ml of either the anti-SHH 5E1 monoclonal antibody (mAb) (DHSB, Iowa, IA, USA) or the isotype-matched anti-cMyc 9E10 mAb (Biomol, Plymouth Meeting, PA, USA). OSC were then processed for paraffin inclusion or snap freezing in liquid nitrogen.

Quantitative invasion assay

Organotypic culture system was set up according to Gaggioli [23]. Briefly, 5.10^5 fibroblasts were embedded in a mixture of collagen I (#354249; BD Biosciences, Oxford, UK) and Matrigel (#354234; BD Biosciences). After 1 hour at 37°C, 4.10^5 keratinocytes were plated on top of the gels, and incubated overnight at 37°C, 10% CO2. Gels were then mounted at the air-liquid interface and fed through capillarity diffusion by cFAD culture medium. After 7 days, the cultures were harvested and fixed using 4% paraformaldehyde, 0.25% glutaraldehyde. The invasion index [1 –(non-invading keratinocytes area/invading and non invading cells area)] values represents the average of 3 total sections from two independent organotypic skin cultures.

Histology and immunostaining

Hematoxylin-Eosin (H&E), immuno labellings on paraffin sections, and indirect immunofluorescence analyses on frozen sections were performed as described [24]. Immunostaining of Laminin B1, β1 Integrin, and P53 were performed on paraffin sections using rabbit anti-Laminin B1 serum (1/400; Novotec, Paris, France); anti-β1 integrin mAb (1/50; clone 4B7, Oncogene Research Products, Boston, MA, USA); P53 monoclonal antibody (1/50; clone DO-7, Dako, Glustrup, Denmark), respectively. Staining was revealed using appropriate secondary antibodies linked to alkaline phosphatase and the Envision kit (Dako, Glustrup, Denmark) followed by H&E counter-staining. Immunostainings of Keratin 10, Loricrin, and Ki67 were performed on air-dried cryosections using the anti-Keratin 10 mouse mAb (1/10; RKSE 60, Sanbio, Uden, The Netherlands), anti-Loricrin rabbit antiserum (1/200, [25]) and Ki67 mAb (1/20, NovoCastra, Newcastle, UK).

Senescence-associated β-galactosidase (SA-β-Gal) activity

Fibroblasts were seeded at a density of 8 500 cells/cm^2 in fibroblasts medium for 48 hours. Fibroblasts were then cultured in FAD medium depleted in calcium and containing 0.5% FCS and conditioned supernatants were collected after 72 hours. WT keratinocytes (2 000 cells/cm^2), SCC13 cells (2 000 cells/cm^2), and WT E6-E7 keratinocytes (650 cells/cm^2) were seeded onto glass coverslips in cFAD for 72 hours. Cells were then cultured in calcium-free FAD medium containing 0.5% FCS for 24 hours before being incubated in the fibroblasts conditioned media for 48 hours. SA-β-Gal activity in cultured keratinocytes was detected using the SA-β-Gal staining kit (Cell Signaling Technology, Danvers, MA, USA) according to the

manufacturer's recommendations. For positive control of SA-β-Gal activity, WT keratinocytes were treated for 2 hours with 200 μM H2O2. After processing, cells were photographed under reflected light. The ratio of SA-β-Gal positive cells / total cell numbers was determined by blind counting of 10 fields / slide by 3 independent experimentators.

Protein extraction and western blotting

For western blot detection of P53, P16, and P21, proteins were solubilized in 8 M Urea lysis buffer (8 M Urea, 50 mM Tris-HCl, 0.1 M β-Mercaptoethanol, 1 mM DTT, 100 μg/ml phenylmethanesulfonylfluoride). Proteins (15 μg) were separated by 8% (P53) or 14% (P21, P16) SDS-PAGE, blotted onto polyvinyl difluoride (PVDF) membrane (Millipore, Corporation, Billerica, MA, USA), and revealed using the anti-P53 DO-7 mAb (1/1000; Dako, Glustrup, Denmark), the anti-P21 EA10 mAb (0.5 μg/ml; Abcam ab16767, Cambridge, UK), or the anti-human-P16 mAb (1/1 000, BD Biosciences, Le Pont de Claix, France). Membranes were re probed using the anti-GAPDH mAb (1/2000; Abcam 9484, Cambridge, UK) or the anti-β-Tubulin TUB2.1 mAb (1/5 000, Sigma Aldrich, St Louis, MO) for loading/transfer controls. Membranes were then analysed using the Luminata crescendo western HRP substrate (Millipore Corporation, Billerica, MA, USA). Quantification of P53 was performed using the ImageJ software. Quantification of P21 and P16 was performed using the Fusion-Capt software (Vilber Lourmat, Eberhardzell, Germany). For detection of SHH, 1μg of concentrated culture supernatants were subjected to 10% SDS-PAGE, blotted, and revealed using the N-SHH 167Ab polyclonal antibody [26]. Quantification of N-SHH was performed using GeneGnome device and GeneTools software (Syngene, Synoptics Ltd, Cambridge, UK).

RNA extraction and quantitative RT-PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed from 1 μg RNA using the Superscript II Reverse Transcriptase (Roche Applied Science, Basel, Switzerland) and random primers. Real Time-PCR was carried out on cDNAs using primers listed in S2 Table and the 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Results were normalized using the GeNorm software (http://medgen.ugent.be/Bjvdesomp/genorm/). Statistical analysis was performed using the Mann-Whitney test. Semi-quantitative PCR were performed using *PATCHED1* primers F: 5′-GATAAGAGCTCCGGGGGATTC-3′ and R: 5′-CACAGTAGCTTAGGCTTCAGCCC-3′, and *GAPDH* primers F: 5'-CCAAGGCTGTGGGCAAGGTCAT-3' and R: 5'-TGACAAGGTG CGGCTCCCTAGG-3' as described [27]. PCR products were quantified after electrophoretic separation in 2% agarose gel, and SYBR fluorescence scanning (STORM; Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software (Amersham Biosciences, Amersham, United Kingdom).

Transcriptional activity of the *PATCHED1* gene promoter

Keratinocytes were seeded at a density of 16 000 cells/cm^2. Transient transfections were performed as described [27]. The RSV-β-Gal plasmid was used as a control to normalize transfection efficiency. After transfection, keratinocytes were maintained for 72 hours in medium conditioned from control or NBCCS fibroblasts and the luciferase and β-Gal enzymes activities were measured using the luciferase assay system and β-Gal enzyme assay system kits (Promega Corporation, Madison, WI, USA) [27].

Reactive oxygen species (ROS)

Accumulation of ROS was examined by using 2',7'-dichloro-fluorescin diacetate (CM-DFHDA) (Invitrogen™/Molecular probes, Eugene, Oregon, USA). Keratinocytes were seeded at 11 000 cells/cm^2 in cFAD for 48 hours. Cells were then incubated in fibroblast-conditioned media for 1 hour and then incubated in CM-DCFHDA in HBSS for 30 min at 37°C. Cells were then washed, dissociated, and resuspended in HBSS. Fluorescence was recorded at an excitation/emission wavelength of 485 nm/530 nm by flow cytometry on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analyses

For the box plot representation, the line in the middle of the box represents the median. The box extends from the 25th percentile to the 75th percentile. The lines emerging from the box extend to the upper and lower adjacent values. Significance of the differences between experimental values measured in NBCCS and in WT fibroblasts was assessed using the Mann-Whitney test.

