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Rémy Bonnavion

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“Study of the effects of *Men1* disruption in mouse pancreatic endocrine progenitors during development and adult life”

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Abstract :

Multiple Endocrine Neoplasia Type I syndrome (MEN1) is a rare hereditary tumoral disease characterized by the apparition of tumors in multiple endocrine organs including the endocrine pancreas. MEN1 patients generally carry a germinal mutation on one allele of the predisposing gene to the disease, the tumor suppressor *MEN1*.

Pancreatic endocrine tumors are rare, slowly evolving and often present with metastasis at diagnosis. These tumors constitute a heterogeneous group defined by their hormonal secretions. Evolution and development of these tumors is far from being understood. The cell of origin of the different pancreatic endocrine tumor types is enigmatic, notably for tumors secreting non-pancreatic hormones such as gastrinomas.

My thesis project was structured toward the characterization of a new murine model allowing the specific disruption of the *Men1* gene in Ngn3⁺ pancreatic endocrine progenitors, the *PancEndoMen1* KO model.

The combined study of this new model and previous model generated in the team, allowed us to demonstrate that pancreatic gastrinomas related to *Men1* inactivation, originate from the endogenous pancreatic endocrine cells. In parallel, our results demonstrated that the mutant mice having *Men1*-deficient Ngn3⁺-progenitors resulted in differential cell proliferation alterations in different pancreatic endocrine cells. Importantly, *Men1*-disruption in either pancreatic endocrine or pan-pancreatic progenitors displayed tumors with impaired differentiation features.

Thus, this thesis works allowed to better characterize pancreatic endocrine tumors histogenesis by addressing the role of pancreatic endocrine progenitors targeted *Men1* disruption during development in tumorigenesis.

Titre : Etude des effets au cours du développement et pendant la vie adulte de l'inactivation du gène *Men1* dans les progéniteurs endocrine pancréatiques.

Résumé:

Le syndrome des Néoplasies Endocriniennes Multiples de type I (NEM1) est une maladie tumorale héréditaire rare caractérisée par l'apparition de tumeurs notamment du pancréas endocrine. Le gène de prédisposition est le suppresseur de tumeur *MEN1*, généralement retrouvé muté sur un des 2 allèles au niveau germinale chez les patients NEM1.

Les tumeurs endocrines pancréatiques sont rares mais souvent métastatiques lors du diagnostic. Ces tumeurs forment un groupe hétérogène de par le type d'hormone qu'elles peuvent sécréter. Le développement et l'évolution de ces tumeurs sont encore très mal compris. L'origine cellulaire des différents types de tumeurs endocrines pancréatiques reste énigmatique, notamment en ce qui concerne les tumeurs exprimant des hormones non-pancréatiques tels que les gastrinomes.

Mon projet de thèse s'est articulé autour de la caractérisation d'un nouveau modèle murin d'inactivation du gène *Men1* spécifiquement dans les cellules progénitrices endocrine pancréatiques Ngn3⁺ (PEPs), le modèle *PancEndoMen1* KO.

Ces travaux nous ont permis de démontrer que les gastrinomes pancréatiques liés à l'inactivation du gène *Men1*, avaient pour origines les cellules pancréatiques endocrines elles-mêmes.

De plus, les souris *PancEndoMen1* KO, développent des altérations de prolifération différentes suivant les lignages endocrines. De surcroît, l'inactivation du gène *Men1* soit dans les cellules progénitrices pancréatiques, soit dans les cellules PEPs conduit au développement de tumeurs caractérisées par une altération de leur différenciation endocrine.

Ainsi, mes travaux de thèse ont permis de mieux renseigner l'histogenèse des tumeurs endocrines pancréatiques en adressant le rôle dans la tumorigenèse de l'inactivation de *Men1* dans les cellules PEPs au cours du développement.

DISCIPLINE Biologie Moléculaire et Cellulaire

MOTS-CLES

MEN1, modèle murin, gastrinome, tumeurs endocrines pancréatiques

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List of abbreviations

ACTH	Adrenocorticotrophic Hormone
AIP	Anterior Intestinal Portal
Arx	Aristaless related homeobox
Ash2L	(absent, small, or homeotic)-like
Atoh1	Atonal homolog 1
ATRX	alpha thalassemia/mental retardation syndrome X-linked
Bcl6	B cell leukemia/lymphoma 6
bHLH	basic Helix Loop Helix
Bmi1	B lymphoma Mo-MLV insertion region 1
BMP	Bone Morphogenetic Protein
Bp	Base-pair
BRCA1	Breast cancer 1
CAG	chronic atrophic gastritis
CCK	Cholecystokinin
CDK	Cyclin Dependant Kinase
Cdkn	Cyclin-dependent kinase inhibitor
Cdx	Caudal type homeobox
CIP	Caudal Intestinal Portal
DAXX	Death-domain Associated protein
DNk	Deoxyribo Nucleic acid
d-NET	duodenal Endocrine Tumor
Dnmt	DNA methyltransferase
ES	Embryonic Stem
EC	Enterochromaffin
ECL	Enterochromaffin-like
eGFP	Enhanced green fluorescent protein
ERK	Extracellular signal-regulated kinase
ER α	Estrogen receptor α
eYFP	Enhanced yellow fluorescent protein
Ezh2	Enhancer of zeste homolog 2
Fox	Forkhead box
Gas1	Growth arrest-specific 1
Gcgr	Glucagon receptor
GEP	Gastro Entero Pancreatic
Gfi1	Growth factor independent 1
GH	Growth Hormone
GIP	Gastric Inhibitory Polypeptide
GLP	Glucagon-like peptide
Glu	Glucagon

GLUT2	Glucose Transporter2
Grg3	groucho 3
hADSC	human Adipose-tissue Derived Stem Cells
Hes1	Hairy and enhancer of split 1
Hlxb9	Homeobox gene HB9
HMT	Histone Methyltransferase
Hnf1 β	hepatocyte nuclear factor 1-beta
Hox	homeobox
HSC	Hematopoietic Stem Cells
IAPP	Islet amyloid polypeptide
IGF	Insulin-like growth factor
Igfbp	Insulin-like growth factor binding protein
Ins	Insulin
Insm-1	Insulinoma-associated 1
JNK	JUN N-terminal kinase
Kb	kilobase
KRAS	Kirsten rat sarcoma viral oncogene
LEDGF	Lens epithelium-derived growth factor
Lgr5	Leucine rich repeat containing G protein coupled receptor 5
LOH	Loss Of Heterozygoty
Maf	Musculo- aponeurotic fibrosarcoma
MAPK	Mitogen-activated protein kinase
MEF	Mouse Embryonic Fibroblast
Meis1	myeloid ecotropic viral integration site 1
MEN1	Multiple Endocrine Neoplasia Type I
MLL	Mixed-lineage leukemia
MPC	multipotent pancreatic progenitor
mRNA	messenger Ribonucleic acid
mTOR	mammalian Target of Rapamycin
Myb	myeloblastosis oncogene
NEC	Neuroendocrine Carcinoma
NES	Nuclear Export Signal
Neurod1	neurogenic differentiation 1
NF	Non-functional
NF1	Neurofibromatosis 1
NFAT	nuclear factor of activated T-cells
Ngn3	Neurogenin 3
NICD	Notch intracellular domain
Nkx	NK homeobox
NLS	Nuclear Localization Signal
Pax	paired box gene
PC	Prohormone convertase

Pdgf	platelet-derived growth factor
Pdgfr	Pdgf Receptor
Pdx1	Pancreatic and duodenal homeobox gene1
PERK	PRKR-like endoplasmic reticulum kinase
PET	Pancreatic Endocrine Tumor
PI3K	phosphatidylinositol 3-kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
Pit-1	Pituitary specific transcription factor 1
PP	Pancreatic Polypeptide
PPAR	peroxisome proliferator-activated receptor
PRL	Prolactin
PRMT5	protein arginine methyltransferase 5
PTEN	phosphatase and tensin homolo
Ptf1a	pancreas specific transcription factor, 1a
PTH	Parathyroid Hormone
PTHrP	parathyroid hormone-related protein
PTN	Pleiotropin
PYY	Peptide tyrosine tyrosine
Rb	Retinoblastoma
RbBP5	retinoblastoma binding protein 5
Rfx6	regulatory factor X, 6
RIP	Rat insulin promoter
RPTP	protein tyrosine phosphatase, receptor-type
Smad	mothers against decapentaplegic homolog
Sox9	SRY-box containing gene 9
STZ	Streptozotocin
Suv39h1	suppressor of variegation 3-9 homolog 1
SV40	simian virus 40
Tag	SV40 Large T antigen
TGFβ	Transforming Growth Factor β
Tsc	Tuberous sclerosis 1
TSH	Thyroid Stimulating Hormone
VHL	von Hippel–Lindau disease
VIP	Vasointestinal Polypeptide
WDR5	WD repeat domain 5
wt	wild-type
WT1	Wilms tumor suppressor protein
ZES	Zollinger-Ellison syndrome

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INTRODUCTION

INTRODUCTION.

PART I the Gastroenteropancreatic endocrine axis: *Gut development.*

1 INTRODUCTION

1.1 PART I THE GASTROENTEROPANCREATIC ENDOCRINE AXIS

1.1.1 GUT DEVELOPMENT.

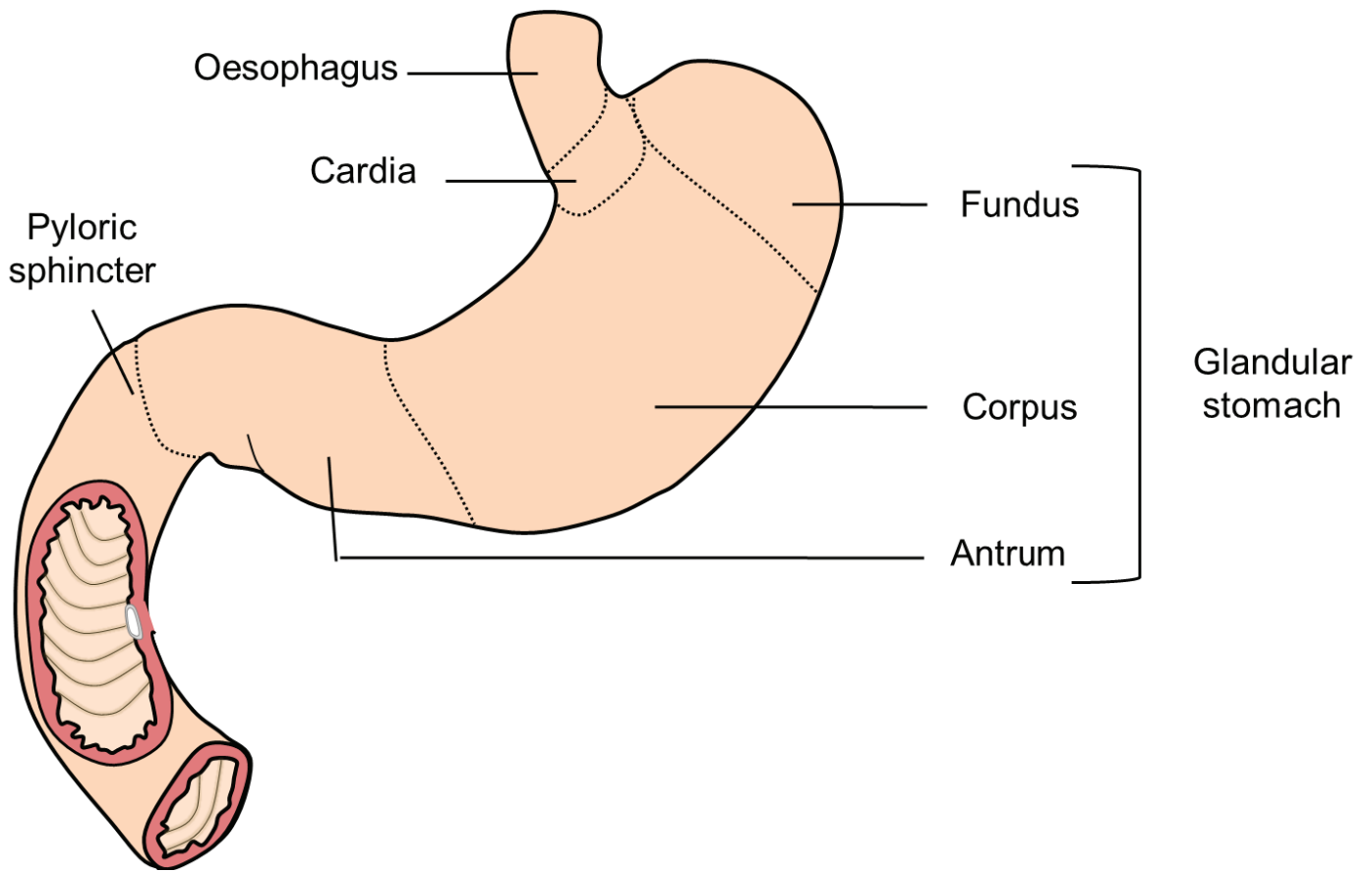
The stomach, intestine and pancreas are different organs deriving from the developing gut during embryogenesis. During early development, the gut forms a tube with a lumen, composed of an outer tissue layer of mainly smooth muscle derived from the mesoderm, and an inner epithelial layer essentially derived from the endoderm excepted for mouth and anal region derived from the ectoderm. The early formation of the tube involves two invaginations of the definitive endoderm occurring at the anterior and posterior part of the embryo called respectively Anterior Intestinal Portal (AIP) and Caudal Intestinal Portal (CIP). Each lip of invaginations then grows and elongates toward each other and meets in the middle of the embryo, with a gut finally closing during the turning of the embryo at E9.0 in mouse (Lewis and Tam 2006). During formation, the gut tube is patternized into three different portions along the Anterior-Posterior axis that are respectively the foregut, midgut and hindgut. This patterning is controlled by crucial transcription factors that are differentially expressed along the antero-posterior axis. *Foxa2* is required for foregut formation (Dufort et al. 1998, McKnight et al.), whereas *Cdx1*, *2* and *4* are necessary for that of the hindgut.

The gut formation is accompanied by the generation of different territories from which will differentiate gut-derived organs. The oesophagus, trachea, lungs, thyroid, parathyroids, stomach, the proximal part of the duodenum, pancreas and hepatobiliary system originate from the foregut. The midgut gives rise to the distal part of the duodenum and small intestine, whereas the hindgut leads to large intestine development (Wells and Melton 1999). Three foregut-derived organs that were studied during the thesis work are introduced more in detail in the following.

1.1.2 THE STOMACH

1.1.2.1 STOMACH DEVELOPMENT

A



B

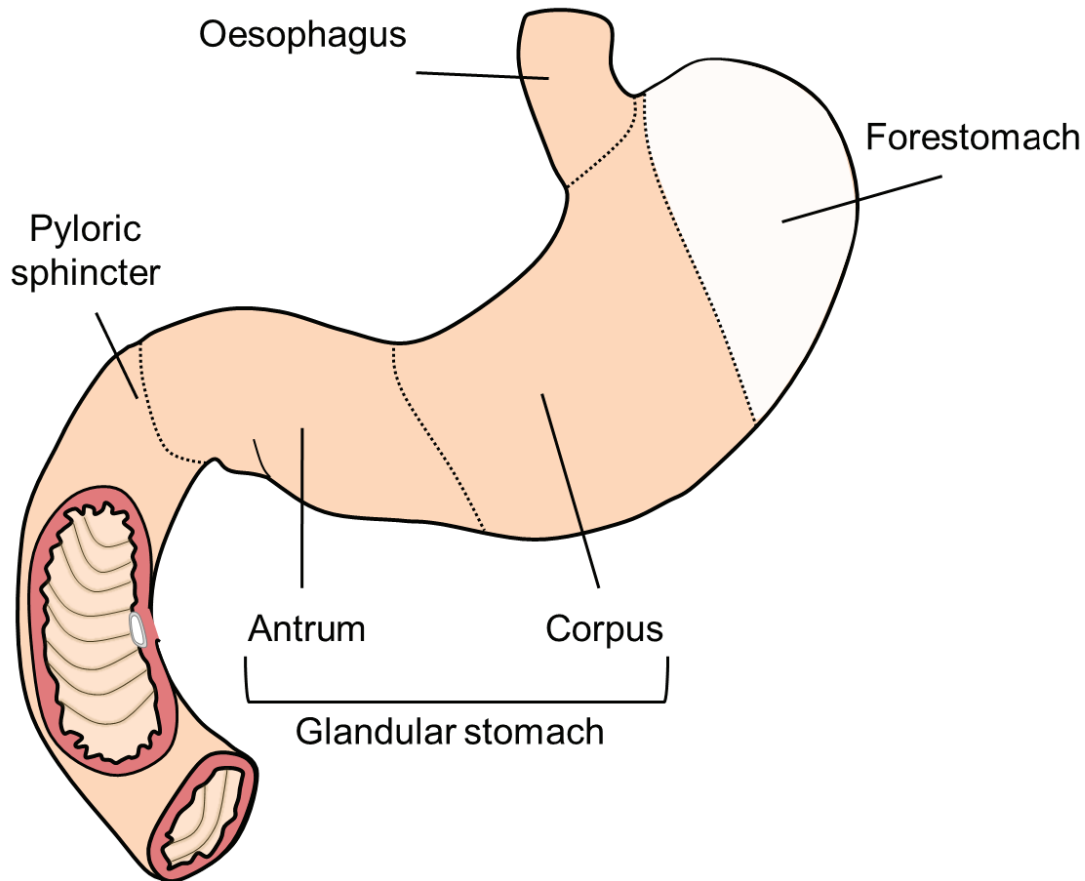


Figure 1. Anatomy of human (A) and mouse (B) stomachs.

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PART I the Gastroenteropancreatic endocrine axis: *The Stomach*

The stomach begins to be formed during antero-posterior regionalization of the gut tube. The cytodifferentiation of the stomach begins at E13.5 in the mouse. At E16.5, the developing stomach is morphologically composed of two regions, the anterior region which will give rise to the forestomach composed of stratified, keratinized squamous epithelium (which does not exist in human) and the posterior part composed of a glandular epithelium (Khurana and Mills 2010). After birth, the stomach development continues and growth rapidly with a peak at three weeks after birth in mouse. The adult stomach epithelium is constantly renewed during life as the intestine (Khurana and Mills 2010).

1.1.2.2 STOMACH EPITHELIUM COMPOSITION

The stomach, as the intestine or colon, is mainly composed of different layers. The exterior one called the serosal layer, is a fine layer of epithelial tissue; then the muscularis externa layer composed of three layers of smooth muscle and enteric nervous system plexi; the submucosa composed of undifferentiated connective tissue and vascular tissue; and finally the most interior layer, the mucosa composed of epithelial cells adjacent to the overlying mesoderm that includes a thin smooth muscle layer called muscularis mucosa (Smith et al. 2000). The stomach in human is regionalized in two functionally and anatomically different zones (Figure 1A). The most rostral part is called the fundus and body (corpus) that contains invaginated thick gastric glands secreting pepsinogen and hydrochloric acid continuous to pits that secrete mucus. The caudal part is called the antrum or pyloric antrum that is also composed of villus-like pit-gland units that secrete mucus and the major part of gastrin (Karam 1999, Smith et al. 2000). The junction with duodenum appears at the most caudal part of the antrum (Smith et al. 2000). In addition, there is another region between esophagus and fundus called cardia which is not well studied (Smith et al. 2000). The development and function of chicken stomach has been much more studied than mammals' stomach. The body and the pylorus regions were shown to be highly similar to chicken proventriculus and gizzard stomach regions (Smith et al. 2000).

In the body/fundus of the stomach the glands forms the oxyntic epithelium which is composed of several cell types (Figure 2 A). Under the pits containing mucus secreting cells, the glands are organized in three parts, the isthmus that contains precursors cells and probably multipotent progenitors, the neck that contains mucus secreting cells, and the base in which reside zymogenic cells (chief cells) producing enzymes such as pepsinogen. Acid-secreting

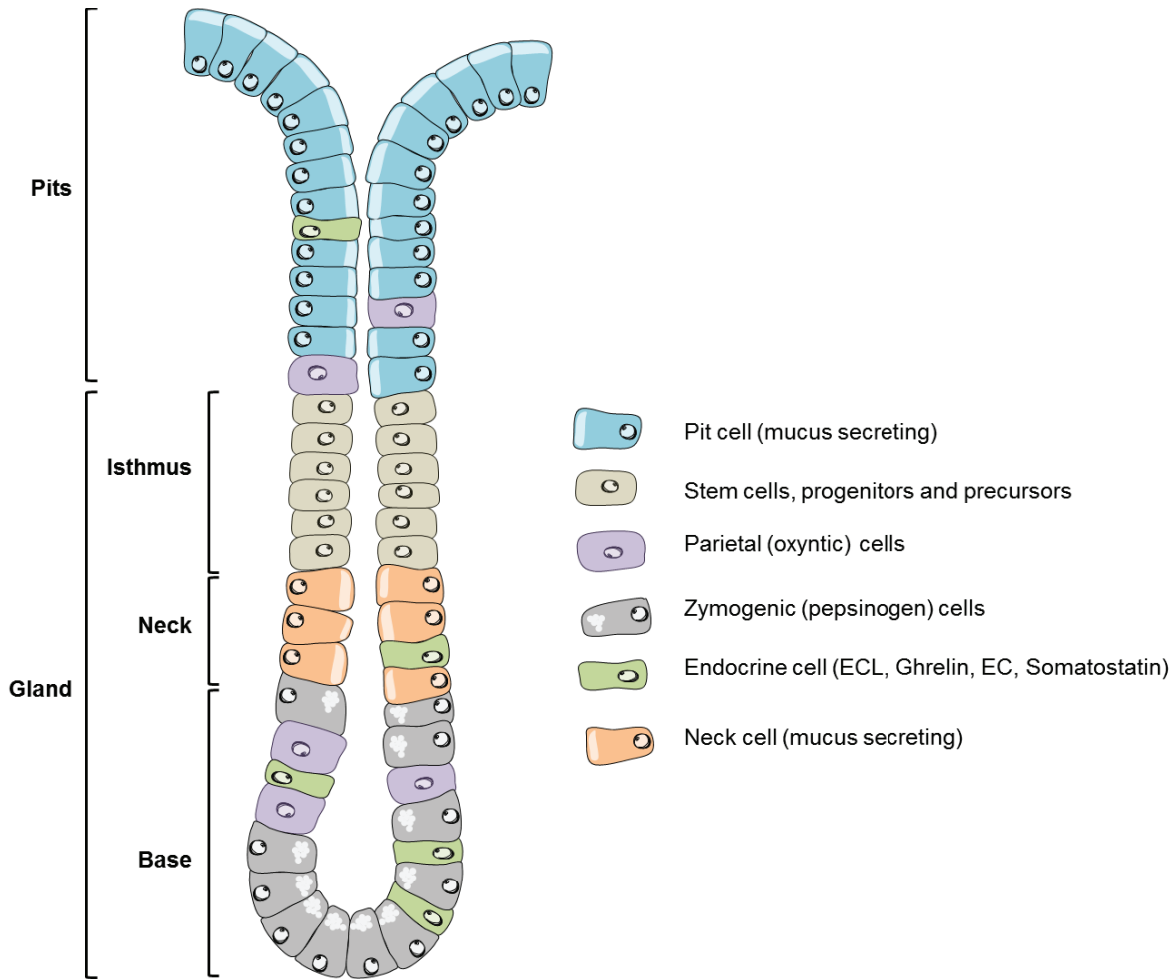
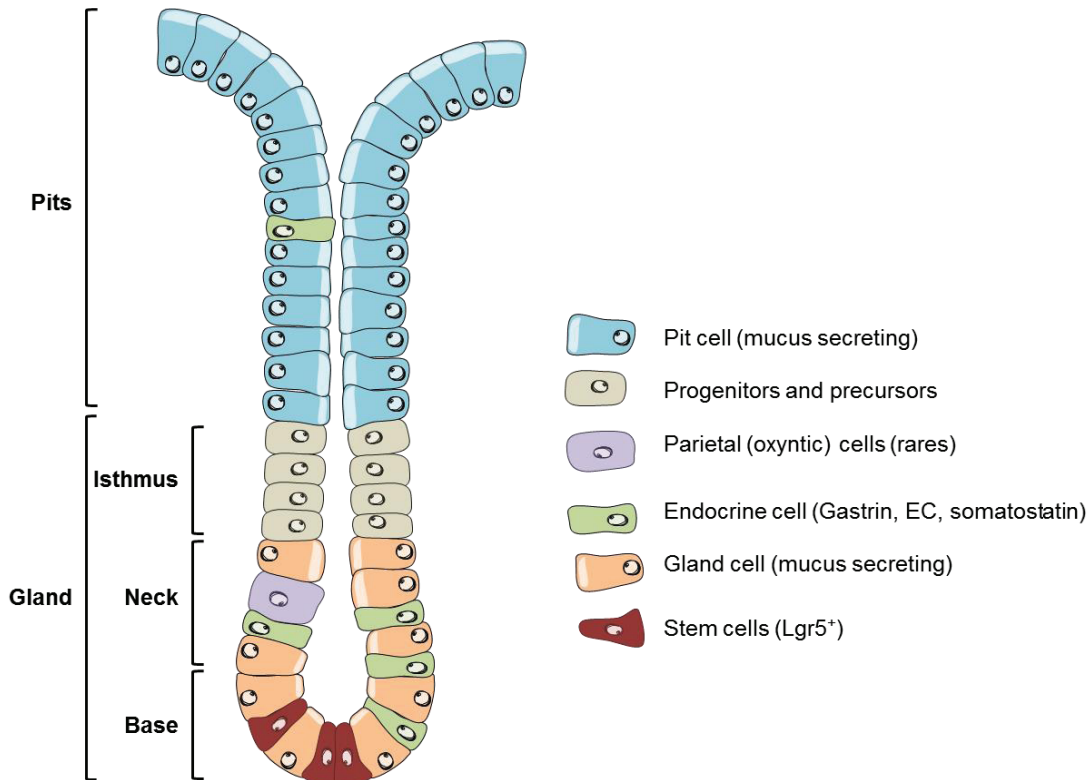
A**B**

Figure 2. Cytoarchitecture of (A)body/fundus gastric pit/gland units and (B)pyloric/antral pit/gland units

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parietal (oxyntic) cells, caveolated (tuft) cells and endocrine cells are dispersed in the gland (Karam 1999, Khurana and Mills 2010).

The pyloric pit/gland units are also similarly regionalized in isthmus, neck and base as in the corpus (Figure 2B). The pit cells secrete mucus. The isthmus is mainly composed of precursor cells. The neck and base are mainly composed of gland mucus producing cells and rare parietal acid-secreting cells. Caveolated cells (tuft cells) and endocrine cells are scattered in the pit-gland pyloric units (Karam 1999, Khurana and Mills 2010). A specificity of antral pyloric glands compared to corpus glands is the presence of $Lgr5^+$ multipotent stem cells at the very base of the glands. These cells give rise to cells that migrate rapidly in the isthmus where they will proliferate and differentiate into all the epithelial lineages of the antrum (Barker et al. 2010).

1.1.2.3 ENDOCRINE CELLS OF THE STOMACH

Several endocrine cell-types are present in the stomach, their distribution being differential between corpus and antrum. The endocrine cells represent 7% of all epithelial cells in the body and 3% in the antrum (Karam 1999).

The histamine-secreting ECL cells (Enterochromaffin-like cells) are the most abundant endocrine population in the corpus/fundus but are absent in the antrum. Gastric ECL cells appear only after birth in the mouse (Jenny et al. 2002). Ghrelin-secreting cells (P/D1) represent about 12% of corpus endocrine cells and are also present but rare in the antrum. The less abundant enterochromaffin (EC) cells secreting serotonin and somatostatin-secreting D cells are scattered through the corpus and antrum. The major endocrine population of the antrum is represented by gastrin-secreting G cells, which are absent in the corpus, residing at the base of the glands (Rindi et al. 2004). Finally, an extremely rare population of glucagon expressing cells that also express ghrelin is found in the corpus/fundus (Solcia et al. 2000).

The mechanisms controlling differentiation of gastric endocrine cells have not been well studied. In the corpus/fundus and the antrum glands, reside endocrine progenitors expressing the bHLH transcription factor Neurogenin 3 (Ngn3). These progenitors originate from multipotent stem-cells localized in the gastric glands. It was demonstrated that all endocrine cell types ($ghrelin^+$, ECL cells, EC cells, $gastrin^+$ and $somatostatin^+$) can be derived from $Ngn3^+$ progenitors by lineage tracing (Jenny et al. 2002). However, if most endocrine

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PART I the Gastroenteropancreatic endocrine axis: *Duodenum*

cells of the antrum derive from Ngn3 expressing precursor, only 40% of body/fundus endocrine cells are deriving from Ngn3⁺ precursors (Schonhoff et al. 2004). Interestingly, it was demonstrated in *Ngn3* null mice, that Ngn3 is necessary for the development of most gastrin⁺ and somatostatin⁺ cells in the stomach whereas ghrelin⁺, EC cells and ECL cells can still develop (Jenny et al. 2002). The precise quantification of the remaining endocrine populations was not assessed, so it is still unknown whether all EC, ECL and ghrelin⁺ cells can develop independently of Ngn3 or that subpopulations sensitive to Ngn3 loss exist or not. However, it was shown that the majority of EC cells require Ngn3 for their development as EC cell number is reduced by 6-folds in the glandular stomach of 2-3-days-old *Ngn3 KO* mice (Lee et al. 2002). Pancreatic duodenal homeobox 1 (Pdx1) is expressed in potential progenitors in antral glands and in 10 to 20% of antral hormone expressing cells (Larsson et al. 1996). Pdx1 is necessary for G cells development in mouse antrum, as the stomach from *Pdx1 null* mice is devoid of gastrin⁺ cells (Larsson et al. 1996). Interestingly, gastric antrum from *Pdx1 null* mice demonstrated a three-fold increase in serotonin producing cells, without change in D cells.(Larsson et al. 1996). The role of Pdx1 in the development of endocrine cells from the corpus/fundus was never assessed.