Results

NBCCS fibroblasts impact homeostasis of wild type epidermis

We first analyzed the potential impact of NBCCS primary fibroblasts on epidermal morphogenesis using OSC. NBCCS fibroblasts from two independent patients bearing distinct *PATCHED1* nonsense mutations (NBCCS6: c.1925dupC; p.Pro643ThrfsX11 and NBCCS10: c.1366delA; p.Thr456ProfsX35) were incorporated in a dermis equivalent (*PATCHED* + /-) overlaid with a stratified squamous epithelium developed from primary WT keratinocytes (*PATCHED* +/+) (NBCCS OSC) and compared to fully WT OSC. Under these circumstances, the presence of NBCCS fibroblasts in the dermis equivalent was accompanied by a decrease in the thickness of the epidermis, the absence of *bona fide* cornified layers and the persistence of nuclei in the upper suprabasal epidermal layers (parakeratosis) (Fig 1). In addition, the

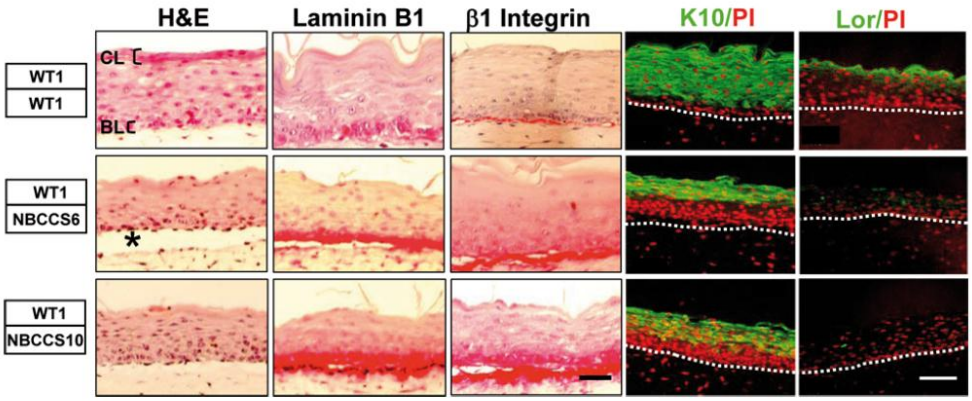

Fig 1. NBCCS fibroblasts alter morphology and differentiation of WT epidermis. WT keratinocytes were seeded onto dermis equivalents comprising skin fibroblasts isolated from either a WT (WT1) donor or from two independent NBCCS patients (NBCCS6 and NBCCS10). H&E staining reveals the absence of cornified layers (CL) and slight epidermal thinning in NBCCS OSC. Note increased accumulation of Laminin B1 and β1 Integrin in the basal layer (BL) and the para basal layers of epidermis. Star (*) points dermo epidermal cleft between the dermal and the epidermal compartments. Also note delayed expression of Keratin 10 (K10) and absence of expression of Loricrin (Lor) in NBCCS OSC; PI, nuclei counter staining using propidium iodide. Dash lines delineate the dermo epidermal junction. Bar: 110 μm

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presence of NBCCS fibroblasts was accompanied by clefts at the dermo-epidermal junction, a feature of human BCCs [28]. In contrast, fully WT OSC showed *bona fide* cornified layers and the absence of such parakeratotic features. Immunolabelling of OSC containing NBCCS fibroblasts indicated that deposition of Laminin B1, a major component of the basement membrane, and of β1 Integrin, a trans-membrane protein essential for the attachment of basal keratinocytes to the basement membrane, were increased compared to WT OSC. In addition, β1 Integrin, which is normally limited to keratinocytes located in the basal layer, extended in the first supra basal cell layers. To confirm the relevance of these results, skin sections from patients NBCCS6 and NBCCS10 were also submitted to histological analyses and labelled using anti-β1 Integrin and anti-Laminin B1 antibodies. Although no gross histological alteration was observed, the apparent amount of β1 Integrin and Laminin B1 was found to be either slightly or more strongly increased in NBCCS, respectively, compared to control skin (S1 Fig).

In NBCCS OSC, labelling of the Keratin 10 (K10), a differentiation marker whose normal onset of expression occurs in parabasal, post-mitotic epidermal cell layers, was extended to the second to third rows of epidermal cells (Fig 1). Finally, Loricrin, a precursor of cornified envelopes in terminally differentiated keratinocytes, [25] became barely detectable or even absent in the presence of NBCCS fibroblasts.

NBCCS fibroblasts provoke stabilisation of P53 in WT keratinocytes

Taken the above data into account, we aimed at measuring the number of cycling epidermal-keratinocytes positive for Ki-67 labelling in the presence of NBCCS fibroblasts within dermis equivalents. Surprisingly however, number of Ki-67 positive keratinocytes was about 10 times lower in the presence of NBCCS fibroblasts than in the presence of WT fibroblasts (Fig 2). To explore the possible mechanism(s) responsible for the decreased proliferation of WT epidermal keratinocytes located in the vicinity of NBCCS fibroblasts, we analyzed the expression of the P53 tumor suppressor protein provided its function as a major effector of cell cycle arrest at G1-S and G2-M check points [29].

In NBCCS OSC, the number of P53-positive nuclei was enhanced 5.5 to 7.5 times (Fig 2A and 2B) as compared to WT OSC. These observations indicated that the presence of NBCCS fibroblasts within the dermis equivalent was sufficient to severely perturb keratinocyte proliferation as well as the early and subsequent steps of epidermal differentiation. Further, due to the 3D architecture of OSCs, these results suggested to us that NBCCS fibroblasts could perturb skin homeostasis through diffusible molecules accommodating a dialog between the dermis and the epidermis.

How NBCCS fibroblasts may alter behavior of epidermal keratinocytes?

Given that NBCCS fibroblasts could impact epidermal homeostasis through diffusible molecules, we assessed in 2D culture conditions expression of secreted proteins as previously reported in NBCCS fibroblasts cultured in 3D dermis equivalents in the absence of epidermal keratinocytes [17]. Under these circumstances, a minimal fraction of 9 of the 18 mRNA signature was differentially expressed (significantly) in WT versus NBCCS fibroblasts (Table 1). Among these mRNAs, the average levels of the anti-angiogenic proteins angiopoietin-like 4 (*ANGPTL4*) and matrix GLA protein (*MGP*) mRNAs were increased by 5.6 ($p<0.025$) and 61.7 ($p<0.05$), respectively, in NBCCS compared to WT cells. As well, relative levels of the C-X-C ligand 12 (*CXCL12*) and fibroblast growth factor 7 (*FGF7*) mRNAs were increased in NBCCS fibroblasts by 4.0 and 2.9 fold, respectively ($p<0.025$). The WNT/-catenin signaling pathway is known to be activated in CAFs [30, 31]. In 2D cultures of NBCCS fibroblasts, 4 mRNAs of the WNT/-catenin pathway were found mis-regulated as in 3D dermis equivalents.

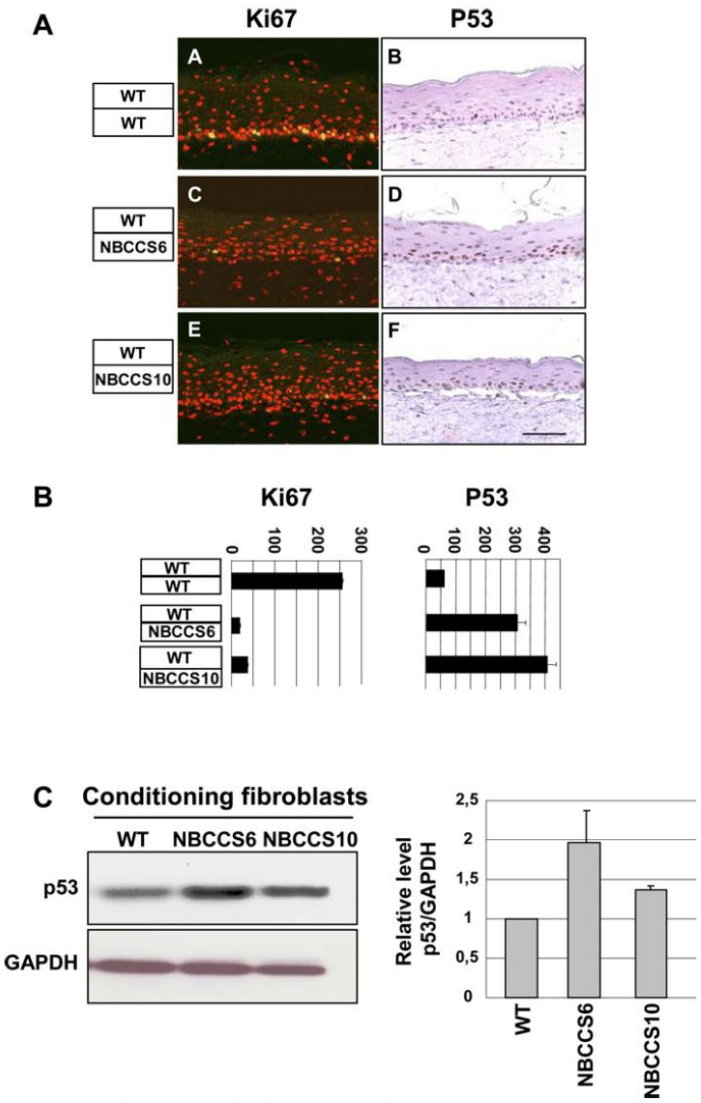

Fig 2. NBCCS fibroblasts alter proliferation of WT keratinocytes in organotypic skin cultures. (A) Rates of cycling keratinocyte (Ki67, green/yellow nuclei), and P53-positive keratinocytes (P53, brown nuclei) were blind counted by 3 independent investigators on sections of OSCs, as indicated. Bar: 170 μm. (B) Ki67- (left panel) and P53- (right panel) positive keratinocytes were counted in the basal layer of 10 different fields spread along 3 independent sections. Error bars indicate the mean ± SD of the counted fields. (C), Western blot analysis shows stabilization of P53 in WT keratinocytes (WT1) treated for 24 h in culture supernatants conditioned by NBCCS (NBCCS6 and NBCCS10) fibroblasts (left panel). Right panel shows levels of P53 relative to GAPDH and expressed as fold induction relative to the WT fibroblasts strain. Error bars indicate the mean ± SD of triplicate experiments.

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Amount of mRNAs encoding *WNT5A* (ligand) and Dickkopf 3 (*DKK3*) (inhibitor) were decreased by 5.0 and 2.0 ($p<0.025$), respectively. Amount of mRNAs of the WNT target genes *WISP2* (WNT1 inducible signaling pathway protein 2) and *ID2* (inhibitor of DNA binding 2) were increased by 4.9 ($p<0.05$) and 3.2 ($p<0.025$), respectively. As in 3D conditions, levels of *COL7A1* mRNA were decreased in NBCCS fibroblasts cultured in 2D conditions compared to control cells (5.0 fold; $p<0.05$). In contrast, mRNAs encoding MMPs (1 and 3), COL11A1, COL3A1, LAMA2, TNC, GREM1, ANGPTL2, and SFRP2 were no longer mis-regulated in NBCCS fibroblasts cultured in 2D conditions while they were in 3D. These results indicated that a fraction of transcriptional deregulations previously described in 3D NBCCS dermis equivalents are conserved under 2D culture conditions, hence allowing fibroblast-led conditioning experiments of epidermal keratinocytes.

NBCCS fibroblasts alter keratinocytes behavior through diffusible molecules

Whether diffusible factors expressed by NBCCS fibroblasts could act on stabilization of P53 in epidermal cells was assessed by treating WT keratinocytes with culture supernatants conditioned by either WT or NBCCS fibroblasts. Western blot analyses showed that levels of P53 were slightly increased in WT keratinocytes treated with culture supernatants of NBCCS fibroblasts (Fig 2C). These observations strongly suggested that NBCCS fibroblasts secrete molecules leading to P53 stabilization and cell cycle modifications in WT keratinocytes. Among the identified factors showing altered expression in NBCCS fibroblasts, ANGPTL4 has been shown to elevate the intracellular O2:H202 ratio in a manner dependent of β-1 and β-4 Integrins and

Table 1. Average mRNA expression levels in WT or NBCCS fibroblasts in either 2D or 3D cultures conditions.

Genes Name	2D		3D	
Average NBCCS / average WT	NBCCS <or> WT p-value		Average NBCCS / average WT	NBCCS <or> WT p-value
MMP1	12.1	NS	8.4	p<0.025
MMP3	4.1	NS	26.8	p<0.025
COL3A1	0.8	NS	0.7	p<0.05
COL7A1	0.3	p<0.025	0.6	p<0.05
COL11A1	7.2	NS	11.5	NS
LAMA2	1.0	NS	0.4	p<0.025
TNC	0.5	NS	1.2	NS
CXCL12	4.0	p<0.025	2.4	p<0.025
MGP	61.7	p<0.05	5.2	p<0.025
ANGPTL2	1.7	NS	3.0	p<0.025
ANGPTL4	5.6	p<0.025	9.8	p<0.025
FGF7	2.9	p<0.025	2.3	p<0.05
GREM1	1.6	NS	1.7	NS
SFRP2	0.7	NS	3.5	p<0.025
DKK3	0.5	p<0.025	0.3	p<0.025
WNT5A	0.2	p<0.025	0.5	p<0.05
WISP2	4.9	p<0.05	2.7	p<0.05
ID2	3.2	p<0.025	2.6	p<0.025

The level of mRNA of the indicated genes was measured by RT-qPCR on the mRNAs extracted from cultures of fibroblasts in 2D conditions or from dermal equivalents (3D) (WT: n = 3; NBCCS: n = 6). The ratio between the average mRNA levels in the NBCCS and the WT fibroblasts and the p-value are indicated. NS: not significant (p>0.05).

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to confer anoikis resistance to tumors cells [32]. Based on these observations, we assessed whether overexpression of ANGPL4 in NBCCS fibroblasts could provoke a paracrine stress in epidermal keratinocytes, hence leading to P53 stabilization. However, quantifications of reactive oxygen species (ROS) in WT keratinocytes conditioned in either primary WT or NBCCS fibroblasts culture supernatants, failed to reveal any difference among our experimental settings (S2 Fig). Finally, in spite of a slight increase of P53 stabilization in WT keratinocytes cultured in medium conditioned by WT or NBCCS fibroblasts, we failed to detect any significant variation in cell cycle features (not shown). Consistently, the check point protein P21, a canonical target of P53, was not increased (but even rather slightly decreased) under our experimental conditions, suggesting that stabilization of P53 in 2-D cultures was not sufficient to provoke significant cell cycle alterations, such as accumulation of keratinocytes in G1 or sub-G1 phases.

Then we hypothesized that NBCCS fibroblasts could alter the behavior of epidermal keratinocytes through a senescent-like mechanism. We measured the accumulation of SA-β-Gal) in keratinocytes with different genetic backgrounds treated with culture supernatants conditioned by WT or NBCCS fibroblasts (Fig 3). Culture supernatants conditioned by NBCCS6 and NBCCS10 fibroblasts induced an increase in the number of SA-β-Gal positive cells in WT1 keratinocytes (1.55-fold and 2.29-fold increases, respectively). In contrast, we did not observe any increase of SA-β-Gal in keratinocytes with P53 altered genetic backgrounds, i.e. in both WT1 E6-E7 or SCC13 keratinocytes.

On the base of the increase of SA-β-Gal in WT keratinocytes conditioned in culture supernatants of NBCCS fibroblasts, we next investigated the role of P21 and P16, two cell-cycle check point regulators that expressions have been described in senescent cells [33]. Surprisingly, however, western blots indicated a marked decrease of P21 and P16 proteins in WT keratinocytes cultured in media conditioned by NBCCS fibroblasts (S3 Fig). These results suggest that the increase of the SA-β-Gal activity described above is not correlated with stabilization of the P21- and P16 check point proteins.

NBCCS fibroblasts impact the SHH/PATCHED pathway in WT keratinocytes

Numerous researches have shown that SHH production could be misregulated in various cancer types, notably those involving alteration of mesenchyme / stromal cells [34]. Expression of SHH was thus analyzed in NBCCS and WT fibroblasts and in keratinocytes cultured in 2D. RT-qPCR analysis indicated the absence of SHH mRNA in keratinocytes and hardly detectable expression in fibroblasts (not shown). In neither WT nor NBCCS fibroblast culture supernatants, the active N-terminal fragment of SHH (N-SHH), could be detected by western blot analyses (Fig 4). In contrast, N-SHH became detectable in culture media obtained after 48 hours of co-cultures of WT keratinocytes with WT fibroblasts (Fig 4B). Secretion of N-SHH was further increased in co-cultures of WT keratinocytes with NBCCS fibroblasts (from x 1.8 to x 2.4).