1.1.3 DUODENUM

1.1.3.1 DUODENAL DEVELOPMENT

The duodenum represents the proximal part of the intestine, directly after the pylorus. When the gut tube is formed, the epithelium derived from the endoderm begins to condense and will form a pseudostratified epithelium at E9.5 in mouse. Then, the developing intestine elongates while both the epithelium and surrounding gut mesenchyme thicken until E13.5. During this period the disorganized pseudostratified epithelium progressively changes to a stratified epithelium where apically located cells are tightly connected by junctional complexes. At E14 in the mouse (9 weeks in humans), the stratified epithelium begins to be massively reorganized to form a simple columnar epithelium surrounding the lumen of the intestine. This process is concomitantly associated with the emergence of intraepithelial cavities (secondary lumina) in the basal part of the stratified epithelium (Matsumoto et al. 2002). The restructuration of the epithelium which progresses in a rostro-caudal manner along the intestine, allows the emergence of villi at E15 in mouse. The latter will further grow secondary to the invagination of surrounding intestinal mesenchyme. Even if, during the villi

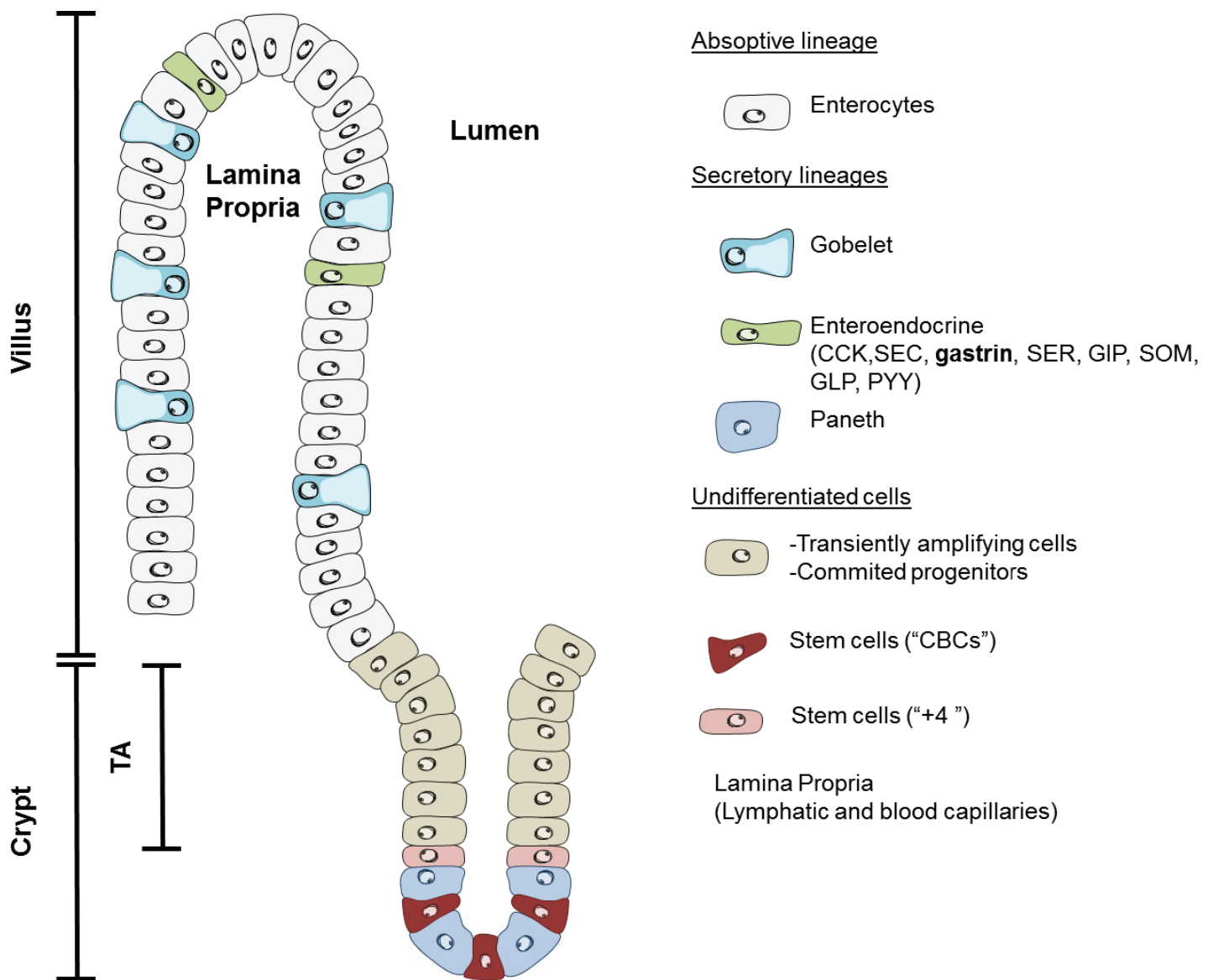


Figure 3. Cytoarchitecture of duodenal Crypt/villus unit.

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emergence, cell proliferation occurs in the whole epithelium, the growth of villi is rapidly supported by the proliferation of cells at their base that is progressively restricted to the intervillus epithelium at E17 and finally crypts of Lieberkühn that develop postnatally in rodents (Hirano and Kataoka 1986) but are already formed during gestation in human (Drozdowski et al.) (Figure 3).

During villi emergence, two groups of differentiated cells emerge in the epithelium, the absorptive lineage represented by enterocytes in duodenum and small intestine and the secretory lineages, respectively. Secretory lineages are composed of goblet cells that secrete mucus; and enteroendocrine cells that secrete different hormones (see next part). In humans, two other secretory lineages, crypt localized Paneth cells that secrete antimicrobial peptides, and tuft cells can be readily found in the developing embryo whereas these populations appear postnatally in the mouse (Figure 3). The origin and process of differentiation of all these post-mitotic differentiated lineages are well studied and described in adult intestine (van der Flier and Clevers 2009). Actually, intestinal crypts are the host of self-renewing adult multi-potent stem cells giving rise to transient amplifying cells that proliferate and then differentiate into enterocytes or different secretory lineages, ensuring the constant renewing of the intestinal epithelium (Spence et al.). However, the existence of such stem cells during embryonic development is not well established in the mouse. In mouse, crypts are characterized by multi-clonality during the first week after birth. This means that several stem cells contribute to the epithelium of a given villi. However, all crypts tend to monoclonality at 2 weeks after birth, that is multiple stem cells of a single crypt are originating from a single stem cells (Shiojiri and Mori 2003). This process may be likely due to the high proliferation rate and fissions of the crypts that allow the generation of new crypts (Spence et al.). This tendency of the crypts to drift toward monoclonality seems to occur also in aged intestine (Snippert et al.).

1.1.3.2 DUODENAL ENDOCRINE CELLS

The endocrine population of the intestine is a discrete one in terms of number as compared with other intestinal epithelial cells, as they represent only about 1% of total intestinal epithelial cells (Schonhoff et al. 2004). Throughout the intestine, enteroendocrine cells comprise at least 10 different types of endocrine cells on the basis of their hormonal secretion (Rindi et al. 2004). The latter are differentially distributed along the proximal-distal axis in the intestine (Aiken et al. 1994). Most enteroendocrine cells are localized in the villi

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but some endocrine cells can be found within the upper crypt and crypt base (Aiken et al. 1994, Sei et al.).

In the duodenum, ghrelin, serotonin, somatostatin, Glucagon-like immunoreactants (such as GLP-1), PYY, gastrin, cholecystokinin, secretin, gastric inhibitory polypeptide (GIP), motilin and neurotensin producing cells can be detected (Rindi et al. 2004), with serotonin⁺ and GIP⁺ cells being the prevalent cells in quantity. The gene encoding motilin is present in humans but neither in mouse nor rat genome. The duodenum is not the main site of expression of GLP-1 nor ghrelin, but is the main site of CCK expression even if the latter is not the main population of endocrine cells of the duodenum. Several reports have demonstrated, by colocalisation experiments and cell ablation studies, that some endocrine cells can be multi-hormonal and co-express different combinations of hormones (Aiken et al. 1994, Egerod et al. , Habib et al.). Aiken and colleagues demonstrated that most crypt localized gastrin⁺ cells were also expressing GLP-1 but not secretin, whereas more than half of villus gastrin⁺ cells were expressing GLP-1 and about 30% of them also co-express secretin in the duodenal mouse mucosa. On the contrary, they found that most serotonin⁺ and GIP⁺ cells were negative for the other tested hormones. However, in contrast with this study, Egerod and colleagues recently demonstrated that GIP⁺ cells also express CCK and secretin in human duodenum by double immunofluorescent stainings, and also provided evidence suggesting the possible existence of GLP and CCK double positive cells in mouse duodenum (Egerod et al.). However, since they did not perform double immunostaining for both GIP and CCK in mouse duodenum, the existence of the endocrine cells expressing both CCK and GIP in mouse duodenum remains to be elucidated. In both mouse and humans, the few ghrelin positive cells in the duodenum do not express any of other gut hormones except motilin in human.

The endocrine cells in the duodenum, as in other parts of the intestine, are all derived from adult stem cells localized at the base of the crypts, and this was definitely confirmed recently by lineage tracing experiments in the mouse (Barker et al. 2007). These stem-cells give rise to the transient-amplifying cells upper in the crypts. They actively proliferate and then engage in a differentiation process leading to the formation of the different lineages of the duodenum (and more broadly of the intestine). However we still do not know if the differentiation process occurs before the TA compartment or after as shown by recent studies (Li et al. 2011, Sei et al. 2011, Buczacki et al. 2013). The differentiation towards the

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endocrine lineage is widely dependent on lateral inhibition mechanisms by Notch pathway. Indeed, it was shown that mice overexpressing the intracellular domain (NICD) of Notch 1 receptor specifically in all the epithelial compartment of the intestine, displayed impaired differentiation of the secretory lineages analyzed (goblet and enteroendocrine) (Fre et al. 2005), via the activation of the nuclear transcription factor RBP-J κ leading to the transcription of *Hes1*, a bHLH transcriptional repressor (Jarriault et al. 1998) that represses the endocrine lineage (and other secretory lineages) cell fate choice. Mice lacking *Hes1* die during embryogenesis, but still develop an intestine with an increase in secretory lineages, specifically in enteroendocrine cells (Jensen et al. 2000). Several lines of evidence indicate that Hes1-mediated repression of secretory lineage is dependent on the inhibition of the expression of the bHLH activator Math1 (also called Atoh1) (Jensen et al. 2000, Kim and Shivdasani 2011). Math1 expression is required for the secretory lineages specification both during development and in adult, and its expression persists in goblet and paneth cells (Yang et al. 2001, Shroyer et al. 2007). Once the secretory lineage is chosen over the absorptive lineage, the enteroendocrine cell fate still has to be engaged. One of Math1 positive target is the zinc-finger transcription factor Gfi1. Several observations led to the concept that in Math1 progenitors, Gfi1 expression favors the differentiation of secretory lineage precursors towards non-endocrine secretory lineages (Paneth and goblet), though this has been recently debated (Bjerknes and Cheng 2010).

Even if the precise molecular mechanisms allowing the allocation of endocrine versus Paneth/goblet cell lineage remain elusive, the intestinal endocrine lineage has been shown to be dependent on the bHLH transcription factor Ngn3. Newborns constitutively lacking *Ngn3* and mice lacking specifically *Ngn3* in the intestine do not develop any endocrine cells in the intestine (Jenny et al. 2002, Mellitzer et al. 2010). Lineage tracing experiments confirmed that all intestinal endocrine cell subtypes were derived from Ngn3⁺ progenitors (Jenny et al. 2002). Conversely, an increase in endocrine cells number and a decrease in goblet cells occur in transgenic embryo expressing *Ngn3* under the widely active *Villin* promoter, (Paneth cells were not studied because of their absence before birth in mouse) strongly suggesting that Ngn3 actively drives uncommitted secretory precursors toward endocrine lineages (Lopez-Diaz et al. 2007). *Math1* mRNA expression was not altered in *Ngn3* KO animals, and goblet and Paneth cell populations are not altered in these mice, suggesting that Ngn3 acts downstream of Math1⁺ progenitors. The importance of Ngn3 in enteroendocrine lineage

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specification has also been reported in human. A severe loss of the endocrine cell population in the intestine, leading to congenital malabsorptive diarrhea was reported in several patients carrying homozygous point mutation in the coding sequence of *NGN3* or biallelic mutations of *NGN3* (Wang et al. 2006, Pinney et al. 2011, Rubio-Cabezas et al. 2011).

Once the enteroendocrine lineage is selected upon *Ngn3* expression, several other transcription factors were shown to be important for the differentiation of different endocrine cell subtypes in the intestine. The bHLH transcription factor *Neurod1* (also denominated BETA2) is necessary for secretin⁺ and CCK⁺ cell lineages in the intestine, since *NeuroD1*^{-/-} mutant mice develop neither secretin⁺ nor CCK⁺ cells in the intestine, whereas other enteroendocrine lineages are unaffected (Mutoh et al. 1997, Naya et al. 1997). *Nkx2.2* was shown to be expressed in 80% *Ngn3*⁺ intestinal progenitors and several endocrine cell-types of the intestine (Wang et al. 2009), and involved in the differentiation of serotonin, CCK, GIP, and gastrin expressing cells as they are absent or severely reduced in the developing intestine of *Nkx2.2*^{-/-} embryos (Desai et al. 2008, Wang et al. 2009). Ghrelin expressing lineage was dramatically increased in these mice, showing that Ghrelin is an independent lineage branching as compared to other endocrine lineages. However, due to postnatal lethality, the role of *Nkx2.2* in the differentiation of intestinal endocrine cells in adult intestine remains to be elucidated. *Foxa1* and *Foxa2* are expressed in the crypts in the intestine and scattered cells of the villi. The concomitant loss of *Foxa1* and *Foxa2* in intestinal epithelium using conditional inactivation led to a loss of GLP⁺ cells and decrease in somatostatin⁺ and PYY⁺ cells differentiation (Ye and Kaestner 2009). *Pax6* deficiency principally leads to GIP⁺ cells loss in the duodenum, while *Pax4* deficient mice lose the expression of endocrine hormones such as serotonin, secretin, GIP, PYY and CCK in the duodenum and jejunum but not in distal parts of the intestine (Larsson et al. 1998). More recently it was demonstrated that *Pax4* deficient mice lost the expression of *Gastrin*, *Somatostatin* mRNA expression in the small intestine whereas *Ghrelin* and *Proglucagon* mRNA levels are increased (Beucher et al. 2012). Interestingly, Beucher and colleagues showed that *Pax4* deficiency led to an increase in *Arx* expression, a transcription factors that is expressed in some *Ngn3*⁺ progenitors and some subsets of early differentiating endocrine cells in the crypts (Beucher et al. 2012). In the same study, the authors showed that mice with disrupted *Arx* had an increase in somatostatin and ghrelin expressing cells without any change in serotonin expressing cells and a sever loss of GLP-1, GIP and PYY expressing cells. *Pdx1* loss led to decrease in GIP, neurotensin, and

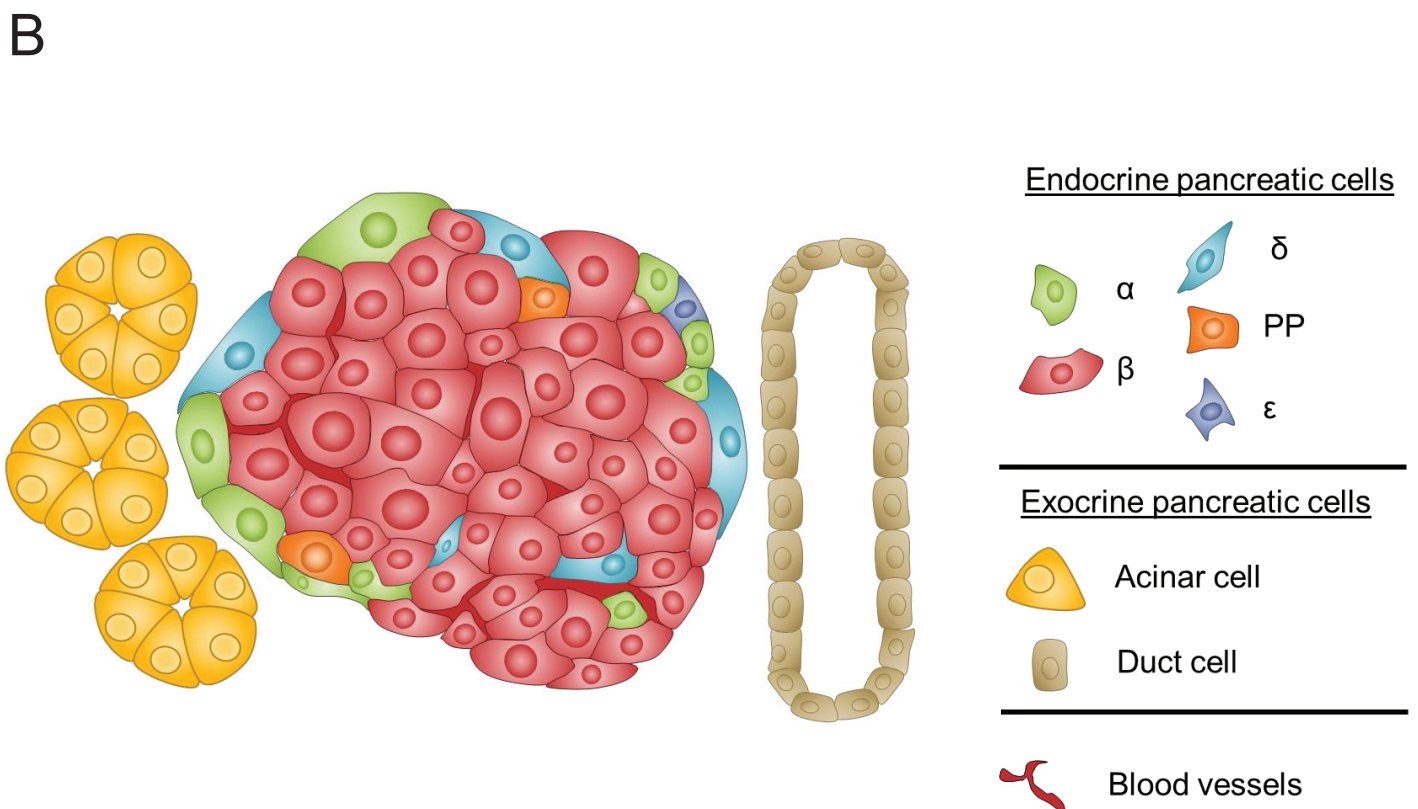
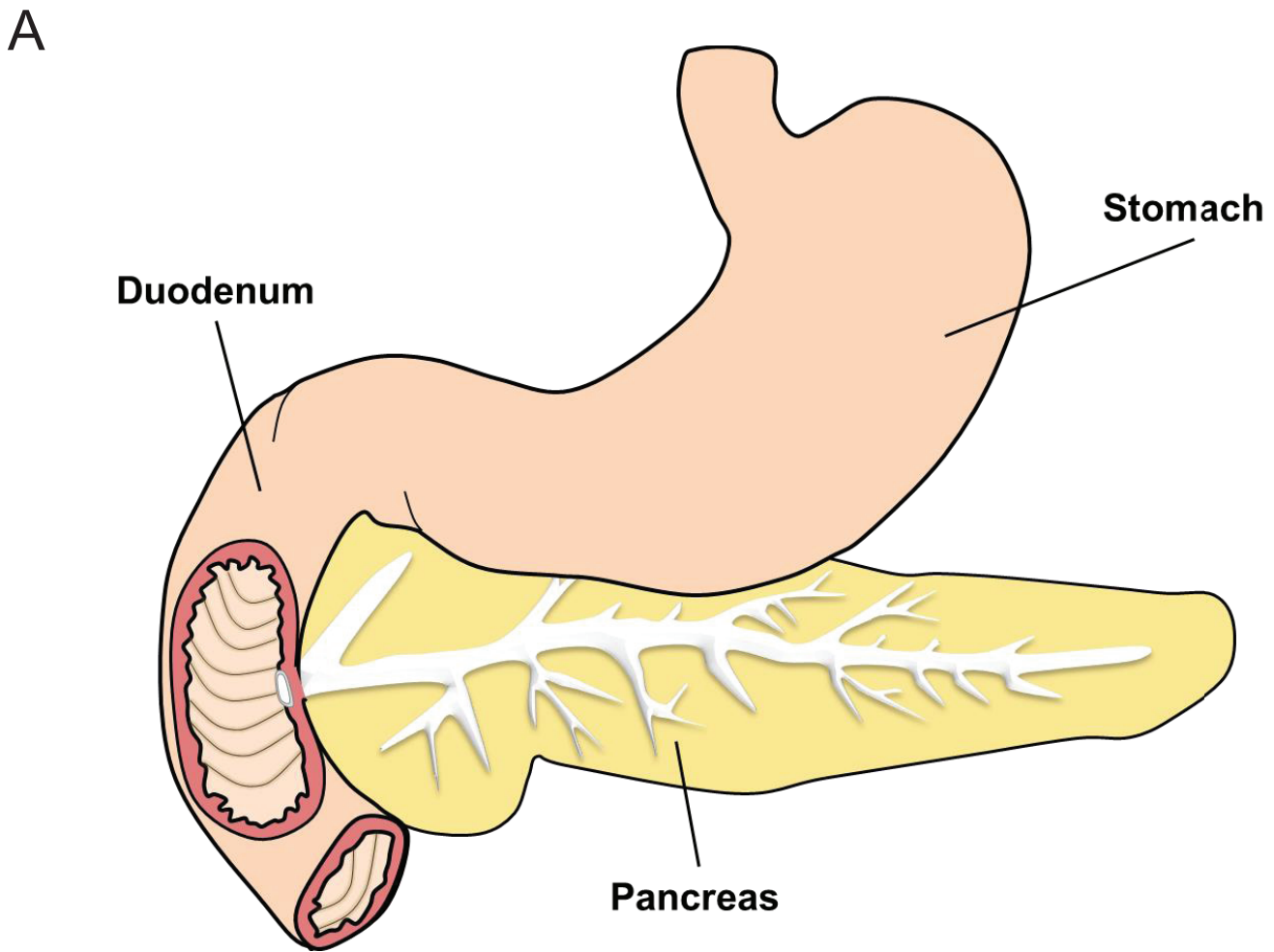


Figure 4. Schematic representations of macro-(A) and microscopic (B) anatomy of the pancreas.

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secretin cells number in the duodenum whereas CCK population is not affected and gastrin cells of the duodenum are lost (Larsson et al. 1996).

1.1.4 THE ENDOCRINE PANCREAS

The pancreas is an organ consisting of different functional units, with the major part being the exocrine pancreas comprising acinar cells and ductal cells (Figure 4A and 4B). The former produce and secrete several digestive enzymes in the lumen of the ducts, while the latter secrete bicarbonates, participating in duodenal pH regulation. The ductal tree is connected to the duodenum, through the main pancreatic duct, in a structure where it joins the common bile duct called ampulla of Vater or hepatopancreatic ampulla. In the exocrine pancreas are dispersed the islets of Langerhans which constitute the endocrine part of the pancreas. The islets are constituted of several endocrine cell types that are defined on the basis of their hormonal secretions. To date, five adult pancreatic endocrine cell-types have been described: α -cells secreting glucagon, β -cells secreting insulin, δ -cells secreting somatostatin, PP-cells secreting the pancreatic polypeptide and the rare ϵ -cells secreting ghrelin (Figure 4B). The islets are intimately associated with blood vessels, but also neurons and stroma, in order to ensure their endocrine functions.

In mammals, the pancreas derives from two different buds that emerge dorsally and ventrally from gut endoderm. Dorsal pancreatic bud will form body, tail and most of the head of the pancreas, whereas the ventral bud will contribute in part to the mature pancreatic head [reviewed in (Pan and Wright 2011)]. The cytoarchitecture of islets largely differs between rodents and human (Figure 5A and 5B). Mouse islets are mainly composed of a dense and large beta cell core with segregation of non-beta cells (such as alpha cells) in the periphery of the islets. In human, non-beta cells are more dispersed in the islets, with beta cells forming contiguous small clusters, providing equal access for all endocrine cells to blood vessels (Brissova et al. 2005, Cabrera et al. 2006, Kim et al. 2009). The proportion of the pancreatic endocrine population is different between species. In mouse, beta cells represent more than 75% of the total endocrine mass, alpha cells account for nearly 20% and delta cells 6%, whereas human pancreas is composed of 55% beta cells, 35-40% alpha cells and 11% delta cells in average (Brissova et al. 2005, Cabrera et al. 2006). In both rodents and human, this

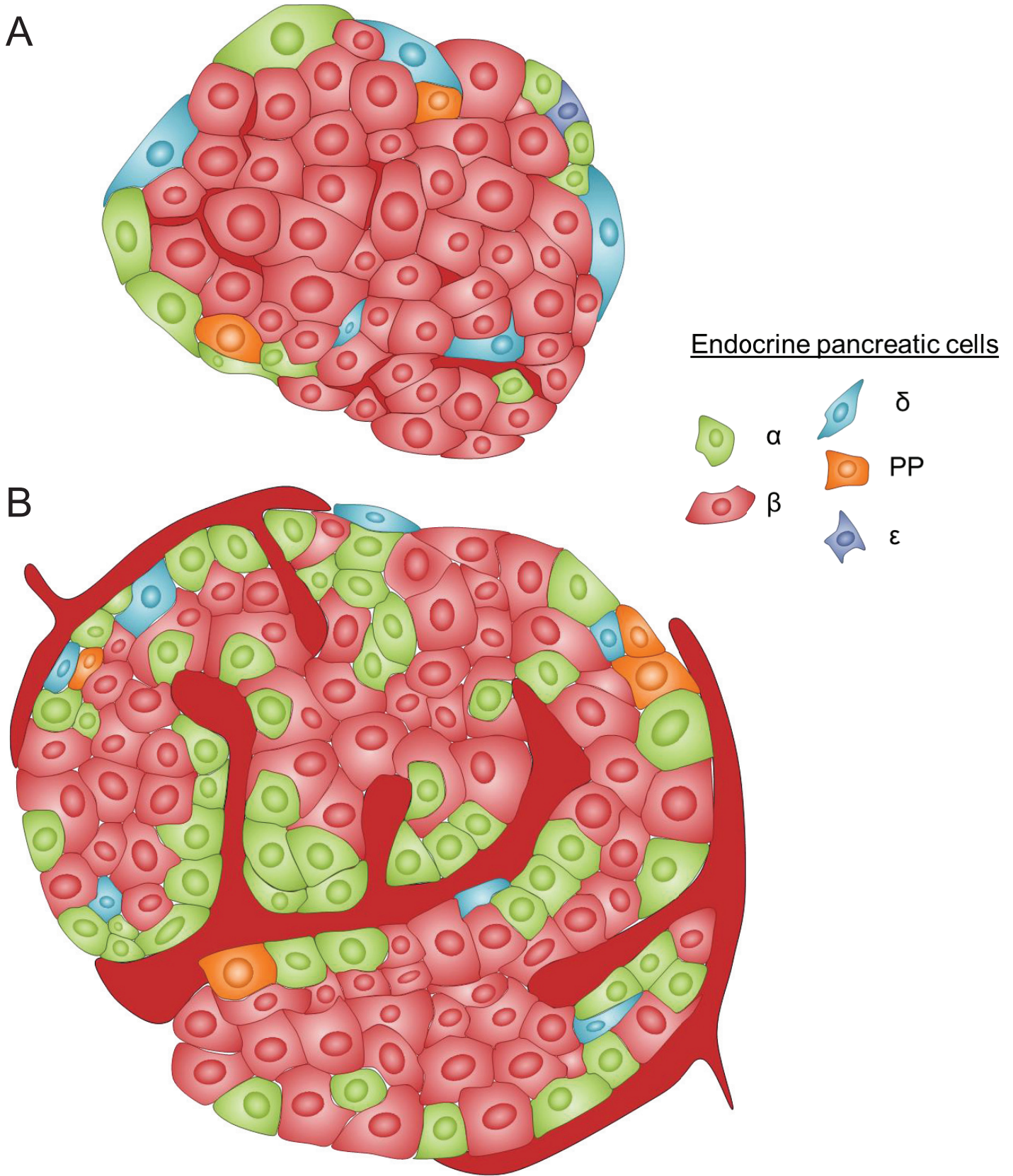


Figure 5. Schematic representation of mouse (A) and human (B) pancreatic islet cytoarchitecture.

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endocrine distribution differs between the different parts of the pancreas according to their ventral or dorsal origin with a highest representation of PP cells in pancreatic region ventrally derived (Suckale and Solimena 2008). In human, this PP rich region was accurately monitored to lie in the uncinate process (Wang et al. 2013). It seems that some insulin⁺ cells that appear between E16.5 and E18.5 would derive from PP expressing cells generated between E14.5 and E16.5 (Johansson et al. 2007), consistent with lineage tracing works (Herrera et al. 1994, Herrera 2000). The specificity of the transgenic *PPY*-promoter driven Cre recombinase has nevertheless never been studied during the pancreas development and the contribution of PP expressing cells to the beta lineage is still not clear. Also, PP expression during pancreatic development remains controversial (Herrera et al. 1991) (Teitelman et al. 1993, Myrsen-Axcrone et al. 1997) (Jorgensen et al. 2007). (Huang et al. 2009). Ghrelin is expressed in different cells during development. The true epsilon lineage that express only ghrelin, appears at E10.5, then increases and is rapidly lost after birth. But most ghrelin⁺ cells are glucagon⁺ from E10.5 to P1 in mouse (Heller et al. 2005), but less frequently seen in the rat (Wierup et al. 2004). Some ghrelin⁺ cells also co-express PP during development and after birth (Wierup et al. 2004). Recently, a lineage tracing study, using a mouse knock-in model in which the *Cre recombinase* gene replaces the *Ghrelin* gene, demonstrated that about 10% of both alpha and PP cells were expressing the genetic reporter at P0, and that, even in adult, 14% of alpha cells and 25% of PP cells were expressing the reporter but no more ghrelin (Arnes et al. 2012). Thus it seems that a subpopulation of alpha and a subpopulation of PP cells are derived from cells that expressed ghrelin (but do not represent epsilon cells) and that these subpopulations which will not express ghrelin anymore are maintained in adult islets. Furthermore, the authors suggest that the decrease in epsilon cells that occurs after birth is not due to the death of these cells but due to a further differentiation into PP cells and extinction of ghrelin expression in these cells.

Other hormones such as CCK and gastrin are transiently expressed during pancreas development. *Gastrin* mRNA can be detected as early as E10 in mouse pancreatic epithelium (Gittes et al. 1993) (Brand and Fuller 1988). Immunohistochemical studies showed that gastrin protein expression is first detected at E15 in rat pancreas in periphery of endocrine clusters (Shimizu et al. 1999). Depending on the study, gastrin expressing cells in islets from Wistar rat pancreases are found until P2 (Shimizu et al. 1999) or until two weeks after birth (Furukawa et al. 2001). However, the total number of gastrin expressing cells rapidly decrease

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after birth. Interestingly, scattered gastrin expressing cells within exocrine pancreas can be detected until 1 month in rat pancreas (Furukawa et al. 2001). Neonatal gastrin production is also observed in human, and was confirmed to be fully processed bioactive gastrin in both rat and human as well as other mammalian species (Bardram et al. 1990). Few or no precise investigations were performed in mouse. Nevertheless it was shown with a transgenic mouse line harboring a BAC construct in which gastrin coding sequence was replaced by the *eGFP* gene that some GFP positive cells are found in neonatal pancreas (Takaishi et al. 2011). It was shown by restaining method that some of these gastrin cells may also express insulin (Furukawa et al. 2001). The functional significance of the transient appearance of gastrin⁺ cells in the pancreas is yet not known.

The highly related peptide hormone CCK was also shown to be expressed in pancreatic islets. Indeed, CCK protein expressing cells could be detected in rat pancreatic islet after weaning (P21) and also at P35 in the same region as beta cells (but was not tested after), but not during development (Shimizu et al. 1999). However this was not confirmed in the mouse, where it was shown indirectly with GFP reporter that CCK expressing cells are not found in adult transgenic *CCK-eGFP* C57BL/6 mouse islets (Lavine et al. 2010). Another study showed that CCK was expressed at E16 in exocrine pancreatic compartment, but after birth and in adult CCK is expressed in virtually all glucagon expressing cells in BALB/c mice (Liu et al. 2001). Thus more studies are needed to better characterize CCK expression in human pancreas and in rodent.

1.1.4.1 MORPHOGENESIS

During regionalization of gut endoderm, the prospective region for dorsal pancreas formation comes in close contact to notochord until E8.5 in the mouse from which it is then separated by the formation of dorsal aorta. At E9.5 (26th day of gestation in humans), the mesenchyme surrounding the prospective dorsal pancreas has condensed, a pancreatic rudiment evaginates in the mesenchyme. Twelve hours after dorsal budding (6 days in humans), at nearly E10, the prospective ventral pancreas and common bile duct that is closely associated with vitellin veins emerge from the ventral gut epithelium. During this process, the ventral pancreatic bud emerges caudally from the common bile duct bud. The two buds grow in the surrounding mesenchyme by elongation of a stratified epithelium delineating a primary central lumen (Villasenor et al. 2010).

Before Secondary Transition

After Secondary Transition

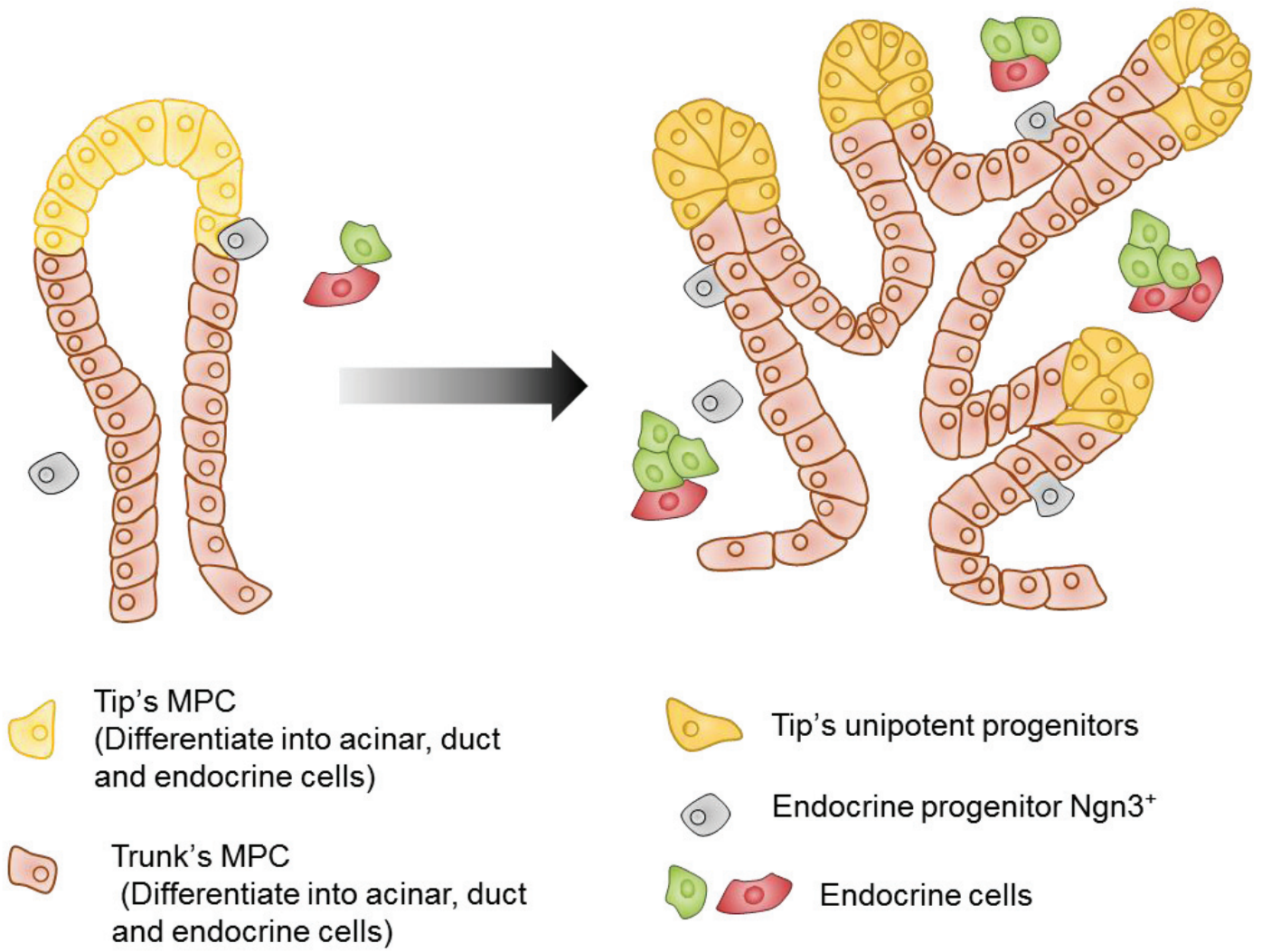


Figure 6. Pancreatic epithelium evolution during embryogenesis.

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As early as E9.5, and until E12.5 occurs the first developmental transition (primary transition) during which proliferate multipotent pancreatic progenitors $\text{Pdx1}^+\text{Ptf1a}^+\text{cMyc}^{\text{High}}\text{Cpa1}^+$ (MPCs) (Zhou et al. 2007) and appear early-differentiated endocrine cells that are mostly expressing glucagon and that can be detected immunohistochemically at E9.5 in ventral pancreas (Jorgensen et al. 2007). This early differentiation process allows the appearance of glucagon⁺ cells, a part of them coexpressing both insulin and glucagon, and having a short life and not contributing to pancreatic endocrine mass at P7 or after weaning (Herrera 2000). These multihormonal cells have also been observed in human at 8 weeks of gestation, but it remains unknown what are their functions and if they contribute to adult endocrine population (Polak et al. 2000). Jorgensen and colleagues showed that cells positive for somatostatin, insulin and ghrelin that do not express glucagon are also readily detectable at E9.5 in ventral pancreas but to a far fewer extent than glucagon⁺ cells (Jorgensen et al. 2007). The contribution of early-differentiated endocrine cells to the pool of endocrine cells after birth is still not completely established and accepted (Gu et al. 2002). During the primary transition, the stratification of the epithelium results in the formation of microlumens following the polarization of some epithelial cells forming rosette-like structures. This then leads to the formation of tree like structures, a process called tubulogenesis, before the end of the primary transition. The branches that are thus formed are separated in two functional domains, the "tip" and the "trunk". MPCs progressively reside in the tips of the branches and are able to give rise to the 3 types of pancreatic cells, acinar, ductal and endocrine cells (Figure 6). The cells in the trunk are restricted to give rise to duct and endocrine cells only. Interestingly it has been proposed and elegantly demonstrated that the total number of MPCs emerging in the two pancreatic buds would determine the final size of the pancreas and that no compensation occurs during development if one part of MPCs is ablated (Stanger et al. 2007).

After E12.5 occurs the secondary transition marked by the fusion of dorsal and ventral pancreatic buds (occurring at 6 weeks of gestation in humans) (Jorgensen et al. 2007) (Pan and Wright 2011). The pancreatic epithelium expands and massive differentiation of the three pancreatic cell types takes place. The tips MPC become restricted precursors with only acinar differentiation potential (Zhou et al. 2007), whereas trunk cells are still able to differentiate in both ductal and endocrine cells (Figure 6). Most endocrine cells constituting the pool of islets are believed to originate from this secondary transition that last until birth in mouse. The

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expansion of the endocrine cell pool (Ngn3⁺) at this secondary transition. These premature endocrine cells are not proliferating at least until E16.5 for beta cells (Zhang et al. 2006). Just before birth, pseudo islets are forming by fission/aggregation mechanisms between alpha and beta cells. Islet formation and shaping will continue after birth to form mature islets (Miller et al. 2009). At the end of embryogenesis, alpha, beta, delta and PP cells highly proliferate. This proliferation rate slightly decrease at birth but alpha and beta cells re-enter high proliferative state, and finally progressively decrease as the endocrine cell mass increase until weaning (Kaung 1994, Zhang et al. 2006, Miller et al. 2009). In contrast delta and PP cells proliferation was shown to be decreasing as soon as birth and seems not to be biphasic as alpha and beta cells, at least in rat (Kaung 1994). In the mouse, postnatal period is also accompanied by functional maturation of islet cells in particular beta cells that will acquire the glucose induced insulin stimulation response. However, it was shown that beta cells can mature and be fully differentiated during embryonic development (Piper et al. 2004). Until the end of life, islet mass increase slowly due to a weak proliferating rate (Rankin and Kushner 2009). Mechanisms controlling islet cells proliferation and death will be discussed in a later part.

1.1.4.2 TRANSCRIPTION FACTORS DETERMINING PANCREATIC LINEAGES SPECIFICATION

The development of pancreatic endocrine cells relies on several extrinsic and intrinsic factors. The importance of several transcription factors has been highlighted to date.

Hlxb9 (Mnx1) is expressed (Harrison et al. 1999, Li et al. 1999) at E8.0 in the prospective dorsal pancreatic bud, later, at E9.5, in the ventral bud (Jorgensen et al. 2007), and is restricted to beta cells in adult pancreas. This transcription factor is necessary for dorsal pancreatic bud development (Harrison et al. 1999, Li et al. 1999).

Pdx1 (Pancreatic duodenal homeobox 1) expression begins at E8.5 at pre-pancreatic regions in the foregut endoderm (Jonsson et al. 1995, Ahlgren et al. 1996, Jorgensen et al. 2007) but is restricted to beta cells in mature mouse pancreas (Jonsson et al. 1995), and to beta cells and centro-acinar cells in human adult pancreas (Park et al. 2011). Low levels of Pdx1 can be detected in early glucagon⁺ cells from the first transition at E10.5 (Mastracci et al. 2013), and is expressed at low levels in the trunk domain during the secondary transition

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(Rukstalis and Habener 2007, Yang et al. 2011). Several mouse models targeting *Pdx1* were generated, and demonstrated that *Pdx1* loss leads to pancreas agenesis but are still able to form dorsal bud rudiments containing some glucagon producing cells and early insulin expressing cells (Jonsson et al. 1995, Ahlgren et al. 1996, Offield et al. 1996). Lineage tracing experiments demonstrated that exocrine, ductal and pancreatic endocrine cells lineages are derived from *Pdx1* expressing cells. However, it seems that *Pdx1*⁺ cells give rise to duct compartment only between E10.5 and E12.5 as demonstrated by conditional lineage tracing of *Pdx1*⁺ cells by administration of tamoxifen at different times (Gu et al. 2002).

Onset of *Ptf1a* expression occurs after onset of *Pdx1* expression. *Ptf1a* is coexpressed with *Pdx1* in dorsal and ventral pancreatic epithelium at E9.5, but is not expressed in other region of the gut endoderm (Jorgensen et al. 2007). In mouse and human adult pancreas, *Ptf1a* expression is restricted to acinar cells (Hald et al. 2008). Genetic lineage tracing showed that all acinar cells are derived from *Ptf1a* expressing cells (however *Ptf1a* is normally expressed in acinar cells, biasing the result), the same as 95% of ductal cells and virtually all pancreatic endocrine cells, excepted for about 25% of alpha cells (Kawaguchi et al. 2002). *Ptf1a* is crucial for the pancreas development as *Ptf1a* knock-out mice only develop a small residual ventral pancreatic bud (Krapp et al. 1998, Kawaguchi et al. 2002). Intrinsically, *Ptf1a* is believed not to be essential for the pancreatic endocrine differentiation as alpha, beta, delta, and PP cells can develop in pancreatic rudiments of *Ptf1a null* mice and in the spleen by migration through the mesenchyme (Krapp et al. 1998). It remains intriguing that lineage tracing experiments demonstrated that the early first wave glucagon producing cells were not derived from *Ptf1a* expressing cells (Kawaguchi et al. 2002). In this case one could speculate that endocrine cells that develop in *Ptf1a null* embryos may result from the differentiation of cells that normally do not derive from *Ptf1a*⁺ cells.

Several other transcription factors have been involved in the development or maintenance of the ensemble of the three pancreatic cell types, such as *Sox9*, *Nkx6.1*, *Nkx6.2* and *Hnf1β* which are co-expressed with *Pdx1* and *Ptf1a* in MPCs. The pancreatic epithelium becomes regionally restricted for the endocrine cell fate differentiation as it organizes in tips leading to acinar cells and bipotent ductal/endocrine trunks since E12.5 (Beucher et al. 2012).

1.1.4.3 ENDOCRINE SPECIFICATION

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The endocrine cell emergence from MPC and bipotent progenitors during first transition or from trunk bipotent progenitors during the second transition largely and indispensably requires Ngn3 expression. Ngn3 can be detected in early dorsal pancreatic endoderm since E8.5 (Jorgensen et al. 2007). In adult normal pancreas, Ngn3 is not expressed even if it has been suggested (Wang et al. 2009). Most Ngn3⁺ cells express high-level-Pdx1 until E10.5, and they express no longer or only low-level-Pdx1 during the second transition (Schwitzgebel et al. 2000, Jorgensen et al. 2007, Nelson et al. 2007 {Rukstalis, 2007 #108}). Ngn3 seems to be expressed in several phases, with a peak at E10.5 during first transition, then decrease and another peak of expression appears during the secondary transition, decreasing after E17.5, being undetectable in adult islets (Apelqvist et al. 1999, Schwitzgebel et al. 2000, Villasenor et al. 2008). Ngn3 expression is essential for all pancreatic endocrine cell-types development as demonstrated by the absence of endocrine cells in pancreas of newborn *Ngn3 null* mice that die of hyperglycemia within 3 days after birth (Gradwohl et al. 2000). However, it was recently reported that some residual glucagon⁺ cells are present at E10.5 and persist until E15.5 in *Ngn3 null* pancreases (Wang et al. 2008), and that rare ghrelin⁺ cells also subsist at P0 in these animals (Arnes et al. 2012). The existence of these cells not deriving from Ngn3⁺ cells in wild-type pancreases remains elusive. Conversely, *Pdx1* promoter-mediated Ngn3 overexpression in transgenic mice leads to hypomorphic pancreas development and differentiation of early pancreatic progenitor pool into endocrine cells (Schwitzgebel et al. 2000). Taken together, most of the above mentioned observations argue for the fact that pancreatic Ngn3⁺ cells are universal pancreatic endocrine progenitors.

The recent report from Herrera's group by clonal mosaic analysis suggests that each Ngn3 expressing progenitors are unipotent and give rise to only one type of endocrine cells. Interestingly, it was shown that the generation of the different endocrine cell types relies on competence windows during pancreatic development, and that each endocrine cell type is generated differentially depending on the stage of pancreatic development. It was demonstrated that conditional and temporally controlled expression of Ngn3 in Pdx1⁺ cells in a *Ngn3 null* background resulted in differential differentiation of each endocrine cell types depending on the stage of induction (Johansson et al. 2007). This study showed that these competence windows were intrinsic to the epithelium with Ngn3⁺ progenitors giving rise to alpha cells starting at E8.7, then acquiring the competence to differentiate into beta cells at E11.5 but more pronounced at E14.5, then PP cells at E12.5 with the highest peak of

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competence at E14.5, followed by late somatostatin⁺ cell competence at E14.5 concomitant with a decrease in the competence of generating alpha cells.

Ngn3⁺ progenitors are not proliferating (Desgraz and Herrera 2009), even if they were erroneously previously thought to be actively dividing because of the reported co-expression of Ki67 with cells expressing *Ngn3* mRNA by *in situ* hybridization (Jensen et al. 2000). It was demonstrated that Ngn3 expression drives cell-cycle exit of pancreatic progenitors by activating the expression of the cell cycle repressor *Cdkn1a* (*p21*) whose expression is later inhibited to ensure normal endocrine cell expansion by proliferation (Miyatsuka et al. 2011). In the same study, the authors also demonstrated that ectopic Ngn3 expression in beta cells after birth dramatically repressed beta cell proliferation that physiologically occurs during postnatal islet development. However, the repression of the proliferation by itself is not important for endocrine cell differentiation, because *Cdkn1a null* pancreases exhibit normal endocrine differentiation (Miyatsuka et al. 2011). In addition, it is now pretty accepted that Ngn3 protein expression is transient and very rapid, lasting less than 24 hours *in vivo* (Desgraz and Herrera 2009, Beucher et al. 2012), strongly suggesting active Ngn3 degradation between the endocrine progenitor state and the endocrine precursor state as recently observed *in vitro* (Roark et al. 2012). Consistent with the short lived Ngn3⁺ progenitors, it was shown *in vitro* that Ngn3 can repress its own expression at transcriptional level, which may participate in the transient Ngn3 expression during endocrine differentiation (Smith et al. 2004). Ngn3 mediates cell delamination from the pancreatic epithelium by positively regulating the expression of the Epithelial-to-mesenchymal transition regulator Snail2 whose expression persists in subsets of endocrine cells hereafter and becomes restricted to insulin⁺ and somatostatin⁺ cells in mature islets (Rukstalis and Habener 2007, Gouzi et al. 2011).

1.1.4.4 PANCREATIC ENDOCRINE LINEAGES SPECIFICATION

Ngn3 expression triggers the expression of several transcription factors necessary for the pancreatic endocrine differentiation, including *NeuroD1* (Gradwohl et al. 2000, Huang et al. 2000), *Islet1* (Gradwohl et al. 2000), *Insm-1* (Mellitzer et al. 2006, Breslin et al. 2007), and *Rfx6* (Smith et al. 2010, Soyer et al. 2010). Several other transcription factors are not the direct targets of Ngn3 but are important for endocrine differentiation.

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NeuroD1 is a direct target of Ngn3. However, its expression seems to be restricted to a subpopulation of Ngn3⁺ progenitors in the developing pancreatic epithelium (Mastracci et al. 2013), and mainly in beta cells in mature adult islet as demonstrated by reporter expression due to lack of specific antibody (Itkin-Ansari et al. 2005). NeuroD1 deficient mice have a reduced pancreatic endocrine cell population but only after E14.5 and mainly due to defect in insulin⁺ cell number (Naya et al. 1997). Importantly, the remaining endocrine cells undergo a massive apoptosis after birth of the mutants but it is still not known if apoptosis occurs in alpha, beta or other endocrine cell-types.

Insm-1 (or *IA1* for *Insulinoma-associated 1*), is also a Ngn3 target and expressed in pancreatic Ngn3⁺ progenitors and likely in all adult endocrine cells (Gierl et al. 2006, Mellitzer et al. 2006). However, the exact pattern of *Insm-1* expression at protein level still needs to be determined. Mice lacking *Insm1* show a slight decrease of glucagon⁺ cells during the first wave and a decrease in insulin⁺ cells after E12.5, but the total absolute quantification of the different types of endocrine cells was not accurately assessed (Gierl et al. 2006). These results were corroborated by *ex-vivo* analysis showing that knocking down *Insm1* in embryonic pancreatic explants resulted in decrease in insulin⁺ and glucagon⁺ cells number {Mellitzer, 2006 #122

Islet1, whose expression is Ngn3 dependent, is expressed in subsets of Pdx1⁺ cells in developing pancreatic epithelium {Du, 2009 #127} and its expression is maintained in adult pancreatic endocrine cells. *Islet1 null* embryos die around E9.5 and do not develop first wave glucagon producing cells in dorsal pancreatic epithelium, but develop a ventral pancreatic bud (Ahlgren et al. 1997). *Islet1*-disruption using *Pdx1-Cre* mediated recombination led to a strong loss in alpha, beta, delta and PP cells and double positive glucagon⁺ ghrelin⁺ cells starting at E13.5, whereas single hormone⁺ ghrelin cells were not affected (Du et al. 2009). No difference in hormone⁺ cells number was detected before E13.5 due to the lack of *Islet1* disruption efficiency. Thus *Islet1* is required for the differentiation of all major pancreatic endocrine lineages.

Rfx6 is another transcription factor regulated by Ngn3, since its expression is lost in *Ngn3*-null pancreas. *Rfx6* is expressed in gut endoderm overlapping *Pdx1* expression, becomes excluded at E10 from multipotent and bipotent progenitors cells, but is expressed later on in Ngn3⁺ cells and hormone⁺ cells during development, and in all adult endocrine islet

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lineages (Smith et al. 2010, Soyer et al. 2010). *Rfx6* deficient mice, albeit normal *Ngn3* expression in the developing pancreas, do not develop any alpha, beta, delta or epsilon cells, whereas PP cells are still differentiating, largely similar to the phenotype reported by the authors for patients with *Rfx6* mutation (Smith et al. 2010). Interestingly, a high proportion of cells stained positively for chromograninA or synaptophysin, but negative for PP, can be detected in mutant pancreases. The endocrine nature of these cells is still unknown even if the study showed that *gastrin* and *VIP* mRNA were rather decreased in *Rfx6*^{-/-} pancreases.

Nkx6.1 is a NK-type homeodomain transcription factor that is expressed in the whole pancreatic epithelium at E10.5 largely co-localizing with *Pdx1*⁺ and *Ptf1a*⁺ cells, but restricted to the trunk domain and excluded from *Ptf1a* tip domain during the secondary transition (Sander et al. 2000, Nelson et al. 2007, Schaffer et al. 2010). *Nkx6.1* is also expressed in *Ngn3*⁺ cells and developing insulin⁺ cells, becoming restricted to pancreatic beta cells in adult islets (Sander et al. 2000). Loss of *Nkx6.1* does not affect alpha, delta, or PP cell population but results in a highly decreased beta cell population from the secondary transition, whereas insulin⁺ cells before E12.5 are not affected (Sander et al. 2000). *Nkx6.1* loss also affects maturation of beta cells but this will be discussed elsewhere. Maïke Sander's group recently demonstrated that *Ngn3*⁺ cell population was reduced by 38% at E10.5 and profoundly decreased until E12.5, but was normal at E14.5 in pancreas of *Nkx6.1* null embryos (Schaffer et al. 2010). More recently, it was reported that targeted disruption of *Nkx6.1* in *Ngn3*⁺ progenitors lead to a differentiation of targeted cells toward non-beta endocrine cells (Schaffer et al. 2013). Thus it remains unknown by which mechanisms the early endocrine cells from first wave are present in normal number in *Nkx6.1* deficient mice.

A related member of *Nkx6* family, *Nkx6.2* is also broadly expressed in the early pancreatic epithelium at E9.5 in same cells as *Nkx6.1*. At E10.5, *Nkx6.2* is expressed in 70% of *Pdx1*⁺ cells but quickly becomes restricted in subsets of pancreatic epithelial cells that mostly express *Pdx1* and is not expressed in *Ngn3*⁺ progenitors (Henseleit et al. 2005). *Nkx6.2* is expressed in some glucagon expressing cells (and some exocrine cells) at E12.5 and to a larger extent at E15.5. Insulin⁺ cells do not express *Nkx6.2* during pancreatic development (other endocrine cells were not assessed). *Nkx6.2* expression is absent from all endocrine cells in adult islets (Henseleit et al. 2005, Nelson et al. 2005). Interestingly, in *Nkx6.1* mutants, *Nkx6.2* whose expression is normal until E10.5 is ectopically expressed in pancreatic epithelial cells that normally express neither *Nkx6.2*, nor *Ngn3*, nor insulin. *Nkx6.2*

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null mice develop a normal pancreas with a normal endocrine cell population (Henseleit et al. 2005). However in double mutants *Nkx6.1^{-/-} Nkx6.2^{-/-}* embryos, insulin positive cells are further reduced as compared to single *Nkx6.1^{-/-}* mutant and a dramatic loss of glucagon producing cells is also observed at E18.5, while early endocrine cells from first wave of differentiation are not affected, neither were somatostatin nor PP and epsilon lineages during whole development (Henseleit et al. 2005, Schaffer et al. 2010). Recent observations from Sander's group demonstrated that loss of both *Nkx6.1* and *Nkx6.2* results in increased acinar cell number at E14.5 due to *Ptf1a* misexpression in trunk domain accompanied with a decrease in *Ngn3⁺* cells in the trunk domain at E12.5 (Schaffer et al. 2010), suggesting that *Nkx6.1* and *Ptf1a* are mutually repressing each other in the late first transition. Altogether, it seems that these factors are necessary to allocate sufficient numbers of endocrine progenitors from MPCs expressing *Ptf1a* to allow normal alpha and beta cells number to differentiate in late first transition and the beginning of secondary transition.

Another member of the Nkx family, *Nkx2.2*, is important for pancreatic endocrine differentiation. *Nkx2.2* is broadly expressed in nascent pancreatic epithelium at E9.5 and becomes restricted to trunks at E12.5 with high expression in *Ngn3⁺* progenitors and low expression in the bipotent progenitors expressing *Nkx6.1⁺ Ngn3⁻*. In mature islets, *Nkx2.2* is expressed in all beta cells, a high proportion of alpha and PP cells but not in delta cells (Jorgensen et al. 2007, Mastracci et al. 2013). Double *Nkx2.2^{-/-} Nkx6.1^{-/-}* mutant mice have the same phenotype as *Nkx2.2* simple mutant mice, showing that *Nkx2.2* is epistatically before *Nkx6.2* in the process of pancreatic differentiation (Sander et al. 2000). *Nkx2.2 null* mice lack beta cells, have a 80% reduction in alpha cells number, and a slightly reduced PP cell population, with no change in delta cells, but a great increase in *ghrelin⁺* cells at E18.5 as compared to control (Sussel et al. 1998, Prado et al. 2004). At E10.5, no *insulin⁺* cells could be detected and decreased *glucagon⁺* cell population was evident. The expression of *Nkx6.1* is totally lost between E12.5 and E18.5, consistent with the absence of *insulin⁺* cells that normally express *Nkx6.1*. In addition, only low-level-*Pdx1* or *Hlxb9* expressing cells were detected at E14.5 (Sussel et al. 1998, Wang et al. 2004). Thus *Nkx2.2* appears to be required for beta cells differentiation and also for part of alpha cell pool, while repressing the epsilon lineage. Interestingly, studies on *Nkx2.2* knock-out mice showed that *Pax6* expressing cells are severely reduced and the expression of *Pax4* at mRNA level is not changed (Wang et al. 2004).

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Pax6 is first detected at E9.0-E9.5 by immunohistochemistry in the pancreatic epithelium (Sander et al. 1997). At E10.5 and E14.5, Pax6 is expressed in all glucagon⁺ cells and some scattered glucagon⁻ cells but the identity of the latter is unknown (but may represent insulin⁺ cells as Pax6 is also expressed in mature islet alpha and beta cells) (Sander et al. 1997, St-Onge et al. 1997, Heller et al. 2004). Some Ngn3⁺ cells express Pax6 (Maestro et al. 2003). *Pax6 null* mice or mice carrying an inactivating mutation in the *Pax6* gene (*Sey^{Neu}*) demonstrate a 4 and 3 fold decrease in glucagon⁺ and insulin⁺ cells respectively at E18.5 with each population severely reduced at E12.5 (Sander et al. 1997). It was nevertheless recently found that ghrelin⁺ cell population (not proliferating) is greatly enhanced in *Pax6* mutant mice pancreases suggesting that the epsilon lineage replaces in part alpha and beta differentiation in *Pax6* mutant mice. Thus, *Pax6* may repress the differentiation of this lineage (Heller et al. 2005). This study highlighted that Pax6 is not required for the endocrine commitment of pancreatic progenitors, but important for the differentiation of alpha and beta cells.

The transcription factor *Pax4* gene seems to be a direct target of Ngn3 *in vitro* (Smith et al. 2003). *Pax4* mRNA or reporter inserted at *Pax4* locus is expressed in the pancreatic epithelium at E9.5, becoming scattered in pancreatic epithelium at E11.5 and increases in the pancreas from E13.5 to E15.5, then decreases until birth. *Pax4* mRNA is detected in some clusters of insulin⁺ cells at birth and is mainly undetectable in adult islets (Sosa-Pineda et al. 1997, Wang et al. 2004). However, studies with a specific Pax4 antibody (Wang et al. 2008) that was used at several times in different recent studies (Collombat et al. 2009, Lu et al. 2010, Yang et al. 2011, Schaffer et al. 2013), showed that Pax4 protein was expressed in beta cells from adult mice (Collombat et al. 2009, Yang et al. 2011), whereas no betagalactosidase expression is detected in islet from *Pax4^{+/-LacZ}* mice (Wang et al. 2004) which may reflect low mRNA expression but high protein expression. It was described that betagalactosidase activity in *Pax4^{+/-LacZ}* mice is detected in Ngn3⁺ pancreatic endocrine progenitors and most betagalactosidase positive cells express Islet1 at this stage (Wang et al. 2004). Later at E15.5, the betagalactosidase activity is detected in Ngn3⁺ cells and also in some insulin⁺ and glucagon⁺ cells, becoming restricted to insulin⁺ cells solely at E18.5. Ngn3 expression is not altered in the pancreases from *Pax4* null mice (Wang et al. 2004). The mutant mice virtually do not develop any insulin⁺ cells (Sosa-Pineda et al. 1997), and delta cells are also absent. However there is a strong increase in glucagon and ghrelin expressing cells (Sosa-Pineda et al. 1997, Prado et al. 2004, Heller et al. 2005, Wang et al. 2008). Importantly, a substantial

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part of glucagon producing cells in *Pax4* mutants are also expressing ghrelin and IAPP, which is normally expressed in beta cells and only rarely in alpha cells. It was shown that at birth, the number of ghrelin⁺glucagon⁻ cells was unchanged, but the double ghrelin⁺glucagon⁺ cell population was increased by nearly 25% as compared to control, which led to conclude that Pax4 does not regulate the true epsilon cell lineage (Heller et al. 2005). Interestingly, most Pax4 deficient ghrelin⁺ cells express low levels of Pdx1 at E17.5 and at birth which is not the case in control mice (Sosa-Pineda et al. 1997, Wang et al. 2008). (Greenwood et al. 2007, Wang et al. 2008). It is important to note that truly assessing Pax4 protein expression during pancreas development is of crucial importance to better study its role.