Since *PATCHED1* is a transcriptional target of the SHH/PATCHED pathway, WT keratinocytes were then conditioned with culture supernatants of NBCCS fibroblasts and levels of *PATCHED1* mRNA in keratinocytes were measured by RT-qPCR. In parallel, WT keratinocytes were transfected with a vector bearing the 4,4 kb 5' regulatory region of the human *PATCHED1* gene upstream the firefly luciferase reporter gene [27] and cultured in the medium conditioned by NBCCS fibroblasts. Fig 5 indicates that both approaches resulted in a 20–30% increase of *PATCHED1* mRNA amount and transcription. These observation were relevant of a modest but reproducible activation of the SHH pathway in WT keratinocytes exposed to culture supernatants conditioned by NBCCS fibroblasts.

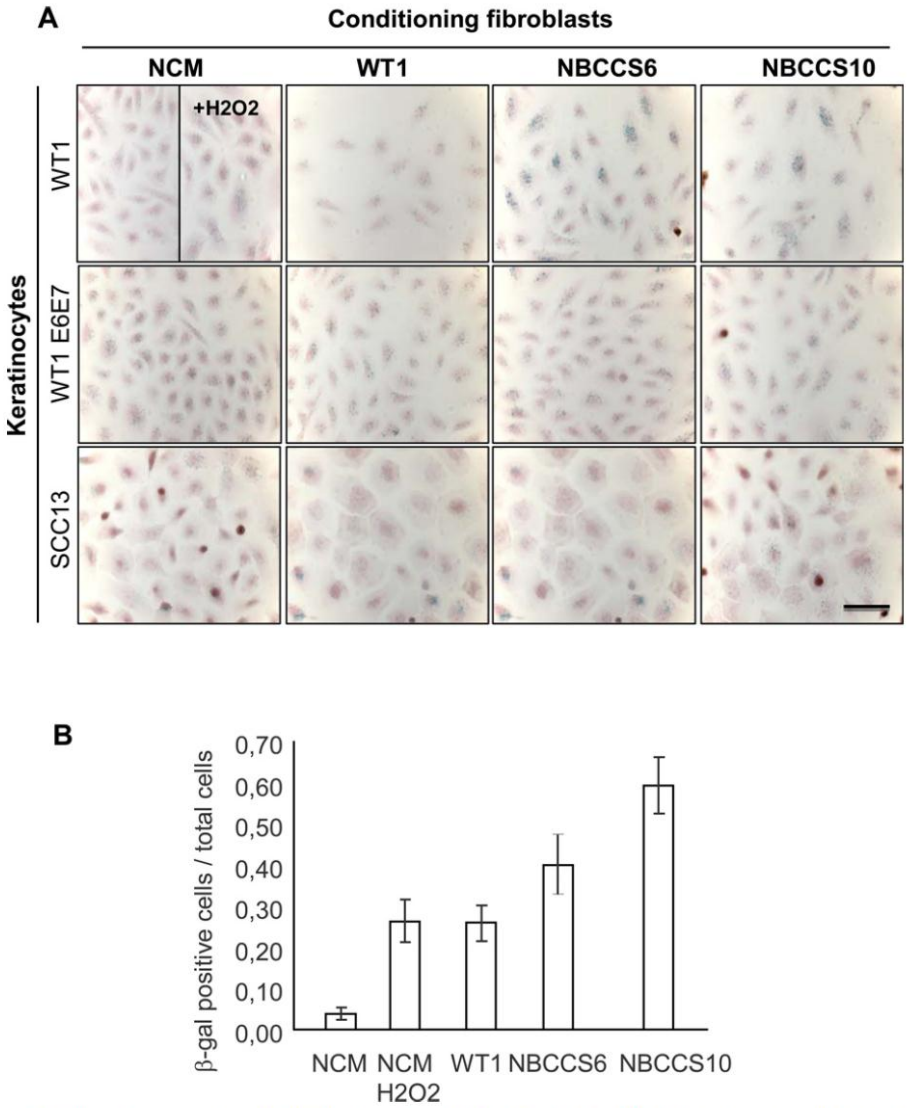

Fig 3. Culture supernatants from NBCCS fibroblasts promote SA-β-Gal activity in WT but not in transformed (E6-E7) or tumoral (SCC13) keratinocytes. (A) SA-β-Gal activity in WT1, WT1 E6-E7, and SCC13 keratinocytes after treatment with either non conditioned medium (NCM), culture supernatant conditioned by WT (WT1), or NBCCS (NBCCS6 and NBCCS10) fibroblasts. (B) SA-β-Gal positive cells were blind counted by three independent investigators. Bars represent the confidence intervals (α = 0.05). Number of SA-β-Gal positive cells was increased in WT keratinocytes treated with NBCCS supernatants. No increase of SA-β-Gal positive cells was detected in both WT1 E6-E7 and SCC13 keratinocytes.

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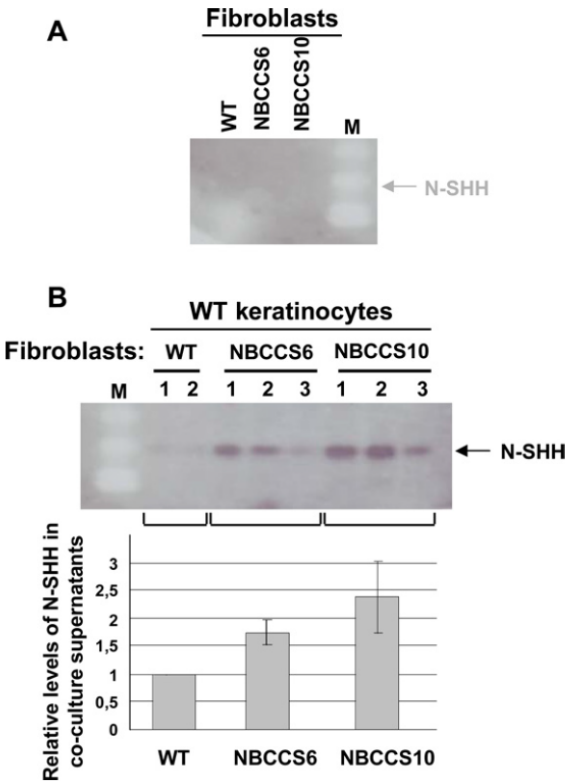

Fig 4. SHH secretion depends on the presence of both fibroblasts and keratinocytes and is further increased in the presence of NBCCS fibroblasts. (A), Western blot analysis of N-SHH secreted from either WT (WT1) or NBCCS (NBCCS6 or NBCCS10) fibroblasts. Secretion of N-SHH was not detectable in culture supernatants of WT and NBCCS fibroblasts. (B), Western blot analysis of N-SHH secreted from co-cultures of WT keratinocytes with WT (WT1) or NBCCS fibroblasts (NBCCS6 or NBCCS10). N-SHH became slightly detectable in the presence of WT fibroblasts (WT1). N-SHH amount was substantially increased in the presence of NBCCS fibroblasts (NBCCS6, NBCC10). Lower panel: quantitation of N-SHH in culture supernatant obtained under the circumstances described for the upper panel. Note that detection of SHH is limited to the N-SHH processed protein (22 kDa, arrow).