Transcription factor *Arx*, localized on the X chromosome, was shown to have an opposite role to Pax4. (Collombat et al. 2003). At E9.5, Arx protein is expressed in a small subset of Pdx1⁺ pancreatic progenitors, and in a small fraction of Ngn3⁺ endocrine progenitors at E12.5 (Mastracci et al. 2011). Through pancreatic development, Arx protein is expressed in most glucagon⁺ cells and in both ghrelin⁺glu⁻ and ghrelin⁺glu⁺ cell populations (within a total of 20% ghrelin⁺ cells at E16.5) (Kordowich et al. 2011, Mastracci et al. 2011). In adult islet, Arx is expressed only in alpha and PP cells (Collombat et al. 2007). It was shown, that Ngn3 is necessary for *Arx* expression as *Ngn3 null* mice do not express *Arx* anymore (Collombat et al. 2003). In *Arx* hemizygous mice (knock-out), the total endocrine cell number is unchanged in 2-day-old pancreas relative to control mice, but no alpha cells are detected, the number of epsilon and PP cells remains unchanged and that of beta and delta cells is increased by 1.3 and 2 folds respectively, indicating a switch of normally alpha cell committed progenitors toward beta and delta lineages (Collombat et al. 2003, Heller et al. 2005, Mastracci et al. 2011). Similar results were obtained in mice with conditional disruption of *Arx* in Pdx1⁺ pancreatic progenitors (Hancock et al. 2010), with the exception that PP cell population also increased. In *Arx* mutants at early stage of endocrine development, glucagon producing cells are detected at E10.5 and also at E12.5, most of them being insulin⁺ at E12.5, but no glucagon⁺ cells could be detected at E15.5 (Collombat et al. 2003). Thus, Arx may not be necessary for first wave alpha cells. Importantly, human boy patients with *ARX* mutation display pancreatic islets containing no alpha or PP cells, whereas insulin and somatostatin populations do not increase and a high proportion of ghrelin⁺ cells is present as in controls where ghrelin cells are expressed in all glucagon⁺ cells (Itoh et al. 2010) (Raghuay et al. 2013). More detailed analyses are required to better understand the precise role of ARX in human.

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The compound *Arx* and *Pax4* KO mice present normal total pancreatic endocrine cell mass devoid of both alpha and beta cells, but a strong increase in PP and delta cells that is not observed in one of the simple mutants (Collombat et al. 2005). In fact, the increase in PP cell population was detectable starting at P2 and was dependent on feeding. Most of these cells were also coexpressing somatostatin, with cell counting of monohormonal PP⁺ cells being unchanged as compared to control. Therefore, delta lineage replaced alpha and beta lineages in double *Arx:Pax4* KO mice but this lineage is not stable after birth and feeding because it starts to express PP. Other genetic interactions were also described for *Arx*. The compound mutants *Nkx2.2 : Arx* show loss of both alpha and beta cells but an increase in somatostatin⁺ and ghrelin⁺ cells and restoration of PP cell number (Mastracci et al. 2011). However, in the double *null* mice, most somatostatin⁺ cells were coexpressing ghrelin and vice-versa (Kordowich et al. 2011, Mastracci et al. 2011) with only one fifth of cells only expressing ghrelin and one fifth of cells only expressing somatostatin. Cells expressing both ghrelin and somatostatin in compound mutants were attributed to originate from ghrelin cells that would ectopically express somatostatin due to loss of *Arx* (Kordowich et al. 2011). It was observed that mice overexpressing *Arx* in *Pdx1* or *Pax6* compartments, or specifically in beta cells during development shifted beta and delta cell lineage toward either alpha or PP cell lineages (Collombat et al. 2007). Interestingly, in these transgenic mice glucagon and PP never colocalized showing that it may exist two subtypes of beta cells with different sensitivity to convert into alpha or PP lineages upon *Arx* misexpression.

MafB is a member of MAF family of transcription factors. During pancreatic development, *MafB* is expressed in subsets of *Ngn3*⁺ cells as early as E13.5 (coexpression experiment was not assessed at earlier stage) and in early glucagon producing cells from first wave and persists in all glucagon⁺ cells through the development. *MafB* is also expressed in insulin⁺ cells at E12.5 until two weeks after birth and is lost from the latter at 3 weeks after birth (Artner et al. 2006). In adult mouse islets, *MafB* is only expressed in alpha cells and occasionally in some PP cells. *MafB*^{-/-} mice that die at birth from apnea, develop an absolute number of endocrine cells comparable to control animals (Artner et al. 2007). The quantity of PP, delta and ghrelin⁺ cells is not altered, whereas a twofold decrease in glucagon⁺ cells and a three-fold decrease in insulin⁺ cells number are observed at E18.5. The hormone negative cells that differentiated in the mutant mice seemed to conserve partially their identity in the absence of *MafB*, as it was for example demonstrated that *Brn4* was expressed in pseudo

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glucagon⁻ alpha cells (Brn4 is a alpha cell specific transcription factor in adult islet). The intriguing point is that a half of glucagon⁺ and less than a half of insulin⁺ cells remain in *Mafb* null pancreases. The authors attributed the insulin⁺ population to be cells that had already expressed MafA, an important regulator of insulin expression in beta cells that is expressed only in late maturation steps of beta cells, whereas the pseudo insulin⁻ beta cells were not expressing MafA (Artner et al. 2007, Artner et al. 2010). However, it is not yet clear how hormone⁻ pseudo alpha or beta cells are formed. It would be interesting to conditionally invalidate MafB expression specifically in developing pancreas at different time points after birth, in order to accurately define the precise role of MafB. The functions of MafB pointed out the difficulty in some instance to clearly dissociate the role of a factor in the differentiation from a progenitor or in the maturation of a specific cell-type. Some crucial transcription factors for pancreas development that have no role in pancreatic endocrine lineage commitment, such as Foxa2 or Pdx1, are important for the specific functions of mature alpha and/or beta cells (Ahlgren et al. 1998, Lee et al. 2005, Gao et al. 2007).

1.1.4.5 MAINTENANCE OF DIFFERENTIATED ENDOCRINE CELL IDENTITY

We have discussed about the essential transcription factors mediating endocrine cell lineages commitment toward the different pancreatic endocrine cell lineages. Most of the phenotypes that we have described were based on the analysis of conventional total knock-out mouse models or conditional disruption during pancreas development. However, the role of many of these transcription factors and other genes has been studied in differentiated endocrine cells (or at least hormone expressing immature endocrine cells), and this led often to uncover their role in the maintenance of cell identity. A differentiated cell is defined by its ability to assure its specific function in a given organ. Consequently, we could consider that each molecular modification altering the defined function of a cell is in fact modifying the identity of the cell. Therefore, the following part will treat genes whose alterations lead to a modification of the nature of the hormone secreted and/or expressed by a given pancreatic endocrine cell.

The role of *Ptf1a* in adult acinar cells has not been much investigated in vivo, principally due to the lack of conditional targeted allele for this gene. However, it was

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recently shown in zebrafish that loss of *Ptf1a* activity from acinar cells can reprogram the cells to pancreatic hormone expressing cells (Hesselton et al. 2011).

Pdx1 controls the identity of adult beta cells. Several models of conditional *Pdx1* disruption in beta cells using *Rat Insulin Promoter (RIP)* driven Cre recombinase mice. These studies showed that *Pdx1* loss in pancreatic beta cells at 3 weeks after birth results in the conversion of beta cells into glucagon expressing cells (Ahlgren et al. 1998) (Gannon et al. 2008). However, the embryonic deletion of *Pdx1* in insulin⁺ cells showed a decrease in insulin expression by immunostaining and an increase in alpha cells and delta cells number that did not expressed the reporter in lineage tracing experiment (ghrelin expression was not assessed), indicating that cell conversion is not involved (Gannon et al. 2008). Interestingly, this differential role of *Pdx1* at different stages of endocrine cells development was also demonstrated recently in mouse with ectopic overexpression of *Pdx1* in either *Ngn3* progenitors or glucagon expressing cells (Yang et al. 2011). Indeed, it was demonstrated in this study that *Pdx1* irreversible overexpression in *Ngn3* progenitors leads to reprogram alpha cells into beta cells but mainly occurring 7 to 12 days after birth, resulting in a nearly complete loss of glucagon expressing cells in adult. Differently, stable overexpression of *Pdx1* specifically in glucagon producing cells did not induce alpha-to-beta conversion, or other pancreatic hormone expression, but induced loss of glucagon and *MafB* expression without loss of *Arx* in the pseudo alpha cells.

The maintenance of beta cell identity also requires the transcription factor *NeuroD1*. Disruption of *NeuroD* in immature beta cells (with the *RIP-Cre* transgene) or in adult islet beta cells (inducible *Pdx1-CreERTm*), induces a loss of *Insulin 1* expression, and an increase by two folds in the number of beta cells expressing NPY and some beta cells also coexpress somatostatin (Gu et al. 2010).

The role of *Nkx6.1* in maintenance of beta cell identity was recently demonstrated (Schaffer et al. 2013). Indeed, *Nkx6.1* loss in insulin expressing cells converts them into somatostatin expressing cells that express *Pdx1* but no longer express insulin. On the contrary, *Nkx6.1* disruption in *Ngn3*⁺ cells which lead to a decrease in beta cell lineage cell fate choice, display *Pdx1*⁻ *MafA*⁻ *Pax4*⁺ insulin⁺ cells with a great proportion of them coexpressing *MafB*, *Arx*, glucagon or somatostatin. It is still however not clear if these *Nkx6.1* deficient insulin⁺ cells in the *Ngn3* mediated *Nkx6.1* disruption model have a maturation deficiency or if they

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arise from conversion of glucagon⁺ and somatostatin⁺ cells into immature insulin⁺ cells in order to compensate the loss of beta cells. In contrast, Nkx6.1 overexpression in alpha cells do not convert them into beta cells (Schaffer et al. 2013).

The conversion of beta cells into alpha cells was also reported in mice carrying a homozygous *Nkx2.2* mutation (Papizan et al. 2011). This effect was attributed to the ectopic expression of *Arx* in *Nkx2.2* mutated beta cells. Indeed, they showed that *Arx* gene expression is normally repressed by the repressor complex formed by Nkx2.2, Nkx6.1, Grg3 and Dnmt3a in wild-type beta cells. Conversely, *Dnmt3a* disruption in beta cells also led to a transdifferentiation of beta cells into alpha cells (Papizan et al. 2011).

Arx appears to be crucial in the regulation of islet cell identity. Indeed, in addition to the previous work, the repression of *Arx* expression in beta cells was also shown to be crucial for the maintenance of beta cell program by several groups, including mice with specific disruption in beta cells of the DNA methyl transferase Dnmt1, a DNA methylation maintenance enzyme. These beta cells convert progressively during aging into alpha cells, mainly due to *Arx* ectopic expression (Dhawan et al. 2011), because of progressive loss of repressive DNA methylation at *Arx* locus after normal division of beta cells. Consistent with this observation, transgenic ectopic overexpression of *Arx* specifically in beta cells, converts them into glucagon⁺ and PP⁺ cells with most converted glucagon⁺ cells being PP⁺. The increase in conversion into PP⁺ cells remains somewhat intriguing. Nevertheless, it seems that the role of *Arx* in the conversion of beta cells into alpha cells is conserved in human (Spijker et al. 2013).

Pax6, was shown to control the maintenance of alpha and beta cell differentiation in adult mouse pancreas with induced systemic disruption of *Pax6* (Hart et al. 2013). Several markers of differentiation are clearly down-regulated in beta cells, a three-fold decrease in insulin⁺ and glucagon⁺ cells, and an increase of 30 fold in the number of epsilon cells (that did not express any other hormones) mainly localized in the periphery of islets, whereas PP expression was not assessed. It seems that no transdifferentiation of alpha and beta cells into ghrelin⁺ expressing cells occurred, raising the question of the origin of these ghrelin⁺ cells that probably arise from PP or alpha cells that have already lost glucagon expression. Moreover, it seems that not all alpha and beta cells are sensitive to loss of *Pax6* four weeks after *Pax6*

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disruption, which could reflect different alpha and beta cell subpopulations within islets or may only be due to a too early analyze.

The maintenance of alpha cell identity is also controlled by the *Men1* tumor suppressor gene. Indeed, our group revealed that *Men1* disruption in alpha cells triggers the transdifferentiation of a substantial proportion of alpha cells into insulin expressing cells (Lu et al. 2010). The transdifferentiation process occurs only two months after birth. Remarkably, a subpopulation of *Men1*-deficient alpha cells does not transdifferentiate and gives rise to alpha cell tumors.

1.1.4.6 MECHANISMS CONTROLLING ISLET CELLS PROLIFERATION.

As previously described, adult islet cell mass is formed by massive differentiation from a pool of Ngn3 progenitors during development and the proliferation of differentiated cells in a narrow time window just before birth until weaning. The endocrine cell mass is then maintained, tightly regulated and expands during adulthood by slow-rate of replication of islet cells (Rankin and Kushner 2009, Salpeter et al. 2010, Stolovich-Rain et al. 2012, Salpeter et al. 2013). Contrary to other organs such as intestine or liver, the maintenance of endocrine islet mass in adult is apparently not supported by significant neogenesis from potential adult stem cells or progenitors (Teta et al. 2007), while several pathological or physiological conditions can influence the proliferation of islets cells such as islet tumorigenesis, obesity and pregnancy. Most of the studies in this field are aimed at understanding beta cell mass expansion in order to improve therapy for diabetes. Consequently, the mechanisms controlling the proliferation of other islet cells are far from being understood with the exception of the recent interest on alpha cells. The known molecular mechanisms controlling beta cell proliferation are deeply described in the recent review from Kulkarni *et al* (Kulkarni et al. 2012). Several genetic evidences demonstrate that islet cell proliferation is differentially regulated during the embryonic stage than in adult life. The works based on *in vitro* experiments with cell lines will not be addressed here, even if we totally agree with the importance of these works, especially for the *ex vivo* expansion of islet cells for transplantation-based therapies.

The *Men1* gene encoding the protein menin (please refer to Part 1.3) is a critical regulator of islet-cell proliferation. *Men1* disruption in either alpha, or beta cells specifically

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using *RIP-Cre*, *Glu-Cre* or *Pdx1-Cre* transgenes, or specifically in adult beta cells using inducible *Pdx1-CreER* induces the proliferation of the target islet cells (Bertolino et al. 2003, Crabtree et al. 2003, Shen et al. 2009, Lu et al. 2010, Shen et al. 2010, Yang et al. 2010). It has been also described that downregulation of menin is important for the compensatory beta cell growth that occurs during pregnancy (Karnik et al. 2007). The effect of *Men1* in the regulation of proliferation seems to be in part due to its known role in the regulation of insulin growth factor pathway (Fontaniere et al. 2006), and cell cycle regulators (reviewed in (Wu and Hua 2011)). However it is still not clear whether *Men1* regulates proliferation of islet cells during development and postnatal period, as this was not studied in these investigations.

Interestingly, several cell cycle regulators were described to regulate islet cell mass. Among CyclinD group (CyclinD1, 2 and 3), it seems that the major regulator of islet cell proliferation is CyclinD2 in normal mice. CyclinD2 begins to be expressed in pancreatic endocrine cells in postnatal period and is decreasing after two weeks (Georgia and Bhushan 2004). Analysis of the pancreases from *CyclinD2 null* animals showed that CyclinD2 is necessary for the proliferation of both alpha and beta cells, 4 days after birth, there was no proliferation, neither the increase of cell mass in *CyclinD2^{-/-}* animals in a C57BL/6 background (Georgia and Bhushan 2004). In a mixed genetic background, Kushner and colleagues however revealed that *CyclinD2^{-/-}* mutants had normal postnatal beta cell proliferation at 2 weeks after birth that is due to compensation by CyclinD1, the beta cell proliferation being impaired only in adult mice at 3 months of age (Kushner et al. 2005).

The cyclin dependent kinase 4 (Cdk4) positively regulates beta cell replication after birth. *Cdk4 null* mice have normal islet cell mass at P5, but subsequent proliferation of beta cells is impaired in these mice and the beta cell mass is dramatically decreased in adult mice, whereas other islet cell-types are not affected (Rane et al. 1999, Martin et al. 2003, Mettus and Rane 2003). Conversely, in mice with homozygous Cdk4 mutations disrupting its interaction with INK4 family (p16, p15, p18, p19) (therefore Cdk4 is not inhibited anymore by these INK4 proteins), the beta cell mass is increased in adult, whereas at P5 the islet cell mass is normal (Martin et al. 2003) even if it has been recently shown that beta cells in these mutants readily proliferate at P1 (Kim and Rane 2011). Altogether, it seems that Cdk4 may regulate beta cell proliferation, except during mid- and late gestation respectively. Calcineurin/NFAT signaling through *Cnb1* was shown to positively regulate postnatal beta cell proliferation at weaning by activating the expression of CyclinD2, Cyclin A2 and FoxM1

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(Heit et al. 2006) (Goodyer et al. 2012). Prenatal and early postnatal beta cell proliferation seems not to be affected by loss of *Cnb1*.

$p16^{\text{Ink4}}$ (*Cdkn2a*) that inhibits Cdk4, is not expressed in the islets of young mice, but its expression progressively increases in aging islet (Krishnamurthy et al. 2004, Krishnamurthy et al. 2006). It was shown that *Bmi1*, which is expressed until 1 month after birth and then decreases with aging, represses *p16* expression (Dhawan et al. 2009). In *Bmi1*^{-/-} mice, islets and exocrine pancreas display a strong expression of *p16* at 2 weeks after birth, accompanied by a half reduction of beta cell proliferation at 4 weeks. Similar results were obtained when *Ezh2* was specifically deleted in beta cells (Chen et al. 2009), suggesting the role played by *Ezh2*-*Bmi1*-*p16* axis in controlling beta cell proliferation starting from late postnatal period. In addition, upstream factors such as *Pdgf* signaling were described as regulating beta cell mass expansion by regulating *Ezh2* expression after birth, mediated by *Erk* and *RB-E2F* pathways. *Pdgfr- α* and *Pdgfr- β* receptors are expressed in neonatal islets but highly downregulated in adult islet cells together with their ligands in a similar way as *Ezh2* (Chen et al. 2011). Beta cell proliferation and mass are decreased by 3 and 2 folds respectively in 2- to 3-week-old mice with specific disruption of *Pdgfr- α* in beta cells. Interestingly, the expression of a constitutive active form of *Pdgfr- α* in islet beta cells results in 2 fold increase in beta cell proliferation and 4-fold increase in beta cell mass at 3 months. The authors also demonstrated that concomitant disruption of the 3 Retinoblastoma family members, *Rb*, *p130* and *p107* in beta cells in adult islets increased their proliferation eventually leading to beta cell tumors (Chen et al. 2011). Also, it was shown that *p38MAPK* regulates *p16^{Ink4a}* and *p19^{Arf}* but only in old pancreatic islets. In mice with a dominant negative allele of *p38MAPK* treated with STZ, islet proliferation was hampered only in old mice (22-month-old) but not in 4-month-old mice (Wong et al. 2009).

$p18^{\text{Ink4c}}$ an inhibitor of Cdk4, negatively regulates islet cell mass (Pei et al. 2004). The role of *p18* in the regulation of different islet cell populations is not clearly defined, but as *p18* regulation of islet cell mass is dependent on Cdk4, we may speculate that *p18* only regulates beta cells proliferation.

Loss of *p27^{Kip1}* (*Cdkn1b*, a cyclin E/Cdk2 inhibitor) in *p27*^{-/-} mice results in proliferation of newly differentiated alpha and beta cells during embryogenesis which normally are quiescent until E16.5-E17.5 (Georgia and Bhushan 2006). However during islet

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cell mass expansion in peri-natal period, *p27* inactivation does not affect the proliferation of expanding alpha and beta cells which was attributed to the fact that CyclinD2 expression during this period may have a major role which predominates on *p27*. However, it seems that after weaning, in adult beta cells at least, *p27* anew represses proliferation.

FoxM1 was shown to regulate beta cell proliferation only after weaning and during pregnancy, by preventing nuclear accumulation of *p27* (Zhang et al. 2006).

Perk kinase is important for beta cell proliferation during late embryogenesis and postnatal periods, since *Perk* null mice and mice with *Pdx1* mediated ablation of *Perk* fail to expand beta cells even at weaning and display profound maturation defects (Zhang et al. 2006). However, as beta cell maturation is severely impaired in *Perk null* mice, it is difficult to know if Perk has a direct role on proliferation or if the proliferation is affected because of maturation defects. Furthermore, beta cell specific disruption of Perk, which was mainly efficient after birth at P4, does not affect their proliferation or their maturation (Zhang et al. 2006 227). Taken together, it seems that Perk may regulate before birth the expression of a critical factor that is necessary for beta cell proliferation.

It has been shown that critical differentiation and maturation factors such as Islet1, *Pdx1* and MafA are related to islet cell proliferation. *Pdx1*-mediated disruption of Islet1, results in a decrease in islet-cells proliferation and an increase in apoptosis in islets after birth (Du et al. 2009). *Pdx1* disruption in beta cells decreases beta cell proliferation at late gestation, whereas it seems that proliferation is not impaired after weaning (Gannon et al. 2008). Ectopic expression of MafA (a beta cell specific transcription factor) in *Pdx1*⁺ pancreatic epithelium impairs the proliferation of pancreatic progenitors by upregulating *p27* and *p57* expression (Nishimura et al. 2009). However, *MafA null* mice do not display significant changes in total islet mass at birth or at 12 weeks but present severe defects in beta cell maturation (Zhang et al. 2005). Thus the role of MafA in beta cell proliferation needs to be more precisely investigated. MafB that is important for the differentiation of alpha and beta cells and restricted to adult alpha cells in the mouse was shown to play a role in beta cell proliferation. The data from our lab indeed demonstrated that MafB expression was reactivated in mouse *Men1* insulinomas and during adaptive beta cell proliferation triggered by pregnancy and diet induced obesity (Lu et al. 2012). This MafB reexpression may play an important role in beta cell proliferation in these cases.

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Several growth factors and hormones were shown to regulate islet cell mass and specifically beta cell proliferation (Nielsen et al. 2001, Tarabra et al. 2012). The importance of AKT/mTOR signaling pathways in regulating beta cell proliferation was highlighted by several mouse models as reviewed in (Blandino-Rosano et al. 2012). It was recently shown that insulin increases alpha cell proliferation in a mTOR dependent manner which does not involve Foxo1 contrary to beta cells (Liu et al. 2011). This effect was corroborated in mice with specific disruption of insulin receptor in alpha cells that have a slight decreased alpha cell mass with aging and hyperglucagonemia but also marked beta cell hyperplasia with evidence of some low-level alpha cell to beta cell transdifferentiation (Kawamori et al. 2009, Kawamori et al. 2011).

Glucose is indirectly involved in beta cell proliferation through glycolysis and body metabolic demand on insulin. The whole is integrated as beta cell “workload” which represents the “net insulin secretion per beta cell” (Porat et al. 2011). It was shown that glycolysis through membrane depolarization increases beta cell proliferation.

Recently, it was demonstrated that betatrophin, a protein secreted by the liver, is a strong inducer of beta cell proliferation which is highly specific to the latter. Its expression is increased during pathophysiological challenges such as pregnancy or obesity but not during regeneration after beta ablation (Yi et al. 2013).

Glucagon signaling appears also as a critical regulator of alpha cell proliferation. Mice lacking functional Prohormone convertase 2 (PC2, Pcsk2) leading to deficiency in *Proglucagon* processing, and thus, mature glucagon but not GLP1, display an increased alpha-cells proliferation in late embryogenesis and at birth resulting in alpha cell hyperplasia. But the proliferation rapidly decreases and is similar to control in adult mice (Furuta et al. 1997) (Vincent et al. 2003). Nevertheless alpha-cell hyperplasia persists in adult mice lacking functional PC2, and an increased delta cell number is also observed along with increased islet neogenesis. Similarly, mice deficient for the *Proglucagon* gene also develop alpha cell hyperplasia and have increased islet expression of *Somatostatin*, *PPY* and *Ghrelin* mRNA (Hayashi et al. 2009). Interestingly, mice lacking the glucagon receptor (*Gcgr*) show overt alpha cell hyperplasia, with an increase in glucagon, GLP1, and somatostatin levels in pancreatic extracts (Gelling et al. 2003), and develop glucagonomas (Yu et al. 2011) (Keddes et al. 2009). Elegantly, it was reported that the specific disruption of *Gcgr* in the liver induces

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a phenotype similar to *Gcgr* null mice, suggesting that increased alpha cell proliferation with disrupted glucagon signaling may be due to an unknown factor released by hepatocytes (Longuet et al. 2013). It would be interesting to further dissect the role of glucagon, insulin and glucose in pancreatic endocrine neogenesis and alpha cell proliferation *in vivo* in the context of alpha-cell deficiency.

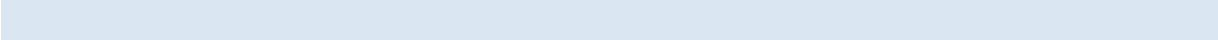


Table 1. Pancreatic endocrine tumors

Tumor	Syndrome Name	Main clinical presentation	Hormone	Incidence	Malignancy % (Jensen et al. 2012)	% of all PETs (Halfdanarson et al. 2008)	MEN1-associated %	Mean size at diagnosis (Kloppel et al. 2011) mm	Age (years) (Halfdanarson et al. 2008)
Insulinomas		Hypoglycemia	Insulin	1-3	<10	30-45	4-5	5-20	30-60
Gastrinomas (pancreatic and duodenal)	Zollinger-Ellison	Pain, diarrhea, ulcer	Gastrin	0.5-2	60-90	16-30	20-25	20 (pancreas)	20-70
VIPomas	Verner-Morrison	Diarrhea, hypokalemia, dehydration	Vasoactive Intestinal Polypeptide	0.05-0.2	40-70	<10	6		20-80
Glucagonomas	-	Rash, glucose intolerance, weight loss	Glucagon	0.01-0.1	50-80	<10	1-20	30-70	40-60
Somatostatinomas	-	Diabetes mellitus, cholelithiasis, diarrhea	Somatostatin	rare	>70	<5	45		30-80
GRFomas	-	acromegaly	Growth Hormone Releasing Factor	rare	>60		16		
ACTHoma	-	Cushing's syndrome	ACTH	rare	>95		rare		
PET causing carcinoid syndrome	-	Carcinoid syndrome (flushing, diarrhea)	serotonin	rare	60-88		rare		
PET causing hypercalcemia (PTHrp-oma)	-	abdominal pain due to hepatic metastases, symptoms due to hypercalcemia	PTHrp	rare	84		rare		
Nonfunctional PETs	-	-	-	-	60-90	25-100%	-	-	20-60
Well differentiated			-PP (60-85%) -calcitonin (42%) -Expression of one or more hormones						
Poorly-differentiated carcinoma			No hormone expression		100%	rare	ND		

1.2 PART II GASTROENTEROPANCREATIC ENDOCRINE TUMORS**1.2.1 GENERALITIES**

Endocrine tumors are also called neuroendocrine tumors because endocrine cells share common markers and similar storage-release organelles with neural cells. Endocrine tumors gather all tumors arising from endocrine glands, such as the adrenal and pituitary glands, and organs with diffuse endocrine system such as the gastrointestinal tract, the pancreas (islets), the lung, the larynx, the thymus and the genital tract. The endocrine tumors are characterized by the expression of NeuronSpecificEnolase or the secretory vesicle components such as Synaptophysin or ChromograninA. These tumors are rare but their incidence is increasing mainly due to better sensitive diagnostic tools. Their prevalence is also increasing, because most of endocrine tumors are benign which is reflected by a high survival rate at 5 years. The most frequent neuroendocrine tumors are gastroenteropancreatic neuroendocrine tumors (GEP-NETs), as they represent about 60 percent of all endocrine tumors (Frilling et al. 2012). Among GEP-NET, more than 73% have an intestinal origin (including colon and rectum), 9% arise from the stomach and 12% have a pancreatic origin or location (Frilling et al. 2012).

1.2.2 TUMOR CLASSIFICATION (OMS, ENET)

GEP-NETs are classified in three grades based on WHO classification. The low grade, grade 1, neuroendocrine neoplasms well-differentiated; the intermediate grade, neuroendocrine neoplasms, grade 2; the high grade, neuroendocrine carcinoma grade 3 subdivided into small cell carcinoma and large cell neuroendocrine carcinoma. The ENET established its own classification based on the WHO classification depending on the KI67 index, tumor size and angio invasion. GEP-NETs can arise sporadically or hereditary. Most of inherited GEP-NETs arise in the context of Multiple Endocrine Neoplasia in patients with germline mutation of the *MEN1* gene with predominance for pancreatic and duodenal endocrine tumors, the remaining cases develop in patients with von Hippel-Lindau syndrome bearing a germline mutation on *VHL* gene, more rarely, in patient with germline mutations in *NF1* or *TSC1-TSC2* (Capurso et al. 2012). Most common oncogenes (*KRAS*, etc) do not seem to have an important role in GEP-NET tumorigenesis.

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PART II Gastroenteropancreatic Endocrine tumors: *Gastroenteropancreatic functional NETs*

1.2.3 GASTROENTEROPANCREATIC FUNCTIONAL NETS

GEP-NETs functioning tumors are endocrine tumors associated with a clinical syndrome due to the excessive hormonal secretion by the tumor.

1.2.3.1 FUNCTIONAL PANCREATIC NEUROENDOCRINE TUMORS

Several subtypes of functioning pancreatic endocrine tumors (functional p-NETs) exist (Table 1). However, certain types of duodenal tumors, such as duodenal-gastrinomas, are often classified as pancreatic endocrine tumors (Halfdanarson et al. 2008, Jensen et al. 2012). The two most common functional p-NETs are insulinomas and gastrinomas.

Insulinomas are the most common functional p-NETs with an estimated incidence of 1–3/million population/year and are characterized by the abnormal expression and secretion of insulin resulting in hypoglycaemia. 90% of insulinomas are benign and appear between 30 to 60 years old of age. In patients with insulinoma, 4% are MEN1. In MEN1 patients, insulinomas represent in average 18% of all pancreatic endocrine tumors (Jensen et al. 2008). Somatic *MEN1* mutations are found in about 10% of sporadic insulinomas (8/81) (Bergman et al. 2000, Moore et al. 2001, Corbo et al. 2010). Loss of 11q13 locus (bearing *MEN1* gene) occurs in 15-20% of insulinomas (Capurso et al. 2012).

Gastrinomas are the second most frequent functional p-NETs, however this is due to the inclusion of duodenal gastrinomas as "pancreatic" endocrine tumors, which seem to be more frequent than their pancreatic counterpart. The incidence of gastrinomas is 0.5–2/million population/year. Gastrinomas are characterized by oversecretion of gastrin leading to the Zollinger-Ellison syndrome (ZES) characterized by peptic ulcers, gastroesophageal reflux and diarrhea. Gastrinomas appear between 20 and 70 years old, and are mainly found within the gastrinoma triangle comprising the proximal part of the duodenum and the pancreatic head. Pancreatic gastrinomas are often malignant at diagnosis, associated with local invasion and/or metastasis in proximal lymph nodes and in the liver. MEN1 syndrome is diagnosed in 20 to 25% of all patients with gastrinomas (Jensen et al. 2012). In MEN1 patients, real pancreatic gastrinomas are rare (0 to 15% of MEN1 gastrinomas), most of them occurring in the duodenum (Jensen et al. 2008). Gastrinomas (duodenum and pancreatic) account for 54% of pancreatic tumors in MEN1 patients (Jensen et al. 2008). The cell origin of pancreatic gastrinomas is intriguingly unknown as gastrin is not expressed in adult pancreas. Somatic

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MEN1 mutations are found in about 50% of sporadic gastrinomas (16/32)(Moore et al. 2001, Jensen et al. 2008, Corbo et al. 2010).

VIPomas are rare functional p-NET with an incidence of 0.05 to 0.2 cases/million population/year. VIPomas secrete the vasoactive intestinal polypeptide leading to clinical symptoms as diarrhea, hypokalemia and dehydration. These tumors are malignant in 40-70% of case and appear between 20 and 80 years of age. MEN1 syndrome is associated in 6% of VIPomas. These tumors represent about 3% of PET in MEN1 patients. The cell origin of these pancreatic tumors is unknown as VIP is not expressed in the pancreas. Sporadic VIPomas harbor somatic *MEN1* mutation in 36% of cases (4/11) (Moore et al. 2001, Corbo et al. 2010).

Glucagonomas are also rare pancreatic tumors that represent less than 10% of all p-NETs with an incidence of 0.01-0.1 /million population/year. The clinical symptoms often comprise rashes and glucose intolerance due to the secretion of glucagon by the tumor. Glucagonomas are malignant in 40-80% of cases and appear between 40 and 60 years of age. MEN1 syndrome is associated in 1 to 20% of glucagonomas (Jensen et al. 2012). About 3% of pNETs in MEN1 patients are glucagonomas (Jensen et al. 2008). Few sporadic glucagonomas were studied for somatic *MEN1* mutations, but the mutations were described in 60% (3/5) of glucagonomas analyzed (Bergman et al. 2000, Moore et al. 2001, Corbo et al. 2010).

Somatostatinomas are even rarer than VIPomas and glucagonomas, and occur both in the duodenum and the pancreas. Somatostatinomas which secrete somatostatin are characterized by occurrence of diarrhea cholelithiasis and diabetes, appearing between 30 and 80 years old. More than 70% of somatostatinomas are malignant. MEN1 syndrome is associated in 45% of somatostatinomas (Jensen et al. 2012). Somatostatinomas are extremely rare tumors in MEN1 patients and account for 0 to 1% of pNETs in these patients (Jensen et al. 2008).

Several other types of functioning pNETs are also rarely reported, secreting either growth hormone, ACTH, serotonin, CCK. Most of these tumors are highly malignant (Jensen et al. 2012).

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PART II Gastroenteropancreatic Endocrine tumors: *Gastroenteropancreatic non-functional NETs*

1.2.3.2 GASTRIC AND INTESTINAL FUNCTIONAL NETS

Most gastric endocrine neoplasms are non-functional and will be treated hereafter.

Duodenal functioning endocrine tumors (functional d-NET) comprise mostly duodenal gastrinomas (48% of all d-NET). Most lesions are generally solitary, with the exception of duodenal gastrinomas in MEN1 patients which are often multiple and small. d-NETs in general are found within the submucosa or mucosa (Delle Fave et al. 2012). Sporadic and MEN1 gastrinomas are often highly metastatic (50 to 90%) to lymph nodes, the later often being bigger than the primary tumor. Liver metastases occur in 7% of MEN patients with gastrinomas (Jensen et al. 2008, Kloppel 2011).

Small bowel (jejunal and ileal) endocrine tumors have an average incidence of 0.4/100,000/year in the world. However, autopsy studies reveal that these tumors are extremely frequent. Most of these tumors are well differentiated serotonin secreting tumors, associated with the carcinoid syndrome due to hypersecretion of serotonin. They are generally localized in the distal part of the ileum. Metastasis is frequently found at diagnosis. These tumors seem not to be related to MEN1 syndrome (Pape et al. 2012). However they are multiple in 26 to 30% of cases and some hereditary forms have been reported but the genetic basis of these hereditary forms is unknown (Kloppel 2011). Colorectal endocrine tumors account for 27% of gastrointestinal endocrine tumors but a high proportion are non-functional (Caplin et al. 2012).

1.2.4 GASTROENTEROPANCREATIC NON-FUNCTIONAL NETS

The majority of gastroenteropancreatic neuroendocrine tumors are considered non-functional, therefore, without being associated with the manifestation of clinical symptoms that could be attributed to the secretion of hormones. These tumors are often hormone-expressing but they either do not secrete it or do not secrete sufficient amount of hormone to cause clinical symptoms. Thus, if they express glucagon for example, they are called "glucagon-producing tumor" rather than glucagonoma. These tumors are often well-differentiated in terms of histopathological aspects. Poorly differentiated neuroendocrine carcinomas exist and virtually never express any hormone. They are thus often diagnosed on the basis of positivity for synaptophysin which is generally the only marker that can classes these carcinomas as neuroendocrine (Nilsson et al. 2006). A gap exists between the clinical

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definition of endocrine tumors and the characterization of cell-types in research. Indeed, as explained in the above, the identity of a normal endocrine cell is defined by the different transcription factors and hormone(s) that it secretes. Thus, a beta cell for example that lose the expression of molecular differentiation marker such as MafA or Pdx1 for example could be considered as altered in its differentiation status. Few studies exist concerning the molecular characterization of endocrine tumors, in order to distinguish what may be the molecular basis of a tumor that make it an insulinoma or a non-functional insulin producing tumor. The rarity of endocrine tumors may explain, at least partially, this lack of characterization as compared to other more frequent tumors or cancers.

1.2.4.1 NON-FUNCTIONAL PANCREATIC ENDOCRINE TUMORS

All endocrine tumors that are not associated with clinical symptoms due to hypersecretion of hormone and PPomas (that generally are not associated with specific clinical symptoms) are classified as non-functional p-NETs (NF p-NETs) (Table 1).

The incidence of NF p-NETs is 1-3/100000/year and 60-90% of the cases are malignant. Microscopic NF pNETs are found in most MEN1 patients with p-NETs (80-100% of case) and large NF p-NETs represent about 13% of p-NETs in MEN1 patients. Most p-NET (superior to 98%) in VHL patient are non-functional. The *MEN1* genes was found to be mutated in 24% of NF p-NETs (32/132) (Moore et al. 2001, Corbo et al. 2010).

A high proportion of NF p-NETs is actually secreting hormones or peptides. They, secrete the pancreatic polypeptide (60-85% of cases), calcitonin (42% of cases) and sometimes ghrelin (5-65%) (Ito et al. 2012). A number of NF p-NETs expresses glucagon, or insulin or other hormones, but either are not secreting sufficient amounts or are not secreting at all. The precise proportion of the different hormones that can be expressed in NF p-NETs is not established as few studies reported the precise immunohistological profile of such tumors. Anlauf and colleagues reported that in two tumor banks comprising a total of 300 NF p-NETs, 3% were expressing gastrin (9/300) (Anlauf et al. 2006). It was found in a study of 114 patients with NF p-NETs that 23% of the tumors displayed multihormonality, which is a little bit lower than 37% found in 86 patients with functional pNETs (Kapran et al. 2006). In a study of 9 MEN1 patients, it was demonstrated that 40% of tumors were multihormonals

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(Kloppel et al. 1986). Among these patients, a high proportion of microlesions expressed glucagon or PP or insulin. Similar results were obtained in more recent studies showing that in MEN1 patients without insulinomas or glucagonomas associated symptoms, a very high proportion of micro and macro lesions expressing glucagon or insulin can be detected (Anlauf et al. 2006, Perren et al. 2007). In a French study, among 23 tumors found in 6 asymptomatic MEN1 patients, 3 stained for insulin, 12 for glucagon and 8 for PP, but none of these stained for gastrin (Le Bodic et al. 1996). However, it was shown that in 10 MEN1 patients, a substantial proportion of pancreatic endocrine tumors displayed focal gastrin stainings in tumors mostly expressing insulin or PP (5/24) (Thompson et al. 1984).

1.2.4.2 NON-FUNCTIONAL GASTRIC ENDOCRINE TUMORS

As gastric endocrine tumors (gastric neuroendocrine tumors, gastric NETs) are predominantly non-functional, the gastric NET denomination will be kept in this part. Gastric NETs can be well or poorly differentiated (called poorly differentiated neuroendocrine carcinomas or NECs). Gastric NET represent 5 to 14.6% of GEP-NETs (Fraenkel et al. 2012) with an incidence of 0.2/100,000/year (Delle Fave et al. 2012). Gastric well differentiated NETs are classes into 3 types.

The Type 1 comprises 70-80% of gastric NETs which arise mainly in women from 50 to 60 years. These NETs occurs in the context of autoimmune or not chronic atrophic gastritis (CAG) of the oxyntic mucosa which is more frequent in women. The decreased gastric pH during CAG leads to hypergastrinaemia. Gastrin will bind to CCK-2 receptors on Enterochromaffin like (ECL) histamine producing cells of the stomach resulting in hyperplasia of these cells. Most type 1 gastric NETs are benign and present as multiple polypoid tumors in the gastric corpus or fundus (0.3-1cm). Metastases of these type 1 gastric NETs are rare (Kloppel 2011, O'Toole et al. 2012). LOH of 11q13 are found in 17 to 73% of type 1 gastric NET.

The type 2 gastric NETs represent 5 to 6% of gastric NETs. These tumors are ECL tumors similar to type 1 gastric NETs but they arise in MEN1 patients with Zollinger-Ellison syndrome. Interestingly, patients with sporadic gastrinomas usually do not develop type 2 gastric NETs reflecting that genetic background may influence type 2 gastric NET

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development. Type 2 gastric NETs occur in corpus and fundus but also in antrum part of the stomach. These tumors are metastatic in 10-30% of cases (Kloppel 2011, O'Toole et al. 2012).

The type 3 gastric NETs represent 14 to 25% of gastric NETs, and are sporadic and solitary. They do not occur in the clinical course of ZES or gastritis. They are composed of cells that secrete 5-HT and not 5-OHT (serotonin) and mostly localized in the gastric antrum. They are the most malignant gastric NETs and they metastasize in 50 to 100% of cases. Poorly differentiated NECs are classified in this type 3 gastric NETs or sometimes denominated as type 4. LOH of 11q13 is detected in 25 to 50% of type 3 gastric NETs (O'Toole et al. 2012).

1.2.4.3 NON-FUNCTIONAL DUODENAL ENDOCRINE TUMORS

Non-functional duodenal endocrine tumors (non-functional d-NETs) are most of time well differentiated and not observed in inherited tumor syndromes such as MEN1, or VHL. Most of them express gastrin. The second most commonly expressed hormone in non-functional d-NETs is somatostatin, then serotonin, pancreatic polypeptide and finally calcitonin (Kloppel 2011).

Gastrin expressing (or also called "producing") NF d-NETs are mainly localized in the proximal duodenum mucosa or submucosa as their functioning counterparts. Lymph nodes or liver metastases are rare for these tumors. Interestingly, Anlauf and colleagues showed that in a collection of sporadic d-NETs, the number of non-functional gastrin expressing tumors was equivalent to that of functional gastrinomas (Anlauf et al. 2006).

Somatostatin expressing NF d-NETs are sometimes miscalled somatostatinomas, but they do not induce diabetes or other clinical symptoms that produce real functional somatostatinomas. They represent about 44% of all duodenal NETs. These non-functional somatostatin expressing tumors arise mainly in the ampulla of Vater and peri-ampullary region and may invade the muscularis propria. These tumors develop in 10% of patients with hereditary syndrome NF1 (neurofibromatosis 1).

Other NF d-NETs are reported such as non-functional serotonin-containing tumours which represent 28% of d-NETs and non-functional calcitonin-containing NETs representing

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9% of d-NETs. Rare case of NF d-NETs can express, insulin, PP, glucagon or ACTH (Delle Fave et al. 2012).

Gangliocytic paragangliomas, commonly found in periampullary region, are tumors composed of endocrine cells that express either PP or somatostatin, ganglion cells and Schwann-like cells. Poorly differentiated NECs are usually found in or near ampullary of Vater. They are aggressive and often present with lymph nodes or liver metastases.

1.2.4.4 NON-FUNCTIONAL SMALL INTESTINE AND COLORECTAL ENDOCRINE TUMORS

In colon, most NETs are actually neuroendocrine carcinomas (NECs) whereas well differentiated NETs are mainly found in the rectum. Rectal net often express Glucagon-like peptides or PP but are classified as non-functional because of the absence of clinical syndrome (Kloppel 2011).

1.2.4.5 MEN1 AND SPORADIC P-NETS

It is well established that alteration of the *MEN1* gene is an important mechanism in pancreatic and duodenal endocrine tumors. Several studies have demonstrated *MEN1* mutations and often LOH of the remaining *MEN1* wild-type allele in sporadic pancreatic endocrine tumors and duodenal gastrinomas. However, other mechanisms than mutations, such as promoter hypermethylation leading to decreased or absent menin expression may also occur in some sporadic p-NETs and duodenal gastrinomas (Arnold et al. 2007). Thus the importance of menin inactivation in pancreatic endocrine tumors may be underestimated. Animal models of *Men1*-deficient pancreatic tumors demonstrated a relative late onset of tumor after *Men1* disruption in pancreatic endocrine cells. This suggests that other molecular events (genetics, epigenetics or biochemics) may also be required to trigger tumorigenesis. In addition, total alteration of *MEN1*, combining frequency of allelic loss of 11q13 (comprising *MEN1* gene) and the frequency of *MEN1* mutations, may represent 25% to 50% of sporadic PETs (Moore et al. 2001, Corbo et al. 2010). Thus it is clear that other factors are important in the initiation and the tumorigenesis process of the remaining sporadic PETs. Interestingly, a recent study conducted on 68 sporadic p-NETs, showed that 44% of tumors harbored a mutation in *MEN1*, 25% in *DAXX*, 17.6% in *ATRX*, 7.3% in *PTEN*, 8.8% in *TSC2* and 1.4%

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PART II Gastroenteropancreatic Endocrine tumors: *Cells of origin of pancreatico-duodenal endocrine tumors*

in PIK3CA (Jiao et al. 2011). Even if this study was of great importance to better understand the genetic basis of pancreatic endocrine tumors, it is regrettably non-informative. Indeed, even if authors state that mainly aggressive tumors were screened for mutations, no description was given on the biology and type of the tumors (functional, non-functional, the type of hormone expressed by the tumor, etc).

1.2.5 CELLS OF ORIGIN OF PANCREATICO-DUODENAL ENDOCRINE TUMORS

1.2.5.1 GENERALITIES

The majority of tumor studies until recently were aimed at understanding the biology of well-established tumors, in order to discover markers of diagnosis and prognosis, and targets for therapies. Finding molecular alterations in tumors also allowed to investigate the role played by these events in initiating tumorigenesis or contributing to tumor progression. Recent advances in technologies allowed demonstrate that each tumor is different for a given type of tumor, which can be attributed to the different selection process and random alterations that lead to tumor development. In addition, recent studies demonstrate frequently seen heterogeneity inside a given tumor, indicating that cancer is not a uniform disease. Among different factors causing tumor heterogeneity, more and more concerns are paid to the cells of origin (Blanpain 2013).

Morphological and/or ultrastructural resemblance of tumor cells with their possible normal counterpart, the shared expression of peptides/proteins that can be detected by immunohistochemistry between a normal cell-type and the tumor cells inside the same organ is so far generally used to establish classifications concerning cell type of a given tumor. A similar approach was used to classify gastroenteropancreatic endocrine tumors but is mainly based on the clinical symptoms due to hormonal secretion, which is a characteristic of functional endocrine tumors. However, these criteria have their limits. For example, it was recently demonstrated in a murine model that pancreatic ductal adenocarcinoma may arise from acinar cells by cell conversion (Kopp et al. 2012). Clearly, cells of origin of a given type of tumor play an important role in the heterogeneity of established tumors, as similar tumors, depending on their cellular origin, may behave really differently in terms of progression and resistance or relapse after treatment. Consequently, deciphering cell of origin of tumors is necessary for more comprehensive and complete understanding of tumor biology. Also, it

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allows to generate adequate *in vitro* and *in vivo* models that faithfully recapitulate key events of tumor initiation, development and progression of a given tumor-type. Meaningful biomarkers can thus be identified to detect neoplasms earlier and appropriate targets be found for early therapeutics before the onset of aggressive disease.

The following approaches are widely used in the study of cells of origin of tumor. Firstly, finding the expression of molecular markers that are normally specifically expressed in a differentiated cell type or progenitor can be informative. The analyses of early lesions in the peritumoral tissues, especially in inherited tumor diseases, provide instrumental clues on the cell of origin of a tumor. Indeed, this can often allow appreciating the lesions with different characteristics in the different stages of tumor progression, in contrast to sporadic cancers (Vortmeyer et al. 2004). The use of genetic lineage tracing of different-cell types in mouse models is a powerful tool to decipher cells of origin of a tumor. Lineage tracing combined with specific targeting a given differentiated cell type or progenitors to disrupt a tumor suppressor or to activate an oncogene in mouse can allow the determination of the susceptibility of the cells to tumor formation and the type of tumor derived.

1.2.5.2 NORMAL DIFFERENTIATED ENDOCRINE CELLS

In the pancreas, functional tumors were until recently mainly considered as originating from their normal differentiated counterpart depending on the main hormone that they secrete (van Eeden and Offerhaus 2006, Ehehalt et al. 2009). Pancreatic beta cells that secrete insulin are considered as the cells of origin of insulinomas, as alpha cells for glucagonomas. In the mouse, several models of GEP-NETs were engineered to date allowing to more precisely define the cell of origin of some of GEP-NETs. The development of insulinomas from beta cells was first demonstrated in a transgenic mouse model expressing the viral SV40 Large T antigen (Tag) under the control of the *rat insulin promoter (RIP)* (Hanahan 1985). The disruption of the *Men1* gene specifically in insulin expressing cells induces the formation of insulinomas (Bertolino et al. 2003, Crabtree et al. 2003). However, since *Men1* disruption or Tag expression can occur initially in non-fully differentiated insulin expressing cells during development in these models, it is not completely understood if fully differentiated adult beta cells are susceptible to insulinoma formation or if only beta cell precursors expressing insulin are at the origin of insulinomas. In addition, in *RIP-Tag* transgenic mice, half of islets display beta cell hyperplasia at 6 weeks and tumors develop in all mice at 12 weeks after birth but in

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relative small number (2 to 10/ animal) compared to the number of hyperplastic beta cells. This could be explained by that either only a few subpopulations of beta cells give rise to tumors or secondary events are needed to trigger insulinoma development (Hanahan 1988). Same experiences targeting glucagon expressing alpha cells demonstrated that alpha cells may be at the origin of glucagonomas (Efrat et al. 1988, Lu et al. 2010, Shen et al. 2010). Interestingly, the *Glucagon* promoter driven Tag expression in glucagon expressing cells, starting at the first wave of pancreatic endocrine differentiation, induces proliferation of alpha cells but only at 5 months after birth, leading to low frequency (less than 1% of islets) of glucagonoma development at 9 to 10 months of age (Efrat et al. 1988).

1.2.5.3 STEM OR PROGENITOR CELLS

In addition, it was also shown that pancreatic endocrine tumors are often multihormonal (Kapran et al. 2006). It is still not known whether the multihormonality is due to multiple occurrences of monohormonal lesions with the same site or because there is initially a common affected cell of origin that gives rise to different types of cells in the tumor. Thus, it is also hypothesized that the cell of origin of GEP-NETs could be a stem-cell, progenitor or precursor. Although such a role of stem or progenitor cells has not been so far experimentally demonstrated in GEP-NET, the following works provided hints showing such a possibility.

In the pancreas from patients developing mixed acinar-endocrine carcinomas, the lesions generally do not stain for any hormone, suggesting either an early progenitor origin of these tumors during embryogenesis or dedifferentiation mechanisms (Klimstra et al. 1994, Ohike et al. 2004). Interestingly, these mixed acinar-endocrine carcinomas generally do not display common pancreatic ductal cancers genetic alterations such as KRAS activation or SMAD4, p16 loss, as same as pancreatic endocrine tumors (Stelow et al. 2010).

Similarly, a pancreatic ductal origin of PETs was suggested in MEN1 patients based on a histopathological study showing the existence of ductal-like early lesions displaying MEN1 LOH (Vortmeyer et al. 2004). The work is further supported by the observation of single endocrine cell closely localized to ducts in mature normal pancreas, which may suggest endocrine neogenesis from adult progenitors from ducts (Yoneda et al. 2013). The significance of neogenesis is highly debated in the field. Recently, it was reported, based on

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the use of a powerful and elegant transgenic mouse model, that neogenesis of beta cells is not likely to occur even during endocrine cell regeneration after injuries, at least in the mouse (Xiao et al. 2013). This report nevertheless confirmed that Ngn3 is reexpressed during pancreatic injuries and colocalize with ductal markers and insulin⁺ cells. The significance of such Ngn3 reactivation is not known but may potentially reflect a dedifferentiation of beta cells which may be necessary to improve their proliferation or survival. In human it was nevertheless recently reported that neogenesis may occur in patients with impaired glucose tolerance and newly diagnosed diabetes (Yoneda et al. 2013).

On the contrary, in the duodenum or intestine, multipotent stem-cells or progenitors may be at the origin of endocrine tumors. Indeed, in these organs, the rapid renewal of endocrine cells suggests that endocrine cell tumors may not arise from fully differentiated endocrine cells but maybe from long-lived stem-cells. In addition, the submucosa localization of endocrine tumors argues for a potential crypt origin of enteroendocrine tumors. However, as it was demonstrated that some differentiated endocrine cells can migrate toward the crypt and not only toward the villi, we could speculate that these endocrine cells could be at the origin of enteroendocrine tumors (Aiken et al. 1994).

The origin of gastrinomas is maybe among the most intriguing enigma, especially in the case of pancreatic gastrinomas. Indeed, in contrast to insulinomas or glucagonomas that have a normal fully differentiated counterpart in the pancreas, gastrin-expressing cells are not found in the adult pancreas. Considering the high frequency of pancreatic gastrinomas in the region derived from ventral bud during development, within the so-called “gastrinoma triangle” it has been speculated that they would derive from stem-cells of embryonic ventral pancreatic bud (Passaro et al. 1998), partly because of their subserosal or submucosal localization. It has not been defined by the authors whether the cell of origin is stem-cells per se or endocrine cells that differentiated from these ventral pancreatic stem-cells or progenitors. Indeed, even if no gastrin⁺ cells can be found in the adult pancreas, gastrin secreting cells are readily detected within the developing and neonatal pancreas (see Part 1.1.4). These gastrin⁺ cells could be subjected to tumorigenesis and subsequent gastrinoma development. Indeed, transgenic mice harboring a human *Gastrin* gene promoter fused to the coding sequence of SV40 large T antigen develop islet tumors (Montag et al. 1993). However, intriguingly, these tumors are insulinomas and do not express gastrin, whereas the Tag keeps being expressed in tumors. *Gastrin* mRNA expression in hyperplastic islets or tumors was not

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assessed. It was not assessed whether the transgene has been directly targeted in Gastrin-expressing islet cells. Tumors do not develop in the duodenum, but interestingly, small G-cell hyperplasia or microtumors are found in gastric antrum in this model (Montag et al. 1993). One may speculate that duodenal G cells may not be targeted by the transgene; or that duodenal G cells are not sensitive to Tag induced tumorigenesis; or that duodenal gastrinomas are unlikely to develop in mice.

1.2.5.4 CELL TRANSDIFFERENTIATION

Interestingly, α -cell specific *Men1* disruption, besides glucagonoma development, also leads to the formation of mixed glucagon⁺/insulin⁺ tumors and insulinomas by transdifferentiation of a subset of menin deficient alpha cells into beta cells (Lu et al. 2010). The occurrence of cells co-expressing both insulin and glucagon were also observed in the heterozygous *Men1*^{+/-} mice supporting the idea that insulinomas in a MEN1 context can have an alpha cell origin (Lu et al. 2010). This work provided experimental evidence allowing to explain the observations made by anatomopathological analysis of pancreas from MEN1 patients that displayed a high proportion of glucagon⁺ microadenomas and micro lesions, whereas macrotumors from the same patients were in majority insulin⁺, suggesting a potential alpha cell origin of insulinomas in MEN1 patients (Perren et al. 2007).

In the pancreas, some observations suggest that gastrinomas could originate from other islet cells. Indeed, it has been reported by Lodish and colleagues a sporadic pancreatic endocrine tumor with characteristics of both insulinoma and gastrinoma. The tumor stained for both insulin and gastrin immunohistochemically, and immunogold electron microscopy studies revealed cells containing the both hormones. The patient presented with both hyperinsulinemia and hypergastrinemia, all along with associated clinical symptoms hypoglycaemia and duodenal ulcer. Historically, hypoglycemic episodes began before symptoms of ulceration, suggesting that the insulinoma potentially originating from a beta cell evolved into a dual gastrin/insulin secreting tumor (Lodish et al. 2008). Another report showed the case of an insulinoma that further evolved in gastrinoma (Mizuno et al. 2001). In addition, it has been shown in a cohort of patients with pancreatic endocrine tumors that a hypergastrinemia with clinical features of a gastrinoma developed after initial PET diagnosis in 4% (13/353 patients) of cases (Wynick et al. 1988). Initial PETs diagnosis before hypergastrinemia apparition was 8 glucagonomas, 3 VIPomas and 2 insulinomas. It has not

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been described if patients were MEN1. It is still unclear from these different observations, whether a transdifferentiation mechanism led to gastrinoma development in patients with a previously diagnosed PET secreting another hormone, or whether gastrin⁺ cells participated in the initial tumor development.

It is noted that little is known about the mechanisms involved in the development of non-functional endocrine tumors, whereas a high proportion of them in the pancreas or duodenum are expressing hormones such as glucagon or gastrin (Garbrecht et al. 2008). It is still currently unknown if non-functional differentiated endocrine tumors (other than PP secreting) could be pre-functional tumors that may evolve into a functional form.




Table 2. MEN1 pathology (tumors and hyperplasia)

Tumor localisation	Tumor type	% of MEN1 patients	% of GEP tumors in MEN1
Endocrine			
Parathyroid	Multiglandular hyperplasia, adenomas (hyperparathyroidism due to PTH secretion)	90-100	
GEP		20-100	
Duodenal	- Gastrinomas	40	20-61
Pancreatic			
	-Non-functional macroscopic PETS	20	0-13
	-Insulinomas	10	7-31
	-Glucagonomas		1-6
	-VIPomas		1-12
	-GRFomas		<1
	-Somatostatin secreting tumors		0-1
	-pancreatic gastrinomas	rare	
Stomach	-Type II gastric NET (non-functional) associated with ZES		
Anterior pituitary		10-60	
	-PRLomas	20	
	-GH	5	
	-GH/PRL	5	
	-ACTH	2	
	-TSH	rare	
	-Non-functional	17	
Adrenal (adrenocortical)		20-40	
	-Non-functional	20-40	
	-cortisol secreting	rare	
	-aldosterone secreting	rare	
	-pheochromocytomas	<1	
Foregut carcinoids			
	-Thymic carcinoid	2-8	
	-Lung (bronchial) carcinoids	2-8	
Thyroid	Thyroid adenomas		
Non-endocrine			
Cutaneous			
	-Skin angiofibromas	85-88	
	-Collagenomas	70-72	
	-Lipomas	30-34	
Central Nervous System			
	-Meningiomas	5-8	
	-Ependyomas	1	
	-Schwannoma	ND	
Smooth muscle			
	-leiomyomas	10	
	-leiomyosarcomas		

1.3 PART III MEN1 SYNDROME

1.3.1 GENERALITIES

Multiple Endocrine Neoplasia Type 1 (MEN1) (MIM#131100) is a hereditary autosomal dominant syndrome. It is characterized by multiple occurrences in a same patient or in several members of the same family of tumors in endocrine organs. The major endocrine organs affected in MEN1 comprise the parathyroids, the endocrine pancreas (and the duodenum) and the anterior pituitary (Table 2). MEN1 patients can also develop less frequently adrenal cortical tumors, foregut carcinoids, and non-endocrine tumors such as lipomas, facial angiofibromas, collagenomas and fibromas.

In MEN1 patients, hyperplastic and tumor lesions of the parathyroids affect virtually all patients (90%), pancreatico-duodenal endocrine tumors occur in 40% of patients, followed by anterior pituitary lesions (functional prolactinomas in 20% of patients, GH secreting, GH/PRL secreting, ACTH secreting, non-functional in 17% of patients) (Falchetti et al. 2009, Thakker 2010). Non endocrine cutaneous tumors are frequent in MEN1 patients with prevalence at 40 years old of 85% for facial angiofibromas and 70% for collagenomas.

1.3.2 THE MEN1 GENE

The predisposing gene to MEN1 syndrome is the *MEN1* gene, identified by positional cloning in 1997 (Chandrasekharappa et al. 1997, Lemmens et al. 1997). It is localized at chromosome 11q13. The gene is considered as a tumor suppressor in affected endocrine organs.

1.3.2.1 ORTHOLOGS, EVOLUTION AND CONSERVATION

The *MEN1* gene is conserved in mammals, notably in the mouse (Bassett et al. 1999, Guru et al. 1999), and orthologs exist in the Zebrafish (Khodaei et al. 1999, Manickam et al. 2000) and *Drosophila melanogaster* (Guru et al. 2001), but neither in *Saccharomyces cerevisiae* nor in *Caenorhabditis elegans*.

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In the mouse, animals with genetic disruption of one allele of the *Men1* gene (heterozygous *Men1* knockout mice) develop a range of endocrine tumors highly similar to what is observed in MEN1 patients, reflecting the conservation of *MEN1* role in mouse (Crabtree et al. 2001, Bertolino et al. 2003, Loffler et al. 2007, Harding et al. 2009).

1.3.2.2 MEN1 TRANSCRIPTS

The *MEN1* gene spanning 9.8 kb is constituted of 10 exons, with the first exon and distal part of the exon 10 being non-coding. The human *MEN1* coding sequence consists of 1830 bp. Two *MEN1* transcripts were detected by northern-blotting in human tissues, a main 2.8 kb transcript expressed in most organs, and a 4.2 kb transcript expressed in thymus and pancreas (Lemmens et al. 1997). In the mouse, the *Men1* gene is localized in the pericentromeric region of the chromosome 19, in a region syntenic to human 11q13 locus, with at least two reported transcripts, a 2.8 kb and a 3.2kb transcripts that result from differential alternative splicing of the noncoding exon 1, containing a coding sequence of 1833bp, and are expressed in most tissues excepted skeletal muscle (Stewart et al. 1998, Bassett et al. 1999).

1.3.2.3 MEN1 GENE MUTATIONS

An extensive landscape of *MEN1* gene mutations has been described to date. More than 1000 germinal mutations and more than 200 somatic mutations (occurring in sporadic tumors) have been reported and lie on the entire *MEN1* gene. About 41% mutations are frameshift deletions or insertions, 23% are nonsense, 20% are missense, 9% affect splice-site and 6% are in-frame deletions or insertions. Interestingly, some mutations in MEN1 patients are not localized in the coding sequence, but rather in the promoter or other untranslated regions. Germline deletions of *MEN1* exons have also been reported. Most mutations are inactivating mutations consistent with the tumor suppressing functions of the *MEN1* gene. No phenotype-genotype correlations have been reported so far, excepted the work recently reported by the GTE showing that mutations affecting the interaction domain of the protein with JunD had a higher risk of death (Thevenon et al. 2013).