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To assess whether SHH secretion by NBCCS dermal fibroblasts could be implicated in epidermal alterations, OSC were developed in the absence or in the presence of the 5E1 anti-SHH blocking antibody. Fig 6 shows that treatment of OSC with the 5E1 blocking antibody (but not an isotype-matched monoclonal antibody) minimized epidermal perturbations observed in the presence of NBCCS fibroblasts. H&E staining revealed increased thickness of the epidermis in NBCCS OSC treated with the blocking 5E1 antibody. Furthermore, labelling of β1 Integrin was normalized to the basal cell layer in the presence of the blocking 5E1 antibody. These data support our observations suggesting that the presence of NBCCS fibroblasts leads to a misactivation of the SHH/PATCHED pathway in the epidermal compartment, and hence, to severe alterations of the behaviour of epidermal keratinocytes.

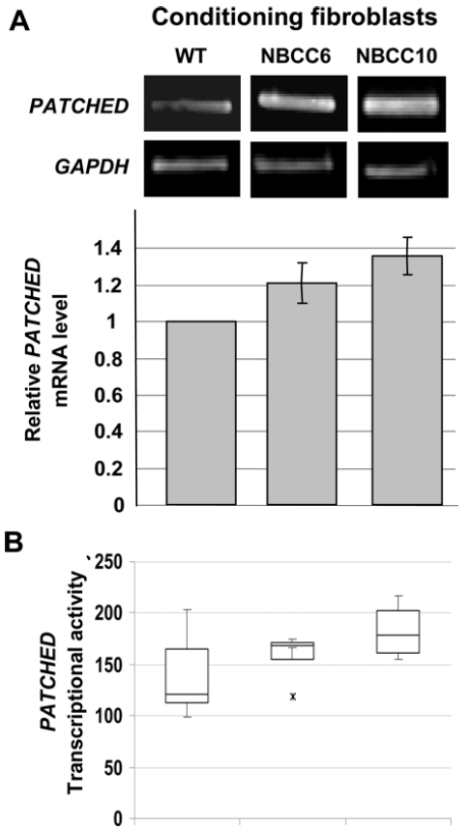

Fig 5. NBCCS fibroblasts stimulate *PATCHED1* transcription in WT keratinocytes. (A), *PATCHED1* and *GAPDH* mRNAs amounts expressed by WT keratinocytes cultured with either WT or NBCCS fibroblasts (NBCCS6 or NBCCS10) were determined after semi-quantitative PCR and agarose gel electrophoresis (upper panel). Relative *PATCHED1* mRNA levels were increased in WT keratinocytes (WT1) treated with culture media derived from NBCCS fibroblasts (NBCCS6 and NBCCS10) (lower panel). (B), Activity of the *PATCHED1* promoter in WT keratinocytes cultured in media conditioned from either WT or NBCCS fibroblasts (NBCCS6 or NBCCS10). Note the substantial increase of the relative transcriptional activity of the *PATCHED1* promoter in WT keratinocytes treated with culture media conditioned by NBCCS fibroblasts. Experiments were performed two times in triplicates, $p<0.09$.

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NBCCS fibroblasts confer invasiveness of keratinocytes with abrogated P53

Although our data showed that P53 is stabilized in keratinocytes in OSC comprising a NBCCS dermis equivalent or after their incubation in culture supernatants conditioned by NBCCS fibroblasts, we failed to identify the exact nature of such a stress. However, since most skin tumors harbor P53 deleterious mutations (IARC TP53 database, R17 November 2013, www.p53.iarc.fr), [35] we aimed at measuring the impact of P53 abrogation in WT and NBCCS keratinocytes exposed to NBCCS fibroblasts in organotypic invasion assays. To do so, NBCCS

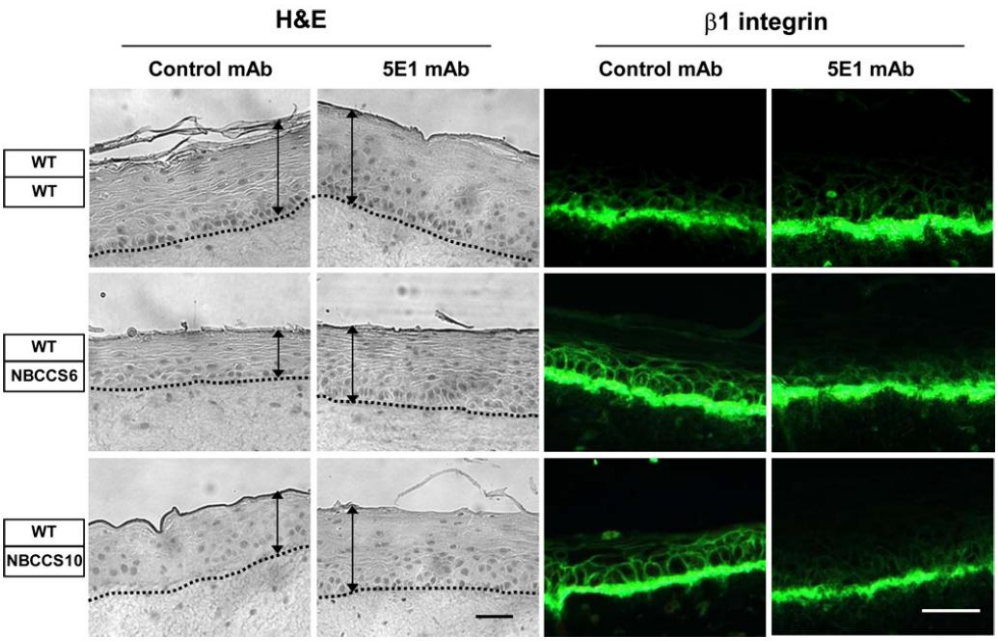

Fig 6. Neutralizing SHH in organotypic skin cultures attenuates alterations of WT epidermis in the presence of NBCCS fibroblasts. OSC comprising either WT (WT1) or NBCCS fibroblasts (NBCCS6 or NBCCS10) were developed in the presence of either the 5E1 anti-SHH blocking mAb (5E1 mAb) or an isotype-matched (anti-Myc 9E10) control antibody (Control mAb). OSC sections were stained with either H&E, or immunolabeled with the anti-β1 Integrin mAb. Note that developing OSC in the presence of the 5E1 antibody improved epidermal atrophy (as pointed out by black arrows) and β1 Integrin delocalization otherwise observed in the presence of NBCCS fibroblasts. Bar: 110 μm

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primary keratinocytes were transduced using retroviral vectors allowing expression of the HPV16 E6-E7 protein, and hence the ubiquitination and proteasome degradation of P53 (Fig 7A). Under these circumstances, both WT E6-E7 and NBCCS E6-E7 keratinocytes became invasive in the presence of NBCCS fibroblasts (Fig 7B and 7C, S4 Fig). Since expression of the HPV16 E6 and E7 oncoproteins not only abrogates expression of P53 but also of the Retinoblastoma 1 protein (Rb1), we also introduced human keratinocytes isolated from human squamous cell carcinoma as a control (SCC13 [36]). Alike WT keratinocytes transformed by the E6 E7 oncoproteins, SCC13 cells became highly invasive in organotypic skin cultures containing NBCSS but not WT fibroblasts (not shown). These observations argue in favor of a central role of P53 in counteracting alterations of the dermo epidermal dialog led in the presence of NBCCS fibroblasts.