In MEN1 endocrine tumors (90%), the loss of heterozygosity (LOH) of the *MEN1* wild-type allele, leading to completely *MEN1* inactivation, can be systematically detected. The LOH seems, therefore, to be an essential event to initiate tumorigenesis, even if a report

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suggest that inactivation of one *Men1* allele in the mouse may lead to haploinsufficiency (Lejonklou et al. 2012).

1.3.3 MENIN PROTEIN

The *MEN1* gene encodes the protein called menin with 610 amino acids in human, whereas mouse menin protein is constituted of 611 amino acids (Stewart et al. 1998).

Menin is expressed in all the tested endocrine and non-endocrine tissues and cell lines (Guru et al. 1999, Wautot et al. 2000). Menin is mainly a nuclear protein but can also be detected in the cytoplasm and membrane in some tissues, cell lines or during cell-cycle progression (Guru et al. 1998, Kaji et al. 1999, Wautot et al. 2000, Cao et al. 2009, Wuescher et al. 2011, Ren et al. 2012). Consistent with its nuclear localization, menin sequence contains in C-terminus two classical nuclear localization signals (NLS), NLS1 and NLS2, which are necessary for nuclear menin expression (Guru et al. 1998), and an accessory NLS domain, NLSa, also localized in C-terminus of menin protein (La et al. 2006). Menin is not a transcription factor *per se* but was shown to be able to directly bind DNA in a sequence independent fashion (La et al. 2004). It was also shown that menin contains two functional Nuclear Export Signal (NES). The NES1 consists of the amino acids 33 to 41 of human and mouse menin, and the NES2 comprises amino acids 258 to 267, the latter containing a LXXLL motif (Cao et al. 2009).

Recently, after years of efforts, the crystal structures of a menin homolog from *Nematostella vectensis* (Murai et al. 2011) and of human menin (Huang et al. 2012), were reported. These studies demonstrated that menin behaves as a scaffold protein. Importantly, the crystal structure of menin in complexes with MLL1 or JUND, the two most studied menin partners (see “Menin functions” part) (Huang et al. 2012). MLL1 and JUND compete to interact with menin as they bind to the same domain of menin protein. In addition, this study demonstrated that menin mutants can still bind target gene *Hoxc8* but are unable to recruit the histone methyl-transferase MLL1 to this target gene. Menin interaction with JUND inhibits JUND activation by JNK and subsequent JUND transcriptional activity, since JUND domains required for interaction with menin and JNK are overlapping (Gallo et al. 2002, Huang et al. 2012).

Table 3. Menin interactions and functions

Interactant	Function of interactants	Function of interaction
Transcriptional regulation		
JunD	Transcription factor	-Repression of JunD transcriptional activity (Agarwal et al. 1999, Gobl et al. 1999, Kim et al. 2003). -Inhibition of JunD activation by JNK by binding competition (Gallo et al. 2002, Huang et al. 2012). -Antagonize JunD mediated osteoblast maturation (Naito et al. 2005). -Repression of JunD mediated <i>Gastrin</i> gene expression in AGS cell-line (Mensah-Osman et al. 2011).
NFκB (p50, p52, p65)	Transcription factor	Repression of NFκB mediated transcriptional activation by an unknown mechanism (Heppner et al. 2001)
Smad1/5	BMP effector (transcription co-regulator)	-Activation of mesenchymal stem cells differentiation into preosteoblasts induced by BMP2-mediated transcriptional activation of Runx2 (Sowa et al. 2003, Sowa et al. 2004). -Positively regulates transcriptional activity of Smad1 in Leydig cells (Hussein et al. 2008).
Smad3	TGF-β effector (transcription co-regulator)	-Mediates TGF-β-induced growth inhibition in anterior pituitary cell lines (Kaji et al. 2001). -Repression of osteoblast maturation by positively regulating TGF-β induced repression of differentiation (Sowa et al. 2003). -Potentially mediates Activin-induced repression of proliferation and Prolactin gene expression in pituitary cell lines (Lacerte et al. 2004). -Potentiates TGF-β-mediated inhibition of myogenesis from mesenchymal cells (Aziz et al. 2009). -Potential positive role in Tgf-β mediated repression of PTH expression and repression of proliferation in primary cells from human parathyroid tumor (physical interaction was not studied) (Sowa et al. 2004).
Runx2	Transcription factor	Activation of mesenchymal stem cells differentiation into preosteoblasts (Sowa et al. 2003).
MLL1	H3K4 tri-methyl transferase of the Trithorax group	-H3K4 trimethylation of target genes (Yokoyama et al. 2004, Huang et al. 2012, van Nuland et al. 2013) and see other interactions in the Table. -Positive regulation of <i>p18</i> and <i>p27</i> expression in MEF cells (Milne et al. 2005) or hepatocytes <i>in vivo</i> (Scacheri et al. 2006) and <i>p18</i> in islet cells <i>in vivo</i> (Kamik et al. 2005{Scacheri, 2006 #475}). -Necessary for oncogene activity of MLL1 fusion proteins driving <i>Hox</i> genes and <i>Meis1</i> positive gene expression in leukemic cells (Chen et al. 2006, Yokoyama and Cleary 2008, Grembecka et al. 2012, Shi et al. 2012, Thiel et al. 2012).
MLL2 (TRX2)	H3K4 tri-methyl transferase of the Trithorax group	H3K4 trimethylation at <i>Hoxc8</i> locus in MEF cells (Hughes et al. 2004, van Nuland et al. 2013)
Rpb2 Ser5^P	RNA polymerase II subunit	Coupling of H3K4 trimethylation and transcription? (Hughes et al. 2004)
c-Myb	Transcription factor	-Recruitment of MLL complex to c-Myb DNA targets such as <i>HOXA9</i> gene locus in hematopoietic and leukemic cells (Jin et al. 2010) -Forms a complex with GATA3, essential for TH2 cell differentiation, and to recruit MLL to <i>GATA3</i> locus in CD4+ effector/memory T cells (Nakata et al. 2010) -Complex formation with GATA3 to recruit MLL to <i>IL-13</i> locus and subsequent transcriptional activation (Kozuka et al. 2011)
Ledgf	Chromatin-associated protein, cofactor	Complex formed by menin/Ledgf/MLL1 is crucial for MLL1 and MLL1 fusion proteins activity (Yokoyama and Cleary 2008) (Huang et al. 2012).
PRMT5	H4R3 symmetric dimethyl transferase	Inhibition of Hedgehog signaling by recruiting PRMT5 to promoters and subsequent repression of <i>Gas1</i> expression (Gurung et al. 2013)
mSin3a	Transcriptional regulatory protein	Recruitment of HDAC to inhibit JunD-mediated transactivation (Gobl et al. 1999, Kim et al. 2003)

SUV39H1	H3K9 methyl transferase	Potential recruitment of Suv39H1 to target promoters (such as <i>Gbx2</i>) and subsequent transcriptional repression by repressive histone mark H3K9me3 in MEF cells (Yang et al. 2013)
ERα	Estrogen nuclear receptor	-Ligand-induced ER α transcriptional activity (recruitment of methyltransferase activity to ER α target genes) (Dreijerink et al. 2006, Imachi et al. 2010). -Possible competition with Tamoxifen binding in breast cancer cells (Imachi et al. 2010). -Upregulation of BRCA1 and Rad51 expression in melanoma cell lines through a MLL 1-dependent mechanism (Fang et al. 2013).
PPARγ	Free fatty acid, prostaglandin nuclear receptor	Essential for PPAR γ mediated differentiation of 3T3L1 and mouse embryonic fibroblast into adipocytes (by recruiting HMT activity to PPAR γ target genes)(Dreijerink et al. 2009).
VitaminD nuclear receptor	VitaminD nuclear receptor	Menin acts as a co-activator, ligand-independent, of VDR-mediated transcription in parathyroid cells (with a potential role of menin-mediated HMT activity at VDR target genes). (Dreijerink et al. 2009)
Skip	Coactivator that enhances transcription from some Pol II promoters. This coactivator can bind to the ligand-binding domain of the vitamin D receptor and to retinoid receptors	Promotes HIV-1 Tat transactivation (Bres et al. 2009)
c-Myc	Possible transcription factor in rodents	Possible role in mouse spermatogenesis (no Pem homolog in human) (Lemmens et al. 2001)
Pem (RhoX5)		Repression of Foxo1 transcriptional activity in hepatic cells (Wuescher et al. 2011)
Foxo1		Indirect interaction in MIN6 cells but weak in MEF cells. Possible role in the regulation of Hlx9 protein expression or regulation of Hlx9 target genes (Shi et al. 2013)
Hlx9	Homeobox transcription factor (repressor)	
Cell signalling		
β-catenin	Cell-adhesion. Transcriptional cofactor	-Nuclear exclusion of β -catenin (Cao et al. 2009) -Coactivation of β -catenin target gene <i>Axin2</i> through H3K4 trimethylation in α and β cell lines (Chen et al. 2008). -Positive regulation of β -catenin transcriptional effect in mediating mesenchymal cells differentiation into osteoblast (Inoue et al. 2011).
TCF3	Transcription factor (effector of β -catenin transcriptional activity)	Coactivation of β -catenin target gene <i>Axin2</i> through H3K4 trimethylation in α and β cell lines (Chen et al. 2008).
IQGAP1	Scaffold protein with GTPase activity (GTPase-activating protein)	Intercellular adhesion by stabilizing E-cadherin and β -catenin at cytoplasmic membrane (Yan et al. 2009)
AKT	Serine/threonine protein kinase	Represses activation of AKT and subsequent pro-proliferative and anti-apoptotic effects. (Wang et al. 2011)
Ask		Repression of ASK induced proliferation. (Schnepp et al. 2004)
Nm23	Nucleoside diphosphate kinase, protein kinase	Activation of menin GTPase activity (Ohkura et al. 2001, Yaguchi et al. 2002)
Ches1 (FoxN3)	Transcription factor. Interacts with SKIP	Important for G1-S arrest in response to DNA damage induced by ionizing radiation in <i>Drosophila</i> and MEF cells (Busygina et al. 2006).
DNA-Repair		
FANCD2	DNA repair	Possible role in FANCD2-mediated DNA repair pathway (Jin et al. 2003).
RPA2	Roles in DNA replication, recombination, and repair and possibly apoptosis and transcription	Unknown function (Sukhodolets et al. 2003)
Cytoskeleton		
GFAP	Type III intermediate filament proteins	Possible sequestration of menin out of the nucleus in glioma cell-lines during S and early G2 phase of cell-cycle (Lopez-Egido et al. 2002)
Vimentin		
NMMHC II-A	Cell structure	Possible role in cytokinesis (Obungu et al. 2003)

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Post-translational modifications of menin protein were reported to occur *in vitro* and/or *in vivo*. Several menin amino acids residues can be phosphorylated in response or not to DNA damage (MacConaill et al. 2006, Francis et al. 2011), but the importance of these phosphorylations are elusive. Sumoylation of non-consensus SUMO sites were also detected on menin, but to which extent sumoylation affects menin function is not known (Feng et al. 2013).

1.3.4 MENIN FUNCTIONS

The biological roles played by menin in an organism is multiple, even if the major *in vivo* biological functions of menin currently known in human consists in repressing endocrine but also non-endocrine tumorigenesis in the organs affected in MEN1 syndrome. Importantly, more and more works provided compelling evidence showing that menin plays critical roles in a variety of biological processes in non-endocrine tissues, including hematopoiesis, osteogenesis, myogenesis, adipogenesis, etc... Many studies demonstrated that multifaceted biological roles of menin rely on its molecular function as a transcriptional regulator (activator or inhibitor), which participates in the control of two crucial aspects of cellular activities: cell differentiation, and cell proliferation and survival. Menin apparently exerts its molecular actions through its physical or functional interaction with numerous partners (Table 3), including those specific to a given cell-type and/or tissue. This may result in the distinct multiple biological effects in different cells.

1.3.4.1 REGULATION OF DIFFERENTIATION

Menin is essential for embryonic development, at least in the mouse, and probably also in human, since the living individual with homozygous inactivation of the *MEN1* gene has never been found so far. Indeed, homozygous *Men1*^{-/-} embryos die at mid gestation during development (Crabtree et al. 2001, Bertolino et al. 2003, Lemos et al. 2009). *Men1 null* embryos show delayed development after E11.5 and they die between E12.5 to E13.5. The major defects are hemorrhages, myocardial hypotrophy, pericardial effusion, neural tube defects (exancephaly) and liver growth retardation. Different analyses done on different mouse genetic background suggested that one possible cause of death in *Men1* null embryos could be in part attributed to pericardial effusion (Lemos et al. 2009). However, mice with

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targeted *Men1* disruption in myocardial muscle, has no effect on development and mice are even viable after birth and fertile (Engleka et al. 2007). Massive apoptosis of hematopoietic cells was observed in E12.5 fetal liver (non-published data of our lab). These data may favor the occurrence of fetal hematopoiesis failure as the major cause of the embryonic death.

1.3.4.1.1 REGULATION OF ENDOCRINE CELLS DIFFERENTIATION

Our laboratory sought to study menin's role in the development of the endocrine pancreas. Menin is expressed in all pancreatic cells during development and in adult mouse pancreas (Fontaniere et al. 2008). *Men1* null pancreases at E12.5 have a noticeable decrease in endocrine cell number and fail to undergo massive endocrine differentiation in *ex vivo* experiments. Moreover, in chimeric mice, the contribution of *Men1* null ES cells to pancreatic endocrine cells is substantially low as compared to other organs such as the exocrine pancreas or the skin, demonstrating that menin is essential for pancreatic endocrine cell development (Fontaniere et al. 2008). As apoptosis is clearly observable in pancreatic endocrine cells from E12.5 *Men1* null embryos, it seems that menin may be required for the survival of pancreatic endocrine cells during embryogenesis. However, the conditional disruption of the *Men1* gene in Pdx1 progenitors cells, and insulin or glucagon-expressing cells during embryonic development using transgenic corresponding Cre mice, demonstrated that the entire development of endocrine pancreas may not be affected and the mice survive and develop pancreatic endocrine tumors (Bertolino et al. 2003, Crabtree et al. 2003, Biondi et al. 2004, Lu et al. 2010, Shen et al. 2010). However, neither the activity and the efficiency of the cre recombinases were assessed, nor different stages of embryonic pancreas development studied in these conditional models (Bertolino et al. 2003, Crabtree et al. 2003, Biondi et al. 2004, Lu et al. 2010, Shen et al. 2010). However, it seems that menin expression is crucial to maintain the identity of a subset of alpha cells as *Men1* disruption in these cells induces a transdifferentiation into beta cells in adult mice (Lu et al. 2010).

Additionally, menin is involved in the regulation of the expression of several adult endocrine cells hormones. Menin was shown to inhibit glucose stimulated insulin secretion and the rat *Insulin2* gene promoter activity in the INS-1 rat insulinoma cell line (Sayo et al. 2002). In the GH3 rat pituitary-tumor derived cell-line, menin overexpression was associated with a decrease in endogenous *Prolactin* mRNA expression (Namihira et al. 2002). Conversely menin was shown in another rat pituitary cell-line to be necessary for Activin-

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mediated downregulation of *Prolactin* gene expression, a possible indirect effect that could be due to the down-regulation of Pit-1, an important positive regulator of *Prolactin* expression (Lacerte et al. 2004). Moreover, menin can repress basal *Parathyroid Hormone (PTH)* gene expression in human primary parathyroid hyperplastic cells, and is also necessary for Tgf- β mediated repression of *PTH* expression (Sowa et al. 2004).

Menin inhibits JunD expression and also interacts physically with JunD to repress JunD mediated *Gastrin* gene expression in AGS cell-line (Mensah-Osman et al. 2008, Mensah-Osman et al. 2011). The reported role of menin as an inhibitor of *Gastrin* gene expression was validated *in vivo* (Veniaminova et al. 2012). In addition, menin overexpression was shown to repress the expression of the *GIP* hormone mRNA in enteroendocrine cell line STC-1 (Angevine et al. 2012).

1.3.4.1.2 REGULATION OF NON-ENDOCRINE CELLS DIFFERENTIATION

Menin regulates osteogenesis *in vivo* and *in vitro*. Indeed, it was shown that menin is necessary for the commitment of mesenchymal stem cells into preosteoblasts (Sowa et al. 2003, Sowa et al. 2004, Naito et al. 2005), menin being crucial for BMP induced transcriptional activation of *Runx2* by interacting with Smad1 and Smad5 resulting in the commitment of progenitors into preosteoblasts. However, once the commitment is engaged, menin represses osteoblast maturation by repressing Smad1/5 transcriptional activity and by promoting Tgf β mediated Smad2 induced repression of differentiation, through its physical interaction with Smad1, Smad5 and Smad3 (Sowa et al. 2003, Sowa et al. 2004). It was also shown that, in addition to its Smad dependent regulation of maturation, menin antagonized JunD mediated osteoblast maturation (Naito et al. 2005), through its interaction with the AP-1 member JunD to represses its transcriptional activity (Agarwal et al. 1999, Gobl et al. 1999, Huang et al. 2012). Menin interacts physically and functionally with β -catenin in mesenchymal and osteoblastic cell lines, by positively regulating β -catenin transcriptional activity (Inoue et al. 2011). In addition, menin seems to be necessary for β -catenin-mediated enhancement of C2C12 mesenchymal cells differentiation into osteoblasts after BMP-2 treatment (Inoue et al. 2011). Conditional *Men1* disruption in neural crest results in cleft palate that may result from defects in osteoblast lineage progression (Engleka et al. 2007).

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More recently, it was demonstrated that during osteoblast induction of human Adipose-tissue Derived Stem Cells (hADSC) menin can indirectly regulate Smad1 expression by activating the transcription of *mir-26a* which represses Smad1 protein expression (Luzy et al. 2012).

Menin was also shown to regulate myogenic commitment from mesenchymal stem-cells. Indeed, menin expression decreases during muscle differentiation (Aziz et al. 2009), consistent with previous reports showing absence or low menin/*Men1* expression in differentiated muscles (Stewart et al. 1998, Guru et al. 1999, Wautot et al. 2000). Ectopic menin expression impairs myogenesis from mesenchymal stem cells, which is mediated by a negative effect of menin/Smad3 complex on differentiation (Aziz et al. 2009). This effect was further supported by the fact that mice with conditional *Men1* disruption in neural crest have an increased number of intercostal muscle fibers, (Aziz et al. 2009).

Menin appears to be crucial for adipocytes differentiation. It was shown that menin interacts physically and functionally with PPARgamma and that menin loss impairs PPARgamma mediated differentiation of 3T3L1 and mouse embryonic fibroblast into adipocytes (Dreijerink et al. 2009). It seems that this role is specific to PPARgamma dependent adipogenesis, as it was demonstrated that menin does not affect BMP-2 mediated adipogenesis from mesenchymal stem cells (Sowa et al. 2003).

Men1 disruption in adult mice decreases the number of peripheral white blood cells and decreases B lineages progenitors, but mice maintain a normal population of upstream hematopoietic progenitors (Chen et al. 2006, Maillard et al. 2009). However, following hematopoietic stress, *Men1* deficient hematopoietic stem cells are severely affected demonstrating the crucial role of menin for hematopoiesis (Maillard et al. 2009). The role of menin in hematopoiesis has been highlighted by its reported physical interaction with the histone methyltransferase MLL (Mixed Lineage Leukemia), the mammalian homolog of *Drosophila* trithorax. MLL1 forms a complex with several proteins, including LEDGF, menin, c-myb, WDR5, Ash2L, RbBP5, which regulates the expression of *Hox* genes (Thiel et al. 2012). Mll1 is critical for the establishment and maintenance of hematopoiesis during development and in adult in the mouse, since *Mll1* disruption impairs more or less hematopoietic stem cells (HSCs) depending on the model used (Hess et al. 1997, Ernst et al. 2004, Jude et al. 2007, McMahon et al. 2007). MLL1 rearrangements in acute lymphoid and

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myeloid leukemias lead to an upregulation of *HOXA9* and *MEIS1* genes expression critical for leukemogenesis. Interestingly, menin was shown to interact physically with MLL1(Yokoyama et al. 2004) and MLL2 (TRX2) (Hughes et al. 2004) , c-Myb (Jin et al. 2010), and LEDGF(Yokoyama and Cleary 2008), positively regulating *Hox* genes transcription in different cell lines. *Men1* disruption in HSCs (Maillard et al. 2009) does not recapitulate the phenotype observed after *Mll* disruption, demonstrating that in basal conditions, Mll can overcome the absence of menin to preserve relatively normal hematopoiesis.

1.3.4.2 REGULATION OF PROLIFERATION AND CELL SURVIVAL

Menin regulates the proliferation of several endocrine cell-types as well as non-endocrine cells.

1.3.4.2.1 REGULATION OF ENDOCRINE CELLS PROLIFERATION AND SURVIVAL

Several murine models aimed at disrupting the *Men1* gene specifically in endocrine compartments demonstrated the putative role of menin in the regulation of the proliferation and tumorigenesis of these cells. The heterozygous *Men1*^{+/-} mice as already described, develop a range of endocrine tumors similar to MEN1 syndrome. Interestingly duodenal or pancreatic gastrinomas only rarely develop or even do not develop at all suggesting a possible resistance of the murine organism to *Men1*-related gastrinoma tumorigenesis (Crabtree et al. 2001, Bertolino et al. 2003, Loffler et al. 2007, Harding et al. 2009). On the contrary, specific *Men1* disruption using Cre/Lox system in pancreatic beta cells (Bertolino et al. 2003, Crabtree et al. 2003, Biondi et al. 2004), pancreatic alpha cells (Lu et al. 2010, Shen et al. 2010), pituitary (due to leakage of Cre transgenes) (Crabtree et al. 2001, Biondi et al. 2004, Seigne et al. 2013), or parathyroid (Libutti et al. 2003), leads to the development of tumors from the targeted cells consistent with a role of menin in regulating proliferation of these endocrine cells *in vivo*.

An interesting study carried out by Thakker group showed that intratumoral injection of adenoviral vectors allowing the reexpression of *Men1* in *Men1* deficient pituitary tumors of *Men1*^{+/-} mice considerably decreased their proliferation after 4 weeks (Walls et al. 2012). This strongly suggests that menin expression by itself is an important gate-keeper of endocrine cell proliferation and that maybe menin loss and subsequent deregulated proliferation is the key

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event required for *Men1* related tumorigenesis. However, more precise and long term studies on the effect of *Men1* gene therapy rescue are required.

It has been shown that menin mediates TGF- β -induced growth inhibition by interacting with Smad3 in anterior pituitary cell lines (Kaji et al. 2001).

Menin positively regulates the expression of the cyclin dependant kinase inhibitors p18^{Ink4c} (*Cdkn1b*) and p27^{Kip1} (*Cdkn2c*) in several endocrine cell types *in vivo* mainly through its interaction with Mll (Karnik et al. 2005, Milne et al. 2005, Schnepf et al. 2006). However, analysis of *Cdkn1b* and *Cdkn2c* mRNA did not reveal significant down-regulation of these genes in human MEN1 pancreatic endocrine tumors (Lindberg et al. 2008). In addition, change in *p27* mRNA expression was not detected in *Men1*-deficient beta cells *in vivo* (Scacheri et al. 2006) and 23% of insulinomas from mice with beta cell specific *Men1* disruption had normal p27 protein expression (Fontaniere et al. 2006).

Menin also regulates cyclin dependent kinases (CDKs) and cyclins expression. Cdk4 expression is increased in mouse *Men1* pancreatic endocrine tumors developed in heterozygous *Men1*^{+/-} mice (Karnik et al. 2005). *Men1* disruption in islet cells also increases the expression of *CyclinA2*, *CyclinB1*, *CyclinB2*, and factors positively involved in cell cycle progression such as *Pbk* and *Mcms* genes (Yang et al. 2010). Importantly, menin was shown *in vitro* to directly repress *CyclinB2* gene expression by promoting the recruitment of negative regulators at *Ccnb2* locus (Wu et al. 2010). In addition, specific beta cell disruption of the *Men1* gene is accompanied with an increase in *CyclinA2*, *CyclinB2* and *CyclinD2* expression in tumors developed in these mice (Fontaniere et al. 2006).

It was shown that menin can interact physically with β -catenin *in vitro* to mediate nuclear export of β -catenin to the cytoplasm (Chen et al. 2008, Cao et al. 2009). β -catenin nuclear accumulation is increased upon loss of menin expression in *Men1* deficient alpha and beta cells *in vivo* (Cao et al. 2009) at early stage, which may be involved in the proliferation of these cells (Dessimoz et al. 2005). In addition, membrane expression of E-cadherin and β -catenin is decreased in *Men1*-disrupted mouse beta cells (Bertolino et al. 2003, Yan et al. 2009). Moreover, menin interacts with the scaffold protein IQGAP1 to mediate intercellular adhesion by stabilizing E-cadherin and β -catenin at cytoplasmic membrane (Yan et al. 2009).

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Menin seems also to play a pivotal role in AKT signaling. It was demonstrated in MEF and HEK-293 cells that menin can bind and sequester inactive AKT, to prevent AKT activation. Consistently, activated AKT^{pS473} staining is increased in all pancreatic islet adenomas analyzed that developed in *Men1*^{+/-} mice, but the hormonal profile of adenomas was not precised (Wang et al. 2011). Considering the important role of AKT in promoting cell proliferation and survival, AKT activation in endocrine cells upon menin loss may play an important role in MEN1 tumorigenesis, but more investigations are needed.

Recently, menin was involved in the repression of Hedgehog signaling in pancreatic beta cells. It was demonstrated that menin interacts with the H4R3me2 methyl transferase PRMT5 to promote repression of *Gas1* expression, a Sonic Hedgehog co-receptor (Gurung et al. 2013). *Gas1* is overexpressed in mice with β -cell specific *Men1* disruption, and treatment of the mice with Hedgehog signaling inhibitors considerably decrease menin deficient beta cells proliferation (Gurung et al. 2013). The role of menin on Hedgehog signaling is straightened by the recent finding that menin represses the transcription of *Gli1*, a major effector of Hedgehog signaling (Gurung et al. 2013).

A role of menin in physiological control of β -cells proliferation was reported by studies on the adaptive β -cell proliferation in mouse. It was shown that menin protein expression is decreased in islet during pregnancy (Karnik et al. 2007, Zhang et al. 2010, Hughes and Huang 2011) and in genetically obese mice (Karnik et al. 2007), thus allowing compensatory beta cell proliferation that occurs in these models. It was shown *in vitro* and *in vivo*, that glucose through Akt signaling (Zhang et al. 2012), and/or prolactin and placental lactogen through increase of Bcl6 (Karnik et al. 2007, Zhang et al. 2010, Hughes and Huang 2011), may play a role in the down-regulation of menin expression during adaptive beta cell proliferation.

Menin was shown to negatively regulate the protein expression but not mRNA expression of *Hlxb9* in an insulinoma cell line by an unknown mechanism (Shi et al. 2013). In this study *Hlxb9* overexpression was shown to induce apoptosis, this effect being rescued partially by *Men1* knock-down. In addition, increased nuclear and cytoplasmic *Hlxb9* is observed in tumoral islets from *Men1*^{+/-} mice. The precise role of *Hlxb9* in MEN1 related tumorigenesis need to be more precisely investigated.

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Menin may regulate proliferation at another level by repressing the expression of several growth factors such as IGF2, Igfbp-2 and PTHrP (La et al. 2004, Fontaniere et al. 2006, Dejeux et al. 2009).

Testis Leydig cells proliferation and apoptosis is respectively negatively and positively regulated by menin (Hussein et al. 2007). Heterozygous male *Men1* mutant mice frequently develop Leydig cells tumors (Bertolino et al. 2003, Loffler et al. 2007). It was shown that loss of menin in these tumors is accompanied by decreased protein expression of TGF- β and BMP effectors Smad3 and Smad1/5 respectively and of the common Smad, Smad4, which may play a role in the development of these tumors (Hussein et al. 2008).

Menin was involved in differential regulation of endocrine cell apoptosis between development and adult islet cells. As already mentioned above, *Men1 null* embryonic pancreases display apoptosis of endocrine cells, but it is still not clear if it is a direct effect of menin absence in endocrine cells or not (Fontaniere et al. 2008). In pancreatic endocrine beta cell lines *in vitro*, menin promotes apoptosis (Sayo et al. 2002, Bazzi et al. 2008). However the role of menin-regulated apoptosis in adult endocrine cells *in vivo* needs further investigations.

Recently, it was demonstrated that menin loss in epithelial cells in the stomach antrum, duodenum and the intestine displayed a decrease in *p27^{Kip1}* mRNA and a two-fold increase in the number of gastrin⁺ cells in the antrum but not in the proximal duodenum of 12 month-old mice (Veniaminova et al. 2012). It is however not clear whether these G cells are *Men1*-deficient and if the increased G cell number results from proliferation or from an increased differentiation of progenitors toward G lineage. In parallel, increased proliferation of crypt cells is observed in the proximal duodenum after menin loss, suggesting that menin may regulate the proliferation of cells of transient amplifying compartment.

1.3.4.2.2 REGULATION OF NON-ENDOCRINE CELLS PROLIFERATION AND APOPTOSIS

While menin plays a crucial role in diverse endocrine cells, it also has important biological functions and specifically regulates cellular proliferation in several non-endocrine cells or tissues which are not part of MEN1 syndrome.

MLL fusion proteins formed upon chromosomal translocations (MLL-AF4, MLL-ENL, MLL-AF9 and others) are important drivers of acute leukemias initiation and

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maintenance by upregulating *HOX* genes and *MEIS1* expression. Menin is required for MLL fusion proteins induced leukemogenesis (Chen et al. 2006, Thiel et al. 2012). This role seems to be due to the recruitment of LEDGF to menin/MLL1 complex (Yokoyama and Cleary 2008). Importantly, inhibitors allowing the loss of menin interaction with MLL and MLL-fusions proteins decrease the expression of MLL/MLL-fusions proteins target genes resulting in a differentiation of leukemic cells and apoptosis, as well as decreased proliferation (Grembecka et al. 2012, Shi et al. 2012). Altogether, these observations demonstrate that in hematopoietic cells, menin behaves as a pro-oncogenic factor by promoting the activity of MLL fusion proteins.

Studies from our group and others also showed a role of menin in the development of hormono-dependants tumors (prostate, breast) (Bertolino et al. 2003, Loffler et al. 2007, Harding et al. 2009, Seigne et al. 2010, Seigne et al. 2013). Menin expression is decreased in subsets of human breast cancers, and *Men1* disruption in mouse mammary gland luminal epithelial cells increases the incidence of mammary intraepithelial neoplasias (Seigne et al. 2013). Interestingly, it was shown that menin interacts with the estrogen receptor α (ER α) and that menin is required for ligand dependant ER α transcriptional activity (Dreijerink et al. 2006, Imachi et al. 2010). Importantly ER α is an important proliferative factor in ER $^{+}$ breast cancers that can be treated by antiestrogens therapy such as tamoxifen. It was shown that in ER $^{+}$ breast cancers treated by tamoxifen, menin positive expression was correlated with a decrease in relapse free survival, likely because of menin's competitive binding to ER with tamoxifen (Imachi et al. 2010). More studies are needed to precisely define the role of menin in breast tumorigenesis.

More and more evidences indicate the importance of menin in suppressing lung tumorigenesis. Indeed, it was shown in lung cancer cell lines that menin positively regulates *Pleiotropin* gene silencing mediated by the Polycomb group protein H3K27 methyl transferase EZH2 by an unknown mechanism (Feng et al. 2010). Menin depletion results in enhanced Pleiotropin (PTN) expression, promoting lung cancer cell proliferation. Several lines of evidence suggest that menin could enhance EZH2 expression. In MLL-MA9 leukemic cells, menin is necessary for MLL-MA9 fusion protein to bind to *EZH2* promoter and subsequent EZH2 transcription (Thiel et al. 2013). However, it was shown that in *Men1 null* MEF cells, *Ezh2* expression is similar to wild-type MEFs, and menin loss also leads to decrease in the binding of *Ezh2* to a target promoter (*Pax2*) (Xu et al. 2011), likely due to

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decreased expression of Wilms tumor suppressor protein (WT1) protein necessary for Ezh2 binding and subsequent H3K27 trimethylation of the *Pax2* promoter. Altogether, these observations demonstrate the indirect but important role of menin in EZH2 mediated gene silencing. It would be crucial to investigate if this role is conserved in endocrine cells and in MEN1 related tumors.

In addition it was demonstrated that *Men1* disruption facilitates KrasG12D mediated lung tumorigenesis in the mouse (Wu et al. 2012). These results are in accordance with previously demonstrated role of menin as a suppressor of RAS-mediated transformation *in vitro* (Kim et al. 1999). Thus, it would be interesting to determine if menin has a role in the initiation or progression of others cancers driven by KRAS mutations, especially in pancreatic ductal adenocarcinomas, since menin expression was shown to be down-regulated in these cancers (Cavallari et al. 2009).

Menin expression is lost in a subset of human melanomas (Gao et al. 2011, Fang et al. 2013), and a study reported possible MEN1 LOH in 4/19 sporadic melanomas (Nord et al. 2000). It has been shown *in vitro* that menin overexpression represses proliferation of melanoma cell lines mainly through repressing gene expression of PTN and its receptor RPTP β/ζ in this cell type and subsequent decreased activation of PI3K and ERK1/2 (Gao et al. 2011).

Menin loss triggered apoptosis during liver development but mainly in liver hematopoietic compartment (Bertolino et al. 2003) (unpublished data of our laboratory). Intriguingly, only a few proportion of *Men1*^{-/-} ES cells contribute to liver cell mass in chimeric mice (Fontaniere et al. 2008), suggesting that menin may have some unknown functions in developing hepatocytes. *Men1* disruption in hepatocytes using *Albumin-Cre* transgene which is active since E19 during mouse embryonic development has no effect on hepatocyte proliferation or death (Scacheri et al. 2004). However menin was shown to have an important metabolic role in hepatocytes (Cheng et al. 2011, Wuescher et al. 2011, Wuescher et al. 2012).

A role of menin in DNA repair and maintenance of genome integrity has been suggested by the description of high rate of chromosomal abnormalities in lymphocytes and skin fibroblast from MEN1 patients (Scappaticci et al. 1991). However such role has been

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demonstrated *in vivo* only in *Drosophila melanogaster* (Busygina et al. 2004, Busygina et al. 2006, Papaconstantinou et al. 2010) and still needs to be confirmed in mammals. Some *in vitro* evidences support a probable role of menin in DNA repair and maintenance of genome integrity by interacting with proteins involved in DNA repair mechanisms (Tomassetti et al. 1995, Ohkura et al. 2001, Yaguchi et al. 2002, Jin et al. 2003, Sukhodolets et al. 2003, Kottemann and Bale 2009, Gallo et al. 2010). In addition, it was shown that in cell lines menin never co-localizes with sites of DNA damage, excluding a direct role of menin in DNA repair. It was suggested that menin's role in DNA repair may be only at transcriptional level (Francis et al. 2011). This was further consolidated recently in melanocytes where menin/Mll complex could be recruited by ER α to upregulate the transcription of *Brcal* and *Rad51*, thus promoting efficient DNA repair (Fang et al. 2013).

1.3.4.3 OTHER MENIN FUNCTIONS.

Several studies involved menin in several function, principally based on its interaction with numerous partners as described in Table 3.



RESULTS

2.1.1 CONTEXT OF THESIS WORK AND QUESTIONS RAISED

As described in the literature review chapter, clinical features of both sporadic and hereditary pancreatic endocrine tumors are extremely varying and complex. The first level of their complexity resides in the occurrence of well differentiated and poorly differentiated islet tumors, two categories of the tumors giving rise to markedly distinct clinical outcomes. Moreover, well differentiated PNETs can either be functional or non-functional, depending on whether they secrete or not an excess of hormone causing clinical symptoms. Furthermore, functional PNETs are subdivided into different subtypes according to the major hormone they secrete or express. Finally, multihormonality of PNETs is far from rare, contributing largely to the heterogeneity of PNETs. The latter is further amplified, intriguingly, by the occurrence of tumors expressing and/or secreting non pancreatic hormones, such as Gastrin or VIP. Consequently, several questions may arise concerning such diversity of the clinical features of islet tumors:

- What are the biological bases of such tumor diversity?
- Does it reside in different cells of origin for each type of tumors, or can it be due to alternative ways of evolution during tumor progression, or both?
- Are there common or specific molecular and cellular mechanisms contributing to each PNET type?

2.1.2 AIMS OF THESIS WORK

In the context of MEN1 disease, PNET initiation is triggered by the loss of function of the remaining wild-type allele of the *MEN1* gene. This event may virtually occur at any time during pancreatic embryogenesis or adult life, and in any cell-type belonging to the endocrine pancreas. Classical and conditional *Men1* KO mouse models have been engineered to date and have proven their usefulness in the understanding of PNETs related to *MEN1* inactivation. However, it is impossible to control and determine the timing of *Men1* disruption in the classical *Men1*^{+/-} model. Furthermore, although the use of conditional *Men1* disruption

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in the mouse allows to precisely define the cell type(s) and the timing of *Men1* inactivation, virtually nothing was known about the tumorigenic potential of *Men1*-disruption before the appearance of differentiated pancreatic endocrine cells. Considering the above mentioned issue concerning the diversity of PNETs, the major aim of this thesis was to investigate the consequences of *Men1* disruption specifically in pancreatic endocrine progenitors. Indeed, we made the assumption that menin loss in pancreatic endocrine progenitors may trigger a more diverse range of PNETs types, as compared to alpha or beta-cell specific *Men1* disruption models.

In addition, it is well known that menin has an important role in maintaining the identity or in controlling the differentiation of several cell-types. Also, as already hypothesized in the literature, PNETs expressing non pancreatic gut hormones (such as gastrinomas) may have a stem/progenitor cell of origin. It is, therefore, relevant for the field to provide concrete experimental clues to better understand tempo-spatial effects of *Men1* disruption in islet cells, including in particular the progenitors.

2.1.3 STRATEGY

In order to specifically disrupt the *Men1* gene in pancreatic endocrine progenitors, we used a novel mouse model based on Cre/Lox system, allowing specific expression of Cre recombinase in Ngn3 expressing pancreatic endocrine progenitors.

2.1.4 RESULTS

The analyses of tumors developed in this model combined with other conditional *Men1* KO models, led to the interesting observation that endogenous Ngn3-derived pancreatic endocrine cells are at the origin of pancreatic gastrin-expressing tumors. The results were reported in the manuscript #1 that is currently in the final stage of preparation.

In parallel, we demonstrated that targeted ablation of *Men1* in pancreatic progenitors in the mouse led to the development of a tumoral phenotype displaying larger features than that described in other *Men1* mouse models. The molecular analyses of these tumors allowed

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to obtain evidence showing that menin deficient insulinomas and glucagonomas display differential abnormalities in signaling pathways. These investigations are included in the Manuscript #2 that needs further experiments before its submission.

2.1.5 RELEVANCE

Our results may open the way for the better understanding of the pathophysiology of pancreatic gastrinomas. In addition, our data concerning the heterogeneity of survival/proliferating pathways among mouse *Men1* PNETs, may help in the better management of PNETs, if they can be validated in patients.

2.1.6 ADDENDUM

In addition to the works presented hereafter, during my thesis, I have had the opportunity to carry out or participate in several other projects related to the study of islet cell biology in normal or pathological conditions. In particular, in an attempt to validate the observations acquired in our mouse models, I observed notable distinct expression of PAX4 and MAFA, both of them being well-known beta-cell markers, in human islets (please refer to the publication 1 in the annexes). Since these works are relatively distant to the main theme of my thesis, or that my contribution was only minor, I decided not to include the data from these works in the Result. Nevertheless, the corresponding published papers can be found in the Annexes.

2.2 MANUSCRIPT #1: INTRA-ISLET PERINATAL GASTRIN-EXPRESSING CELLS SERVE AS THE CELL OF ORIGIN OF PANCREATIC GASTRIN-EXPRESSING TUMORS.

2.3 MANUSCRIPT #2: *MEN1*-DISRUPTION IN NGN3⁺-PROGENITORS REVEALS ISLET TUMOR HETEROGENEITY BOTH IN TUMOR TYPES AND MOLECULAR ALTERATIONS

SHORT COMMUNICATION

Intra-islet perinatal gastrin-expressing cells serve as the cell of origin of pancreatic gastrin-expressing tumors

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ABBREVIATIONS: NPG, neonatal pancreatic gastrin.

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ABSTRACT

Pancreatic gastrinomas develop either hereditarily in patients with Multiple Endocrine Neoplasia type 1 or sporadically, although, intriguingly, no gastrin expressing cells are found in normal adult pancreas. Previous studies described the existence of Gastrin expressing cells in developing and neonatal mammal pancreases that disappear rapidly after birth. However, neither their origin, nor their relation with other islet-cells was clearly defined. By using cell lineage-specific gene ablation approach, we provided compelling evidence showing that gastrin-expressing cells in the pancreas, found from E12.5 till P7 days, are derived from pancreatic Ngn3⁺-progenitors. More importantly, the majority of them co-express glucagon, with 1/25th co-expressing insulin, indicating that they are a temporary subpopulation of both alpha and beta cells. Consistent with their ontogeny, *Men1*-disruption in both Ngn3-progenitors, and beta and alpha cells led to the development of pancreatic Gastrin-expressing tumors. Our data provide insight into histogenesis of MEN1 pancreatic gastrin-expressing tumors.

INTRODUCTION

Gastrinomas are endocrine tumors secreting peptide hormone gastrin, usually associated with Zollinger-Ellison syndrome (ZES) characterized by recurrent peptic ulcers and gastroesophageal reflux due to gastrin-induced gastric acid hyper-secretion (Zollinger 1987). Most adult gastrin-producing cells are found within gastric antrum and, to a much lesser extent, in the proximal duodenum in mammals. Intriguingly, the majority of primary gastrinomas, about 70%, is found located in the duodenal submucosa, the remaining third within the pancreas, and only rarely in stomach (Tartaglia et al. 2005). These tumors can develop either sporadically or hereditary in patients with Multiple Endocrine Neoplasia type 1 (MEN1, OMIM131100). MEN1 patients develop multiple tumors in endocrine organs principally affecting parathyroids, pituitary and gastroenteropancreatic tract. Gastrinomas are the most common functioning tumors of the gastroenteropancreatic axis in MEN1 patients (Halfdanarson et al. 2008), displaying as multiple small lesions in the duodenum and, in rare case, in the pancreas. Sporadic gastrinomas are often solitary tumors originating both in the duodenum and pancreas, with the etiology of these tumors being poorly understood. Interestingly, somatic *MEN1* mutations and LOH at *MEN1* locus on 11q13 have been detected in a substantial proportion, approximately 30%, of both sporadic duodenal and pancreatic gastrinomas (Debelenko et al. 1997, Zhuang et al. 1997). In addition, we reported previously the occurrence of gastrin expressing pancreatic tumors in about 15% of heterozygous *Men1* mutant mice (Bertolino et al. 2003), further confirming that *MEN1* inactivation plays a crucial role in gastrinoma pathogenesis.

Deciphering the cell of origin of cancers and tumors is currently a highly active field (Blanpain 2013), due to its importance not only for better understanding tumor biology but also for tumor treatment with targeted therapy. The histogenesis of gastrinomas is a longstanding intriguing question (Passaro et al. 1998) that is still unanswered especially for

pancreatic gastrinomas. To determine the cells of origin of the latter, being the most aggressive form as compared to their duodenal counterparts, is of special interest, since gastrin expressing cells are not found in human and rodent adult pancreas. Nevertheless, several studies described Gastrin expression, both at mRNA and protein levels, in developing and neonatal mammal pancreases, which decreases rapidly after birth (Brand and Fuller 1988, Bardram et al. 1990, Gittes et al. 1993, Furukawa et al. 2001). Analyses of the lysates from rodent neonatal pancreas established that gastrin was fully processed and active in the pancreas at this stage (Bardram et al. 1990). Consistently, Takaishi and colleagues demonstrated, by the use of transgenic mice harboring the *GFP* gene whose expression is driven by the mouse gastrin promoter, that GFP expression could be targeted to pancreatic cells in embryonic and neonatal pancreases until 2 days after birth (Takaishi et al. 2011). Passaro *et al.* postulated that stem cells formed in ventral pancreatic bud could be the cell origin of pancreatic gastrinomas found in the triangle area, based on the clinical features of the tumors (Passaro et al. 1998). Indeed, pancreatic endocrine cells derive from the differentiation of Ngn3 expressing progenitors, while gastric and duodenal Gastrin-expressing cells also derive from endocrine progenitors expressing Ngn3 situating at the corresponding tissue (Jenny et al. 2002). This may argue for that pancreatic gastrin-expressing cells may also arise from Ngn3⁺ pancreatic endocrine progenitors. However, the existence of the transient pancreatic Gastrin-expressing cells remains so far poorly defined, and the evidence showing their cell origin is totally lacking. Altogether, the above mentioned observations prompted us to better define gastrin-expressing cells in the pancreas and determine the cells of origin of MEN1 pancreatic gastrinomas.

METHODS

-Animal study approval

All animal maintenance and experiments were performed in accordance with the animal care guidelines of the European Union and French laws and were validated by the local Animal Ethic Evaluation Committee (Protocol CLB 2010/016).

-Mouse studies

Ngn3^{tTA} knock-in mice were generously provided by Dr Jan.N. Jensen (Wang et al. 2009). The tetO-cre mouse line (B6.Cg-Tg(tetO-cre)1Jaw/J MGI:2679524) was purchased from Charles River France. *Men1^{F/F}-RIPCre⁺* (β *Men1 KO*) (Bertolino et al. 2003), *Men1^{F/F}-GluCre⁺* (α *Men1 KO*) mice (Lu et al. 2010) and *R26^{eYFP}* mouse line were already described (Srinivas et al. 2001). *Ptf1aCre* knock-in mice (Kawaguchi et al. 2002), were generously provided by Dr Christopher V.E. Wright.

-Immunohistochemistry and immunofluorescence

Neonate and adult pancreases were fixed in Neutral buffered formalin (Lilly's fixative) for 2 hours at room-temperature or 18 hours at 4°C respectively, and then were embedded in paraffin. Serial section of 3µM were prepared, mounted on Superfrost Plus glass slides, and subjected to deparaffinization and rehydration. Endogenous peroxidases were quenched in 3% H₂O₂ solution for 30 minutes at room-temperature. Heat-induced epitope retrieval was performed by immersion in Antigen Unmasking Solution (H-3300, Vector) in a microwave oven. After blocking with antibody diluent (DAKO) sections were incubated overnight with a primary antibody. For immunofluorescence, stainings were revealed with Cy-3 or Cy-5 Tyramide amplification kit (PerkinElmer) according to the manufacturer's instructions with prior incubation with appropriate biotinylated secondary antibody, or incubated with appropriate Alexa488-555-or-647 coupled secondary antibodies (Life Technologies) for 1 hour. For immunohistochemistry (IHC), after 1st antibody incubation, biotinylated secondary antibody was applied for 1 hour and revealed using ABC-DAB system (Vector Lab). Images were captured on a TCS-SP5 confocal microscope (Leica-Microsystems) or a Zeiss 780

confocal microscope. Cell counting was manually performed with ImageJ cell counter module (U. S. National Institutes of Health, Bethesda, Maryland, USA) on multiple channel pictures. Graphs and results of counting are represented as mean±S.E.M.

-Antibodies.

Antibodies used in the study were, anti-glucagon (G2654, Sigma), anti-insulin (A056401, DAKO), anti-somatostatin (sc-7819, Santa Cruz), anti-PP (IMG-3835, Imgenex), anti-Ghrelin (Kind Gift from Dr Catherine Tomasetto), anti GFP recognizing eYFP (A-6455, Life Technologies), anti-Chromogranin A SP (20085-Immuno, Immuno Star), anti-Gastrin (NCL-GASp, Novocastra), anti-gastrin antibody (serum 2717, Dr. Jens F. Rehfeld), anti-CCK antibody (Dr J. Chayvialle), anti-CCK antibody (detects proCCK, sc-20937, Santa Cruz), anti-Menin (A300-105A, A300-106A, Bethyl), anti-Ngn3 (BCBC AntibodyCore AB2774), anti-Ki-67 (sc-7846, Santa Cruz), anti-MAFA (ab26405, Abcam), anti-PDX1 (ab47383, Abcam), anti-MafB (IHC-00351, Bethyl) and anti-Pax4 antibody (Kind gift from Dr Beatriz Sosa-Pineda (Wang et al. 2008)). Donkey secondary antibodies coupled with either Alexa 488, 555, or 647 were used (Life technologies, Jackson ImmunoResearch). For Immunohistochemistry or immunofluorescence with TSA, biotinylated secondary antibodies from horse or donkey were used (Vector Lab).

RESULTS

Pancreatic gastrin⁺ cells represent subpopulations of alpha and beta cells at birth.

We first tried to better characterize the population of mouse neonatal pancreatic gastrin-expressing (NPG) cells, since very little is known about this transient cell population. Immunofluorescence (IF) analyses with an anti-gastrin antibody demonstrated that scattered cells with positive cytoplasmic staining could be detected in the pancreas of mouse neonates (Figure1A). We quantified the proportion of these NPG cells relative to the total endocrine

population at birth by performing double IF staining for gastrin and ChromograninA, a marker of endocrine cells (Figure1A). NPG population represented 4.3% of total endocrine cell number (Figure 1D) at P0, but became undetectable at P7 (data not shown), consistent with what was reported in the literature. Then, we further checked the existence of Gastrin⁺ cells in mouse developing pancreas. Gastrin⁺ cells were readily detected but rare at E12.5 and the population was markedly increased at E14.5 in the wild-type mouse developing pancreas (Figure 1F). The related peptide hormone CCK was not expressed at P0 in the pancreas but was detectable in the duodenum at birth as was Gastrin (Supplemental Figure 1), supporting the specificity of the above detected NGP cells.

Furukawa and colleagues observed previously that some gastrin⁺ cells were also positive for insulin staining by restaining method (Furukawa et al, 2001). By double-IF staining, indeed, we could detect in rare case cells expressing both gastrin and insulin (Figure1B and 1E), accounting for $4.4\% \pm 1.8$ of total NPG cells, while most of NPG cells were negative for insulin. Since we observed that most NPG cells localized to the periphery of the developing endocrine islets, where most of alpha cells resides in mouse pancreas, we sought to determine if these NPG cells could express glucagon. To our surprise, a very high proportion, $85.5\% \pm 7.3$, of NPG cells was found to also express glucagon (Figure1C and 1E). Thus it appears that NPG cells represent subpopulation of both developing alpha and beta cells. We performed double staining of gastrin with other pancreatic hormones and we detected that somatostatin⁺ cells did not express gastrin at P0 (Supplemental Figure 2). In order to identify if cells only expressing Gastrin and none of other pancreatic hormones, we performed double staining with gastrin antibody and a cocktail of pancreatic hormones (insulin, glucagon, somatostatin, PP and ghrelin). A small proportion ($3.3\% \pm 1.3$) of gastrin expressing cells was indeed negative for the hormonal cocktail (Figure1E). Analyses of transcription factors expressed by NPG cells showed that they displayed high MafB

expression and some also expressed low-level of Pdx1 at P0 (Figure 1G), whereas the expression of neither MafA nor Pax4 was detected. These data argue for the fact that NPG cells are immature pancreatic endocrine cells.

Taken together, our data allowed defining embryonic and neonatal pancreatic gastrin-expressing cells as the subpopulations of both insulin- and glucagon-expressing cells.

Pancreatic gastrin⁺ cells originate from pancreatic-progenitors within the developing pancreas

Considering that all alpha and beta cells are derived from Ngn3⁺ endocrine progenitors, we suspected that NPG might also be derived from the differentiation of pancreatic progenitors, as virtually all NPG cell expressed either glucagon or insulin. In order to determine if NPG derive effectively from pancreatic progenitors, instead of resulting from the migration of gut progenitors or gut differentiated cells as hypothesized previously (Passaro et al. 1998), we performed lineage tracing experiments with *Ptf1aCre⁺-R26^{eYFP}* mice, since *Ptf1a*-expressing cells during development only give rise to pancreatic acinar, ductal and endocrine cells (Kawaguchi et al, nature genetics 2002). Using double IF staining, NPG cells were found to also express the YFP reporter in *Ptf1aCre⁺-R26^{eYFP}* mice (Figure 2A), confirming thus our hypothesis. Then, we further checked whether *Men1*-disruption specifically in pancreatic progenitors can target pancreatic Gastrin⁺ cells. Using *Men1^{F/F}-Ptf1aCre⁺* mice (*PancMen1* KO), we found that indeed, the expression of menin, the protein encoded by the *Men1* gene, was lost in a substantial proportion of NPG cells (Figure 2B and E). We next genetically traced the progeny of Ngn3⁺ pancreatic endocrine progenitors using *Ngn3^{wt/tTA}-tet-Ocre-R26^{eYFP}* mice. As demonstrated in figure 2C, YFP expression could be detected in NPG cells. In addition, as in control neonatal mouse pancreases, gastrin⁺ cells could be detected in pancreas of *Men1^{F/F}-Ngn3^{wt/tTA}-tet-Ocre* mice (*PancEndoMen1* KO) as shown on Figure 2D.

Analysis of these gastrin⁺ cells showed that the expression of menin was lost in about 20% of NPG cells from *PancEndoMen1* KO mice (Figure 2C and E). Taken together, our results indicate that NPG cells are derived from the pan-pancreatic (Ptf1a⁺) and pancreatic endocrine (Ngn3⁺) progenitors.

Pancreatic gastrinomas development upon *Men1*-disruption in both Ngn3⁺ pancreatic progenitors and insulin- or glucagon-expressing cells (Figure 3)

Having identified that NPG cells were derived from pancreatic progenitors, we hypothesized that the Ngn3⁺ endocrine progenitors could be cells of origin of pancreatic gastrinomas. To test this hypothesis, we carried out *Men1*-disruption in Ngn3⁺ progenitors. Histological analyses of pancreas dissected from aged *PancEndoMen1* KO mice showed that 12-month-old mutant mice displayed a high proportion of endocrine lesions. Tumor lesions exhibited a complete loss of menin expression as expected (Figure 3), and most of them were positive for insulin staining by IHC (the detailed data concerning the analysis of other islet tumors will be published elsewhere). Interestingly, by using gastrin antibody, several tumors were positively stained (Figure 3), whereas CCK was not detected with two different antibodies in these gastrin immunoreactive tumor cells (Data not shown). The frequency of gastrin⁺ lesions in *PancEndoMen1* KO mouse model is reported in Table 1. Unexpectedly, we found that gastrin⁺ tumors were also insulin⁺ with a much lower intensity as compared with other insulin⁺ tumors negative for gastrin and other hormones (Figure 3).

Since NPG cells co-express insulin or glucagon at birth, we next sought to determine whether perinatal *Men1*-disruption specifically in insulin⁺ or glucagon⁺ cells led to gastrin⁺ tumor development. Careful analysis of menin deficient lesions from *Men1^{F/F}-RipCre* mice (β *Men1KO*) at 12 months of age revealed the occurrence of gastrin⁺ lesions (Figure 4A). The cells expressing gastrin in tumors were also expressing insulin, but not glucagon, as

demonstrated by triple IF staining (Figure 4B). Three of four analyzed mice developed at least 1 gastrin⁺ tumor with a total of 5 gastrin⁺ lesions on 106 lesions analyzed (see Table 1). Molecular characterization of gastrin⁺ tumors from *PancEndoMen1* KO and *βMen1* KO demonstrated that gastrin expressing tumors were Pdx1⁺, consistent with a beta cell-gastrin⁺ origin of these tumors. Nevertheless, Ki67 analyses did not revealed any difference in proliferation as compared to other insulin expressing tumors (Figure 4D). In parallel, the analyses of tumors developed in the mutant mice where the *Men1* gene was specifically disrupted in glucagon⁺ cells (*αMen1* KO mice) demonstrated a low occurrence of gastrin⁺ lesions in 12-18 month-old *αMen1* KO mice (Table 1).

DISCUSSION

By better defining the pancreatic gastrin-expressing cells, and direct cell-specific *Men1* ablation, our study provides the evidence showing that both embryonic and neonatal gastrin-expressing cells and their progenitors can serve as the cells of origin of pancreatic gastrin-producing tumors. The data documented in this study provide insight into our knowledge on islet biology and a better understanding of histogenesis of pancreatic gastrinomas.

NPG cells are derived from pancreatic Ngn3⁺-progenitors and disappear before weaning

The cells of origin of pancreatic gastrinomas remained so far elusive. Moreover, although pancreatic gastrinomas are described in general as monohormonal tumors, clinical observations often reported them displaying co-expression of other islet-hormones (Kapran et al. 2006). Furthermore, the attempt to generate *Men1*-deficient mouse gastrinoma has been unsuccessful, hampering detailed experimental analyses. The latter situation is at least partially due to our poor understanding of gastrin-expressing cells in the pancreas. Albeit the reported existence of Gastrin expressing cells, it has been unknown where they are derived

from and what their relation with other islet cell populations is. In our current study using several mouse models combined with different analyses, we found that 1) the neonatal pancreatic Gastrin-expressing (NPG) cells are derived from pancreatic Ngn3⁺-progenitors; 2) they can be found at E12.5 till P7 days; 3) the majority of them co-expressing glucagon, and the remaining 1/25th co-expressing insulin, with only about 3.3 % Gastrin+ cells without coexpressing other islet hormones. Therefore, we defined, for the first time, the NPG cells as temporal sub-populations of islet cells, with pancreatic Ngn3⁺-progenitors as their common cell origin.

During the preparation of the current manuscript, we saw the publication of the work by Suissa and colleagues (Suissa et al. 2013). Using different mouse models and cell lineage tracing, they detected the existence of embryonic and perinatal gastrin-expressing cell population in the pancreas and determined these cells a distinct cell lineage derived from pancreatic Ngn3⁺ progenitors. Indeed, our data are in consistency with the cell origin and the appearance, as well as the disappearance of this cell population described by their study. The discrepancy between our work and theirs resides in whether they belong to a subpopulation of alpha or beta-cells, or rather than an independent lineage. We believe that the finding of the presence of only a few Gastrin+ cells without co-expressing other islet hormones at perinatal stage, the period, unfortunately, not having been analyzed in Suissa's study, and of the occurrence of Gastrin+ lesions in both *aMen1* KO and \square *Men1* KO mice support our definition of NGP cells. It remains unknown how these cells evolve, once they no longer express Gastrin, and whether they die or they entirely differentiate into alpha or beta-cells. If it is the latter case, do they keep on being, even without expressing gastrin, as or potentially as a subpopulation respectively in alpha and beta cells. It would be interesting in the future to identify the mechanisms, intrinsic or environmental, controlling their formation, relation with their counter part and their disappearance. It may be worth mentioning that recent studies

using human islet cells revealed that there are distinct subpopulations in both alpha and beta cells.

The newly defined NPG cells are reminiscent of another islet-cell lineage, Ghrelin-expressing cells. The latter also starts to appear at the developmental stage and disappear at neonatal period. Moreover, they more frequently co-express glucagon. Although our data do not support that they belong to the same subpopulation, their co-existence during the same period in the pancreatic islet, more often in alpha-cells, may suggest that both may play some relevant biological roles during the neonatal period. Several groups reported that *Gastrin*-disruption in the mouse did not result in any overt defect in endocrine pancreas development (Boushey et al. 2003, Cowey et al. 2005). Nevertheless, considering the known growth promoting of Gastrin in adult tissues and crucial developmental and maturation events in islets at the late embryonic and neonatal period, we cannot exclude that Gastrin-expressing subpopulation may somehow participate in the regulation of islet cell proliferation or maturation.

***Men1*-disruption in Ngn3⁺-progenitors and perinatal Gastrin⁺ cells led to pancreatic gastrinoma development**

Interestingly, targeting distinct islet cell lineages, including in particular Ngn3⁺-progenitors, alpha and beta cells, triggered gastrinoma development in all used different mouse *Men1* models. This is in total consistency with the ontogeny nature of the above mentioned newly defined NPG cells: a Ngn3-derived temporal subpopulation in both alpha- and beta-cell lineages. Remarkably, the frequency of gastrinomas found in each of the above mentioned models approaches closely to the proportion of gastrin-positive cells found during the late embryonic and neonatal period. Interestingly, the similar situation may occur in human, since

Anlauf and colleagues reported that in two tumor banks comprising a total of 300 NF pNETs, 3% were stained positive for gastrin (9/300).

The *Men1* gene is reported being mutated in about from one fourth to one thirds of sporadic gastrinomas. Our works using mouse models confirmed more specifically the oncosuppressive role of the *Men1* gene in NPG cells. Obviously, other genetic and epigenetic factors may also be involved in the tumorigenesis of these cells. Up to date, there are no reports specifically addressing this issue. Recently, using exome sequencing, the study by Jiao and colleagues identified several factors, including the *MEN1* gene, which were found mutated in a substantial proportion of analysed islet tumors (Jiao et al. 2011). The finding of the ontogeny nature of NPG cells and their relation with other islet cell lineages led us to speculate that the same genes may also play a role in the tumorigenesis of these cells. Interestingly, several studies previously highlighted the difference in molecular markers and altered gene expression between gastrinomas developed in the pancreatic and those in the duodenum, suggesting that distinct molecular mechanisms could be involved in gastrinomas developed in these two different but close anatomical sites. Further studies using mouse models could be of help to gain more experimental evidence on this issue.