Discussion

BCCs are skin cancers that exceptionally metastasize but may present with a high potential of local invasiveness. Since the early 60^s, [37, 38] it has been hypothesized that growth of BCC cells depends on specific micro environmental conditions. This notion is further supported by the fact that in contrast to SCC cells, [36] BCC cells cannot be propagated *ex vivo*. Here, we present experimental evidences showing that dermal fibroblasts isolated from non-involved

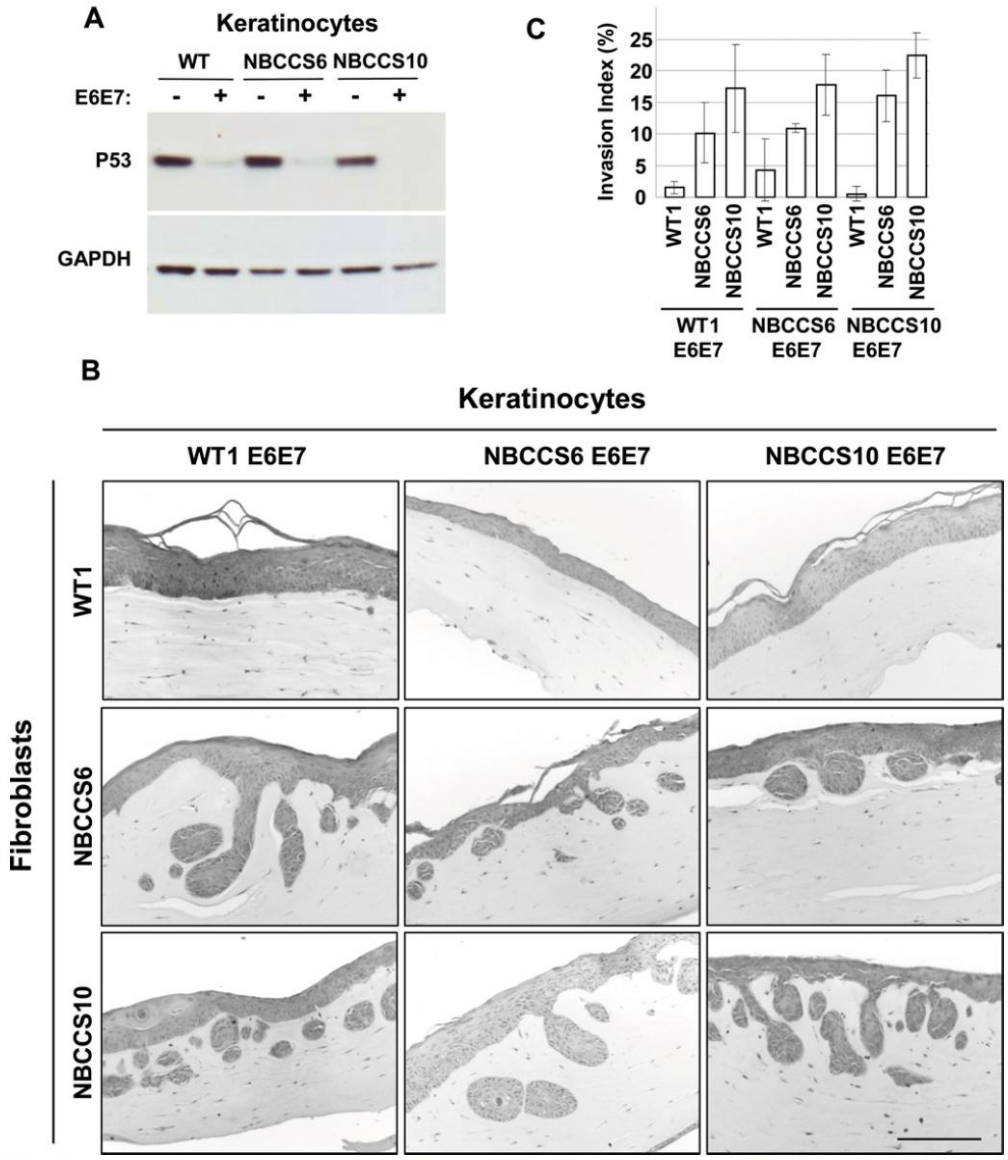

Fig 7. Abrogation of P53 in NBCCS keratinocytes confers invasive properties in the presence of NBCCS fibroblasts. (A) Western blot analysis of P53 showed a strong attenuation in WT (WT1), NBCCS6 and NBCCS10 keratinocytes transduced with a retrovirus expressing the HPV16 E6 and E7 oncoproteins. (B) Representative images of H&E coloration of paraffin-embedded sections of organotypic invasion assays composed of either WT E6-E7 or NBCCS E6-E7 keratinocytes overlaying a dermis equivalent comprising either WT or NBCCS fibroblasts. Bar: 200 μm. (C) Invasion index of keratinocytes from assays as in (B). Values represent the average of 3 total sections from two independent organotypic cultures. Bars represent the confidence intervals, $\alpha = 0.05$.

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non photo-exposed skin biopsies from NBCCS patients severely impact behavior of WT keratinocytes in OSC. Most importantly, our data indicated that the presence of primary NBCCS dermal fibroblasts in dermis equivalents resulted in P53 stabilization and cell cycle perturbations, a situation reminiscent of keratinocytes response toward genotoxic stress.

In a previous study we showed that primary NBCCS fibroblasts expressed *ex vivo* and, in a cell autonomous manner (i.e. in absence of keratinocytes), a transcriptional signature of 18 genes [17] closely similar to that reported in fibroblasts associated to human BCCs [18]. Importantly, a portion of this signature was conserved irrespective of the 2D or 3D culture conditions, suggesting that *PATCHED1* heterozygosity constitutively impacts gene expression in NBCCS fibroblasts. The transcriptional signature of NBCCS fibroblasts included genes associated with tumor cell invasion, metastasis, and neoangiogenesis. Notably, NBCCS fibroblasts expressed higher mRNA levels of the anti-angiogenic protein ANGPTL4 which is known to limit tumor cell motility and invasiveness [39] and the invasive potential of BCC cells, as well [40, 41]. Also, we noticed an important decrease of *COL7A1* mRNA in NBCCS primary fibroblasts. Deleterious mutations of *COL7A1* have been associated with the development of aggressive SCCs in patients suffering from the recessive Hallopeau-Siemens dystrophic epidermolysis syndrome (HS-RDEB) [42] while its restoration delayed tumor cell dissemination *in vitro* and *in vivo* [43]. In HS-RDEB fibroblasts, abrogation of COL7A1 expression has also been shown associated with increased levels of *WNT5A* mRNAs [43]. *WNT5A* expression was also found augmented *in vivo*, in the stroma from pancreas cancers [31] and in fibroblasts surrounding both cutaneous SCC and BCC cells [44]. In spite of these observations, NBCCS fibroblasts exhibited decreased levels of *WNT5A* mRNAs, while, paradoxically, some markers of the activation of the WNT pathway (*WISP2* and *ID2* mRNAs) where found increased. The mechanisms by which heterozygosity of *PATCHED1* in NBCCS primary fibroblasts orchestrate the interplay between the SHH and WNT pathways then deserves further research.

Experiments in transgenic mice have shown that development and maintenance of BCC-like lesions depend on either ectopic and sustained secretion of SHH in the basal germinative epidermal keratinocytes, [45, 46] or from overexpression of GLI1 [47] or of GLI2 [48]. Our results in human cells indicated that *SHH* mRNAs were undetectable in keratinocytes and barely detectable in fibroblasts cultures. Surprisingly enough, and for the first time, cultures of WT keratinocytes with WT fibroblasts resulted in detectable SHH secretion. The amount of secreted SHH was further increased in co-cultures of WT keratinocytes and NBCCS fibroblasts. These results strongly suggested that NBCCS fibroblasts play a key role in the regulation of the SHH/PATCHED pathway in epidermal keratinocytes. In good agreement with these results, we showed that expression of endogenous *PATCHED1* mRNA and the activity of the 5' regulatory region of the *PATCHED1* gene were slightly but reproducibly increased in WT keratinocytes maintained in the presence of culture supernatants conditioned by NBCCS fibroblasts. Ectopic expression of SHH in murine basal keratinocytes has been suggested to result in increased proliferation and tumor development [11]. Together with clinical observations showing that NBCCS BBCs may occur in non photo-exposed skin areas, these observations suggest that NBCCS fibroblasts could play a decisive role in the onset of BCCs. It is very important to notice that, due to the failure to grow BCC cells in culture, we could not assess the impact of NBCCS fibroblasts on BCC cells behavior under *in vivo* settings, i.e. using mouse as a vector of BCCs development.

In addition to these observations, OSC composed of NBCCS fibroblasts and WT keratinocytes exhibited abnormal traits of epidermal stratification and differentiation with thinning of the epidermal compartment. The presence of NBCCS fibroblasts also resulted in the formation of dermo-epidermal clefts, a feature commonly found at the periphery of human BCC *in vivo*, [28, 49] but not described in non-lesional skin from NBCCS patients [5]. It is conceivable that

dermo-epidermal clefts found in OSC were to be connected to our observations showing accumulation of MMP1 and MMP3 mRNAs in NBCCS primary fibroblasts [17].

In addition to epidermal thinning and dermo-epidermal clefting, severe alterations of the stepwise program of epidermal differentiation were noticed. β1 Integrin was extended to the suprabasal epidermal layers, where it is generally low or undetectable, while K10 Keratin was found delayed (K10) and Loricrin severely decreased (Loricrin) in the presence of NBCCS fibroblasts. Morphological defects and terminal differentiation were then partly normalized in the presence of the SHH-blocking antibody 5E1, further supporting the idea that over secretion of SHH by NBCCS dermal fibroblasts in the presence of WT keratinocytes may affect epidermal homeostasis. Traits of delayed differentiation in sporadic skin tumors or in psoriasis skin lesions [50] are usually associated with hyper proliferation. Unexpectedly however, in spite of delayed epidermal differentiation, we observed a sharp decrease of Ki67-positive keratinocytes overlaying NBCCS fibroblasts, indicating that NBCCS fibroblasts alter proliferation of WT keratinocytes. Together with decreased numbers of Ki67-positive cells, the P53 protein was found stabilized in epidermal cells either overlaying a NBCCS dermis equivalent or following incubation in culture supernatants of NBCCS fibroblasts. In spite of P53 stabilization, no sign of apoptosis was detected in sections of OSC containing NBCCS fibroblasts (not shown). In addition, in 2D culture conditions, we failed to detect any increase in the stabilisation of P21 which is a direct transcriptional target of P53. These observations suggest that although they promote P53 stabilization in WT keratinocytes, culture supernatants of NBCCS fibroblasts are not able to activate the P21 checkpoint in 2D culture conditions, and/or in the absence of a fibroblast-keratinocyte dialog, as observed for N-SHH (over)expression (in NBCCS fibroblasts). Since stabilization of P53 results from exposure to genotoxic stresses [29] including ROS, [51, 52] we hypothesized that NBCCS fibroblasts could be responsible for such a stress in keratinocytes. Under our experimental settings, no ROS accumulation could be detected in WT keratinocytes treated with culture supernatants conditioned by NBCCS fibroblasts. However, in the frame of the same experimental design, WT keratinocytes cultured in NBCCS fibroblasts conditioned medium exhibited substantial increase of SA-β-Gal activity. In spite of this, neither the P16 nor the P21 markers of senescence [53] were stabilized under our experimental conditions, but rather appeared in reduced amounts. Further analyses should indicate whether NBCCS fibroblasts provoke an atypical mechanism of senescence irrespective of 2D or 3D cell culture conditions, or, alternatively, that triggering of a *bona fide* senescent mechanism depends on the presence of keratinocytes (in 3D organotypic skin cultures).

The mechanism by which secretory components from NBCCS fibroblasts can affect expression of gatekeeper proteins such as P53 in keratinocytes deserves further investigations. Nevertheless, we observed that strong attenuation of P53 expression in keratinocytes immortalized by the oncogenic HPV-16 E6-E7 proteins elicited epidermal invasions in the presence of NBCCS fibroblasts. Altogether, these observations suggested that fibroblasts-led stabilization of P53 in keratinocytes ensues from a yet unidentified genotoxic stress. In NBCCS patients, dermal fibroblasts could thus further promote the development of numerous BCCs before or after abrogation of P53 following an environmental mutagenic stress, as reported in more 50% skin cancers.

In conclusion, our observations further document the role of the SHH/PATCHED pathway in cutaneous homeostasis in the human. We propose that susceptibility of NBCCS patients towards BCCs could be partly due to a "pre activated state" of stromal fibroblasts. Normalizing fibroblasts "pre activation" using appropriate pharmacological substances could improve the high susceptibility of NBCCS patients toward BCCs development and, presumably, of human cancers associated to unbalanced SHH/PATCHED pathway.

Supporting Information

S1 Fig. Alteration of early markers of epidermal differentiation in NBCCS skins *in vivo*. Paraffin sections of non photo-exposed skin from WT (WT1), NBCCS6 and NBCCS10 patients were subjected to immunolabelling for Laminin B1 and β1 Integrin, as indicated. Note the increased deposition of both β1 Integrin and Laminin B1 as observed in OSC containing NBCCS fibroblasts. Bar: 100 μm.
(TIF)

S2 Fig. Effects of NBCCS culture supernatant on ROS production in WT keratinocytes. The accumulation of ROS in WT keratinocytes was measured using the fluorescent dye DCFH-DA and FACS analysis. Upper panels, WT keratinocytes without treatment (Control). WT keratinocytes treated with non-conditioned fibroblast medium (NCM) or non-conditioned fibroblast medium + H202 (NCM + H202) as positive control of induced-ROS production. Lower panels, WT keratinocytes treated with culture media conditioned by either WT (WT1) or NBCCS (NBCCS6, NBCCS10) fibroblasts. Pre-treatment of WT keratinocytes with NBCCS culture supernatants did not led to detectable increase of ROS production.
(TIF)

S3 Fig. Effect of NBCCS culture supernatants on P21 and P16 proteins expression in WT and WT E6-E7 keratinocytes. Western blot analysis showed decreased expression of P16 and P21 in WT keratinocytes treated with culture supernatants conditioned by NBCCS fibroblasts. P16 was also decreased in WT1 E6-E7 cells. P21 was not expressed in WT1 E6-E7 keratinocytes. Right panels show levels of P21 and P16 relative to Tubulin and expressed as fold induction relative to the WT fibroblast strain.
(TIF)

S4 Fig. Full sections organotypic invasion assays. Images were taken all along the length of the organotypic sections and assembled using Image Composite Editor (ICE) software.
(TIF)

S1 Table. Patients and cells characteristics.
(DOCX)

S2 Table. Specific TaqMan® primers used for quantitative real-time PCR analysis.
(DOC)

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Author Contributions

Conceived and designed the experiments: YG MR FB SR SA MG-M TM. Performed the experiments: YG FB EB-V SB SS NS SR SA MG-M. Analyzed the data: TM YG FB MR EB-V SR SA MG-M. Wrote the paper: TM YG FB MR SR SA MG-M. Produced the anti-SHH antibody and set up conditions of use: MR. Contributed to clinics, patients recruitment and care: MFA.

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ANNEX II: Conference publications

1) Role of Xeroderma pigmentosum fibroblasts in squamous cell carcinoma invasion

SI Al-qaraghuli, S Rouanet, C Gagioli, J Albrengues, M Goncalves-Maia, T Magnaldo and Y Gache Institute for research on cancer and aging, Nice, France (ESDR Annual Meeting Abstracts) 135 (Sup 2):109 (2015).

2) Contribution des fibroblastes dermiques de patients XP-C dans l'invasion tumorale

S. Al-qaraghuli[1], Y. Gache[1], C. Girard[1], G. Gendronneau[1], M. Ohanna[2], C. Bertolotto[2], R. Ballotti[2], T. Magnaldo[1]. *[1]IRCAN, Nice et [2]C3M, Nice, France.* Vol 140 - N° 12S2, 2013 (Congrés de CARD, Paris).

3) Deciphering of molecular events occurring in keratinocytes upon solar simulated radiations

S Rouanet, Y Gache, M Goncalves-Maia, **SI Al-qaraghuli**, F Pedeutour and T Magnaldo IRCAN, Nice, France (ESDR Annual Meeting Abstracts) 135 (Sup 2):355 (2015)

4) Analysis of melanoma development with the xeroderma pigmentosum model

Y Gache,1 **SI Al-qaraghuli,1** S Rouanet,1 M Goncalves-Maia,1 M Ohanna,2 C Bertolotto,2 R Ballotti2 and T Magnaldo1 *1 IRCAN - INSERM1081 - CNRSUMR7284, Nice, France and 2 C3M, Nice, France.* (ESDR Annual Meeting Abstracts) 135 (Sup 2):355 (2015)

1) Role of Xeroderma pigmentosum fibroblasts in squamous cell carcinoma invasion

SI Al-qaraghuli, S Rouanet, C Gagioli, J Albrengues, M Goncalves-Maia, T Magnaldo and Y Gache Institute for research on cancer and aging, Nice, France (ESDR Annual Meeting Abstracts) 135 (Sup 2):109 (2015).

Squamous cell carcinoma (SCC) is the most frequent metastatic skin cancer. The etiology of skin SCC is linked to exposure to genetic stress, notably ultraviolet radiation (UVR). Xeroderma pigmentosum type C (XP-C) is a rare genetic disorder characterized by a severe susceptibility to particularly aggressive SCCs following minimal exposure to UVR, hence compromising the life expectancy of patients. XP-C cells are deficient in the nucleotide excision repair mechanism (NER) of DNA lesions introduced at bipyrimidine sequences upon UVR exposure. In addition, we reported that XP-C dermal fibroblasts constitutively expressed a phenotype resembling that of stromal fibroblasts associated to cancer cells (Carcinoma Associated Fibroblasts, CAFs) with accumulation of reactive oxygen species and over expression of matrix metalloproteinase 1. We further explored the phenotype of XP-C fibroblasts. We show here that they constitutively overexpress hepatocyte growth factor/scatter factor (HGF/SF). In organotypic skin cultures, XP-C fibroblasts promoted the invasion of SCC cells. Also, scratch healing of SCC cells was enhanced in culture supernatants of XP-C fibroblasts through a mitogenic effect connected to increased ratio of SCC cells in the G2-M phase of the cell cycle. Blockage of c-MET activation prevented invasiveness of SCC cells within dermal equivalent through inhibition of p38 activation by XP-C fibroblasts culture supernatants. Our data indicated for the first time that absence of XPC in fibroblasts leads to overexpression of cell growth stimulators such as HGF and other factors (MMP1) responsible for the formation of a microenvironment permissive towards SCC cells proliferation and invasion. Therapies targeting XP-C fibroblasts may be considered as a way to control cancer in XP patients as well as in patients from the general population suffering from aggressive cancers.

2) Contribution des fibroblastes dermiques de patients XP-C dans l'invasion tumorale

S. Al-qaraghuli[1], Y. Gache[1], C. Girard[1], G. Gendronneau[1], M. Ohanna[2], C. Bertolotto[2], R. Ballotti[2], T. Magnaldo[1]. *[1]IRCAN, Nice et [2]C3M, Nice, France.* Vol 140 - N° 12S2, 2013 (Congrés de CARD, Paris)

Le développement et les propriétés invasives des cancers épithéliaux sont liés à l'âge et aux facteurs environnementaux tels que l'exposition aux UV. Il a été montré que le développement des cancers épithéliaux implique un dialogue entre cellules tumorales et fibroblastes du stroma. Nos objectifs sont d'analyser les mécanismes contribuant au développement des cancers épithéliaux en prenant comme modèle les cellules de patients atteints de xeroderma pigmentosum (XP-C). XP-C est un syndrome génétique dû à un défaut de réparation par excision de nucléotides (NER) des lésions induites dans l'ADN par les UV. Dès le plus jeune âge, les patients présentent une forte prédisposition aux SCC (squamous cell carcinoma) et mélanomes malins. Nos études mettent en évidence un rôle important des fibroblastes XP-C dans la progression tumorale. Les fibroblastes, isolés de biopsies de jeunes patients XP-C prélevées dans des zones non photo-exposées, présentent un phénotype âgé avec une morphologie dendritique, une accumulation des espèces réactives de l'oxygène, des altérations mitochondriales. Leur correction génétique ne permet pas la normalisation de ce phénotype. Les fibroblastes XP-C et leurs surnageants promeuvent la migration et l'invasion de cellules de SCC et de la lignée de mélanome Mel501 *in vitro.* Les Mel501 traitées avec les surnageants des fibroblastes XP-C deviennent tumorigènes chez les souris. Ces résultats suggèrent que les fibroblastes XP-C sont capables de provoquer la tumorigenèse. Les analyses moléculaires et biochimiques nous permettront d'identifier les facteurs responsables de la formation d'un microenvironnement permissif en absence de XPC.

3) Deciphering of molecular events occurring in keratinocytes upon solar simulated radiations

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Xeroderma pigmentosum (XP) is a rare, recessive genodermatosis characterized by high photo-sensitivity and susceptibility to the development often before the age of 8 of malignant epidermal carcinomas in photo exposed skin, in absence of photoprotection, leading to compromised health conditions and life expectancy. This disease is due to defective nucleotide excision repair of DNA lesions occurring upon solar radiation. XP-C is the most frequent form of the disease in our countries. In our laboratory, we use XP-C primary skin keratinocytes to model genomic hypermutability as well as molecular and cellular events leading to aggressive cutaneous cancers. As chronic solar irradiations are responsible for the majority of SCC, which relative frequency is much higher in XP patients than in general population, we analyzed the effects of chronic solar simulated radiations (SSR) on WT and XP-C keratinocytes in culture, using chronic low dose of single SSR per week for 8 weeks (100 J UVB/m2). We have shown the emergence of atypical clones of XP-C keratinocytes populations while SSR irradiation of WT cells resulted in clonal transition. Atypical clones exhibited significant increase in growth and clonogenic potential compared to the WT suggesting a selective advantage of growth after iterative irradiation (IRR) of XP-C cells (from 10% after 4 IRR to 25% after 8 IRR). Karyotype analyzes of XP-C clones revealed normal chromosomal distribution, suggesting occurrence of point mutations rather than gross genetic rearrangement. Cell cycle analyses of those clones showed higher rates of cells in G2/M phases. We are currently analyzing 1) the genetic status of 400 oncogenes and tumor suppressor genes frequently mutated in tumor cells, and 2) the patterns of gene expression in those clones. Deciphering molecular and cellular modifications occurring in cells during iterative exposure toward UV carcinogenesis will be a great help to better diagnose the evolution of skin cancers and to develop drugs targeting the specific pathways affected in both XP patients and in individuals of the general population

4) Analysis of melanoma development with the xeroderma pigmentosum model

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Our objectives are to study the mechanisms implicated in the development of human cancers among which squamous cell carcinoma (SCC), and malignant melanoma (MM) of the skin account for the most aggressive neoplasms. Increasing evidences support the idea that stromal cells, notably dermal fibroblasts, promote tumor cells aggressiveness and invasive capacities. Xeroderma pigmentosum (Group C, XP-C) is a rare genetic disorder characterized by defective nucleotide excision repair (NER) of UV-induced DNA lesions and severe proneness toward aggressive skin cancers. To shed light on the agressiveness of XP skin cancers, we hypothesized that XP-C fibroblasts could play a prominent role through pejorative stroma-cancer cells interactions. We found that XP-C fibroblasts present features of cancer associated fibroblasts (CAF) towards epidermal keratinocytes in absence of genotoxic stress. Here, we analyzed the capacity of XP-C fibroblasts to promote migration and invasion of cells derived from human MM. Our results show that the secretome of XP-C fibroblasts elicited migration and invasion of the non-tumorigenic Mel501 melanoma cells *in vitro*. Treatment of Mel501 cells with culture supernatant of XP-C fibroblasts but not of their genetically counterparts was sufficient to promote tumorigenesis in mice. Screening of molecules secreted by XP-C fibroblasts identified Hepatocyte Growth Factor/Scatter Factor (HGF/SF) and CXCL12/SDF1 as potential pro-invasive/tumorigenic protagonists. Mel501 cells cultured in culture media conditioned by primary XP-C fibroblasts showed activation of the c-Met receptor and of the downstream p38 and JNK effectors, as well. Depletion of HGF in XP-C culture media conditioned by XP-C fibroblasts supressed c-Met activation. Our studies demonstrate for the first time that XP-C fibroblasts play a decisive role in melanoma progression and identify factors responsible for the formation of a permissive microenvironment.

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