Better ontogeny knowledge of islet cells could be of help for understanding clinical islet tumor features

The finding of gastrinomas in Ngn3⁺-progenitors derived tumors demonstrate that, apparently, *Men1*-disruption in these progenitors does not impair the fate decision made for Gastrin⁺ subpopulations. At the same time, importantly, we noticed that islet Gastrin-expressing lesions found in our models displayed only feeble gastrin expression compared with the neonatal pancreatic gastrin-expressing cells. Considering the low frequency of their

occurrence and the lack of pathological lesions in the stomach in mutant mice, hypergastrineamia was even not suspected. Consequently, the lesions could be considered non-functional. Mensah-Osman and colleagues reported that menin negatively regulates the *Gastrin* gene, by using mainly *MEN1* overexpression (Mensah-Osman et al. 2011). Their description is consistent with other studies reporting that menin exerts negative regulation of other hormones, such as insulin and prolactin. Intriguingly, we noticed that *Men1*-disruption has never been reported to lead to hormone overexpression in every single *Men1*-deficient cell, even mutant mice have in general the increase in blood hormone levels because of massively increased number of tumor cells. Taken together, our data suggest that *Men1* inactivation in pancreatic Gastrin-expressing cells should promote their proliferation, whereas it does not favor their endocrine functions. However, the role of menin in regulating *Gastrin* gene expression needs to be further clarified.

Intriguingly, we noticed that the majority of the gastrin⁺ islet-lesions observed in our *PancEndoMen1* mutant mice co-express insulin, although 80% of gastrin⁺ cells co-expressing rather glucagon. It is worth mentioning that in this mouse model, although a similar proportion of alpha and beta-cells had the *Men1* gene disrupted at 1 month of age, only few glucagonomas were detected in 12-month-old mutant mice (data not shown). Thus, it is likely that Gastrin⁺Glucagon⁺ subpopulation may be less sensitive to *Men1*-disruption, compared with Gastrin⁺Insulin⁺ subpopulation. Indeed, although rare, several clinical reports described the cases where gastrinomas occurred simultaneously or right after an insulinoma development (Mizuno et al. 2001). However, we found a substantial proportion of human Glucagonomas having Gastrin-expressing cells dispersed within the tumors (data not shown), suggesting that islet-tumors co-expressing gastrin and glucagon may be more frequently occurring. They could be neglected because of the lack of clinical symptom and of specific but sensitive antibodies for Gastrin detection.

In conclusion, the current study provides meaningful insights into ontogeny of islet NPG cells and histogenesis of pancreatic gastrinomas. Our data pave the way for further dissect the molecular mechanisms controlling the development and the tumorigenesis of the pancreatic gastrin-expressing cells. They would be also of help for the conception of new strategies for diagnosis and treatment of the disease.

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Table 1 Recapitulation of Gastrin expressing lesions in different models of *Men1* disruption

Model	n mouse with gastrin lesion/ n total mice	n Gastrin+ lesion / n Total lesions (%)
<i>Men1</i> ^{+/-}	1/5	1/17 (5,9%)
<i>PancEndoMen1</i> KO	2/4	2/70 (2,9%)
β <i>Men1</i> KO	3/4	5/106 (4,7%)
α <i>Men1</i> KO	1/6	1/34 (2,9%)

FIGURE LEGENDS

Figure 1. Neonatal gastrin expressing cells represent subsets of alpha and beta cells.

(A) Immunofluorescence (IF) colocalisation of gastrin and ChromograninA in the pancreas of newborn wild-type mice. Scale bar = 20 μ M (B) Graph representing the proportion of gastrin expressing cells among endocrine cells at birth. (C) Double IF stainings with antibodies directed against Gastrin and Insulin in pancreas of newborn wild-type mice. Scale bars = 20 μ M (D) Double immunostaining by immunofluorescence with antibodies against gastrin and glucagon in pancreas of newborn wild-type mice. Scale bar = 20 μ M (E) Graph representing the percentage of glucagon, insulin, or any of the pancreatic hormones (cocktail, see results part) expressing cells among the gastrin expressing cells at birth in wild-type mouse pancreas. (F) Representative IHC stainings for gastrin in wild-type mouse pancreas at E12.5 and E14.5. Scale bar = 50 μ M. (G) Double immunostaining by immunofluorescence with antibodies against gastrin and the indicated transcription factor in pancreas of newborn wild-type mice. Scale bars = 10 μ M.

Figure 2. Pancreatic progenitors targeted *Men1* disruption results in loss of menin expression in NPG cells. (A, C) Representative IF staining for gastrin and eYFP in the neonatal pancreas of control *R26^{eYFP}* mice and *Ptf1aCre⁺-Rosa26^{eYFP}* mice (A) or *Ngn3^{wt/TA}-tet-Ocre-R26^{eYFP}* mice (C). Gastrin expressing cells express the genetic tracer eYFP. Immunofluorescence stainings for menin, gastrin and glucagon in the neonatal pancreas of (B) *PancMen1 KO*. (D) *PancEndoMen1 KO*. Scale bars = 20 μ M. (E) Graph representing the percentage of gastrin⁺ cells which have lost menin expression at birth in the models analyzed. Scale bars= 20 μ M

Figure 3. *PancEndoMen1* KO mice develop tumors expressing gastrin. IHC stainings for the indicated factors in 12-month-old Control and *PancEndoMen1* KO mice. Note that the tumor located on the right express gastrin whereas the tumor on the left does not. Scale bar = 50 μ M

Figure 4. β *Men1* KO mice develop tumors expressing Gastrin. (A) Immunohistochemical detection of gastrin in 12-month-old control and β *Men1* KO mice. Gastrin is not expressed in normal islets of control mice. Most lesions in β *Men1* KO mice do not express gastrin (left), whereas subsets of tumors express Gastrin at high (middle) or low levels (right). Scale bars = 50 μ M. (B) Triple IF stainings for insulin, glucagon and gastrin in 12-month-old mice of the indicated genotype. Gastrin expressing cells in tumors from β *Men1* KO mice also express insulin. (C) IF staining for Pdx1, Gastrin and DAPI. The right-handed lesion do not express gastrin but express Pdx1 whereas the left-handed lesion express gastrin and Pdx1. (D) Graph representing the percentage of Ki67⁺ tumoral cells in insulin+gastrin- and insulin+gastrin+ tumors counted from both 12-month-old β *Men1* KO and *PancEndoMen1* KO mice. $n \geq 5$ tumors.

Supplemental Figure 1. Representative IHC stainings for gastrin and CCK in wild-type mouse pancreas and duodenum at birth. Scale-bar = 50 μ M

Supplemental Figure 2. Gastrin is not expressed in somatostatin⁺ cells at P0. Representative IF stainings for gastrin and somatostatin at birth in wild-type mouse pancreas. Scale-bar = 25 μ M

Figure 1

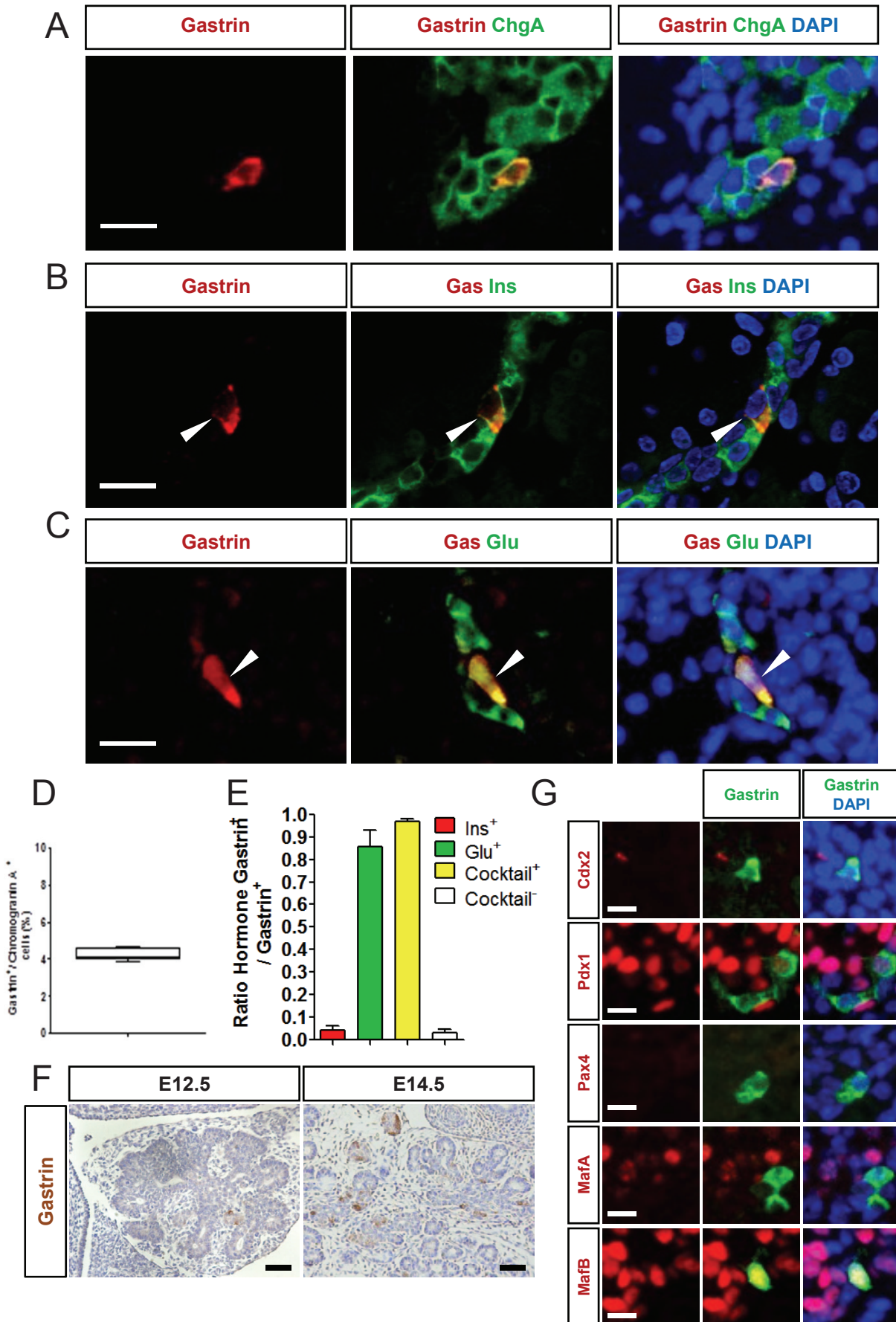


Figure 2

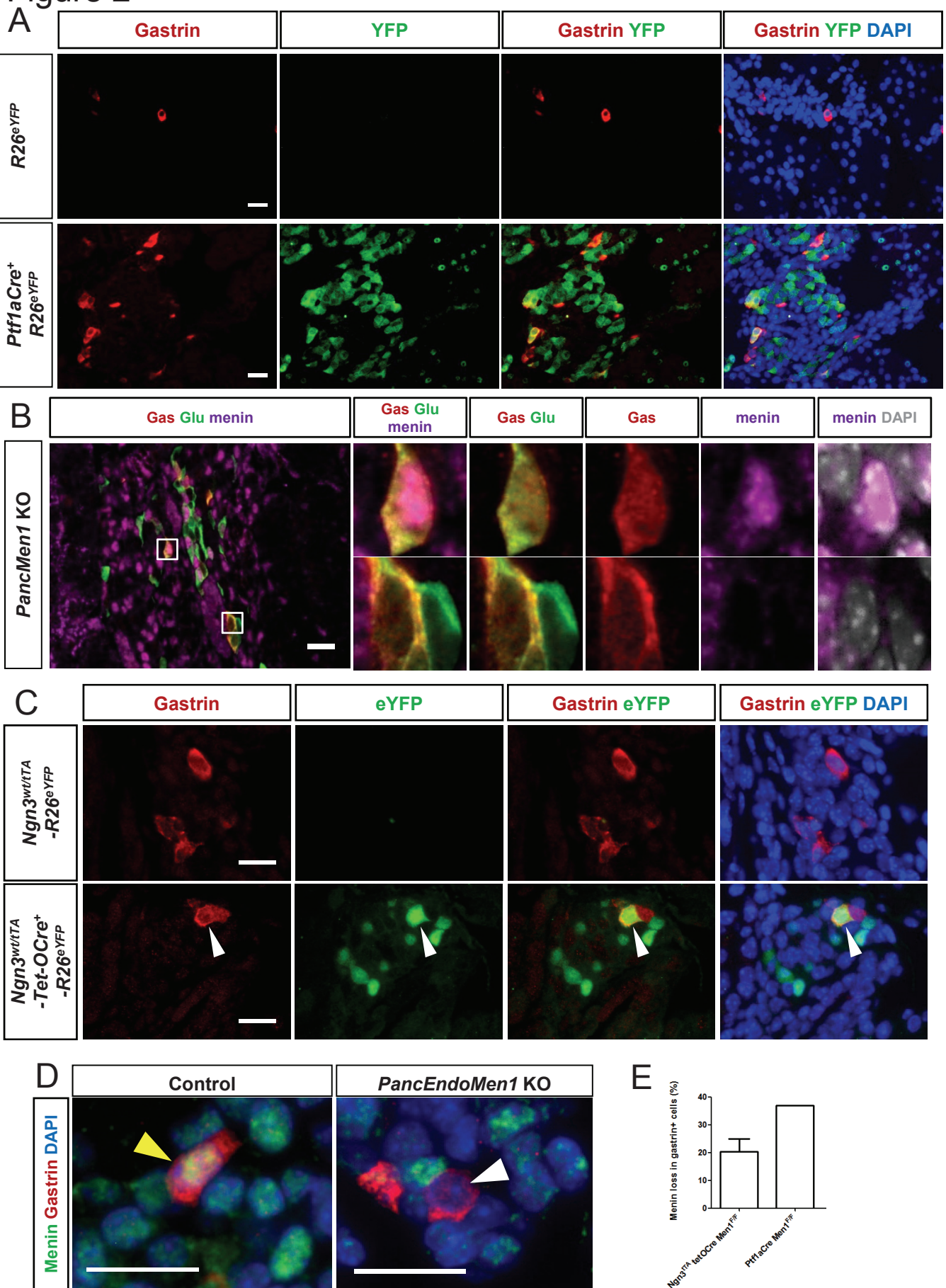


Figure 3

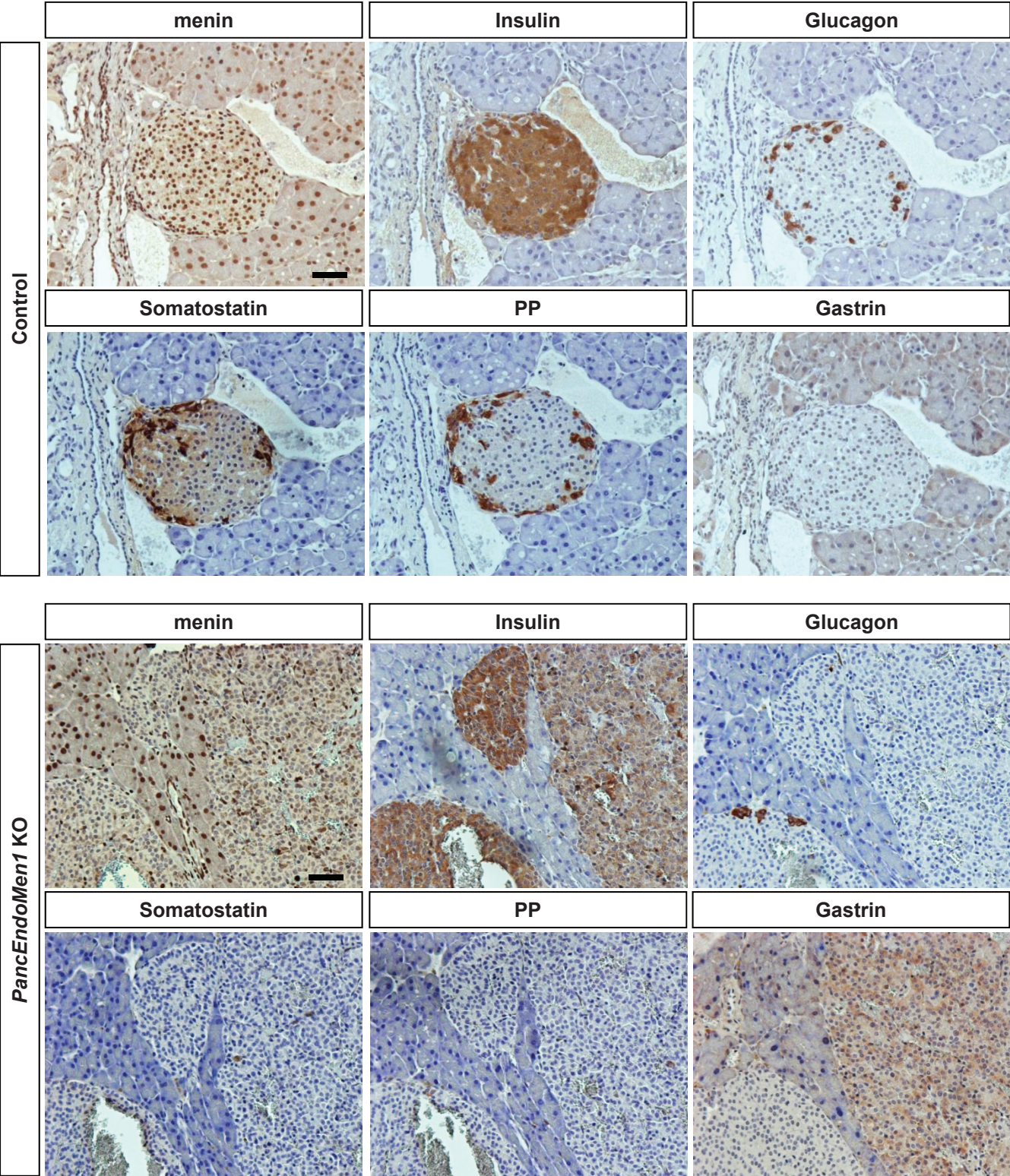
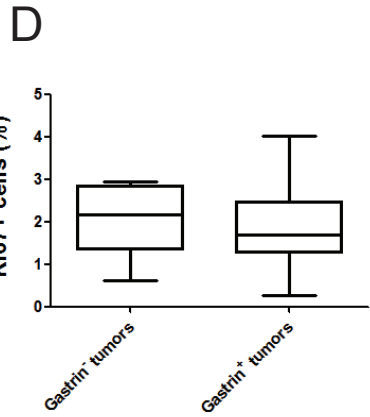
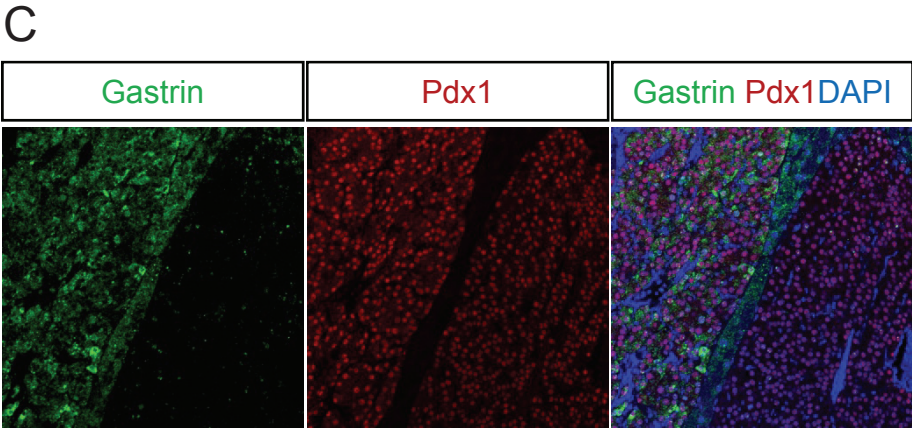
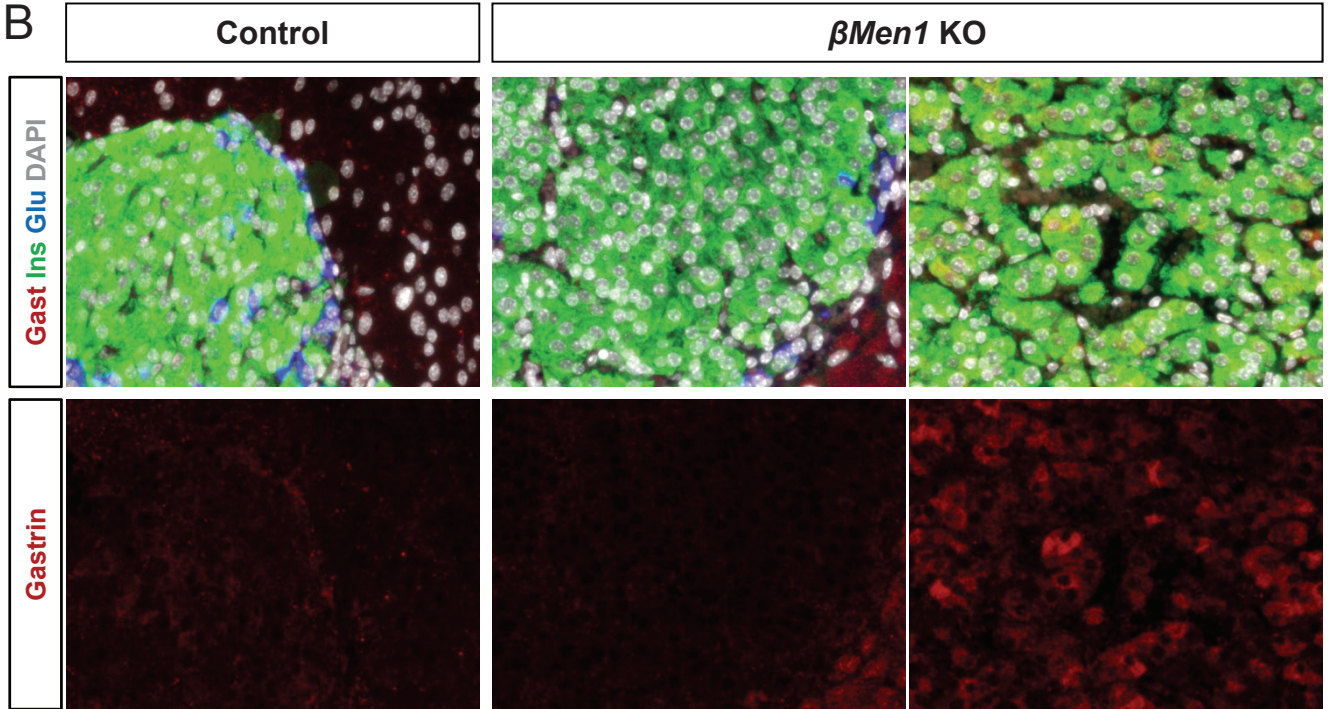
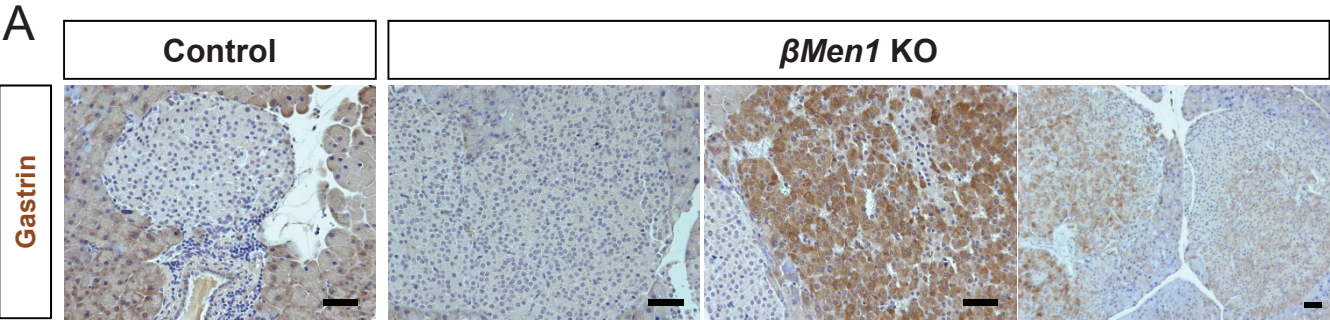
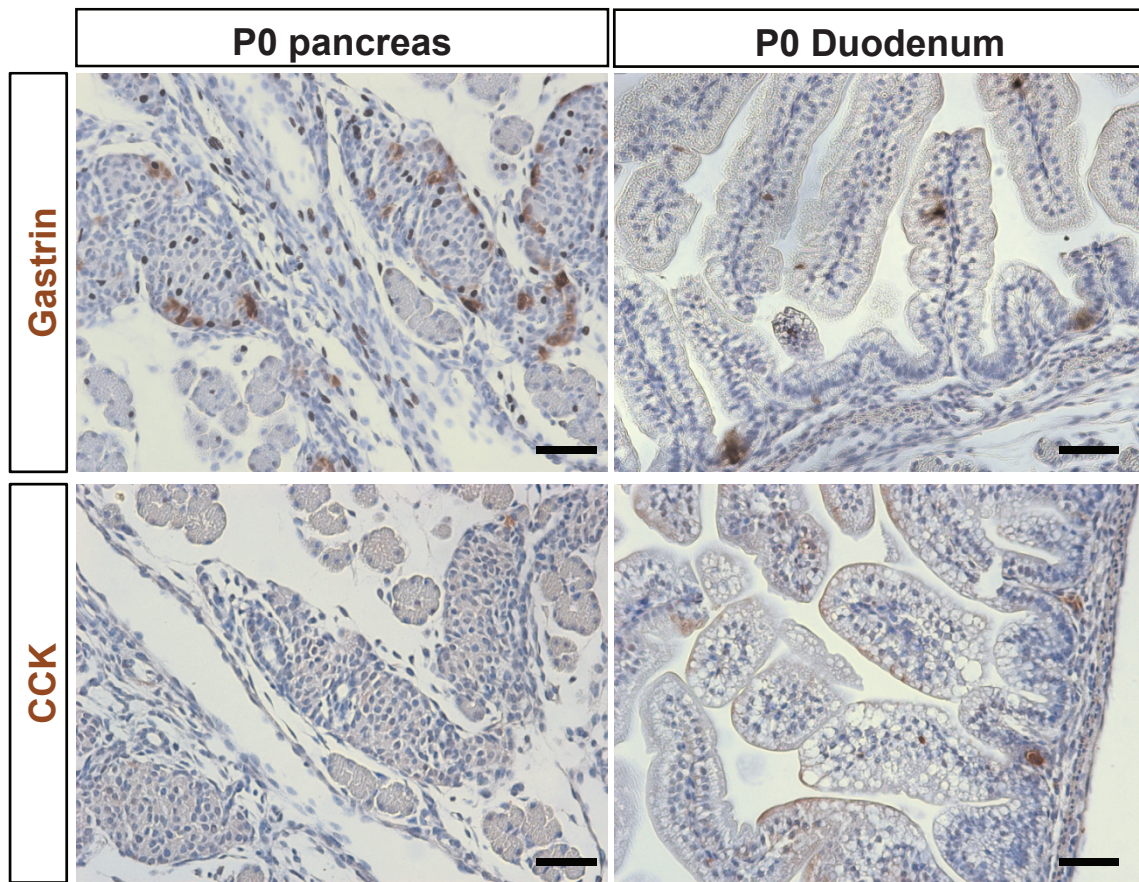


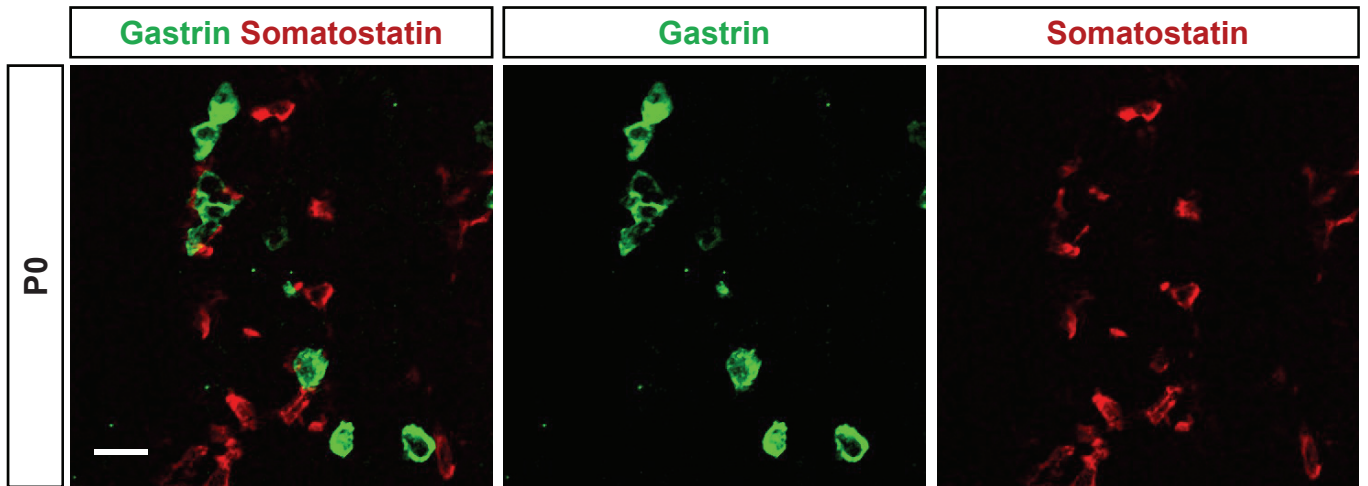
Figure 4



Supplemental Figure 1



Supplemental Figure 2



***Men1*-disruption in Ngn3⁺-progenitors reveals islet tumor heterogeneity both in tumor types and molecular alterations**

SHORT TITLE: Ngn3-progenitors and islet tumor heterogeneity

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ABBREVIATIONS: KO, knockout; MEN1, multiple endocrine neoplasia type 1; Ngn3, neurogenin 3; PCR, polymerase chain reaction.

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ABSTRACT limit 260 words

Background & Aims: Pancreatic neuroendocrine tumors (panNETs) are clinically complex tumors, with slow evolution but frequent metastasis at the time of diagnosis because of their often silent development. The endocrine tumor suppressor *MEN1* gene, encoding menin protein, is involved in several sporadic panNETs and those in patients with hereditary Multiple Endocrine Neoplasia syndrome Type I. To learn more about the complex nature of these tumors, especially concerning the occurrence of non-functional and multihormonal panNETs, we sought to investigate the consequences of *MEN1* inactivation in pancreatic progenitors and different derived pancreatic endocrine lineages.

Methods: We generated and studied new conditional Knock-Out (KO) mouse models where the *Men1* gene was disrupted in all pancreatic endocrine cells by targeting the pancreatic endocrine progenitors expressing Ngn3 and the pan-pancreatic progenitors expressing Ptf1a.

Results: Our results demonstrated that the mutant mice having *Men1*-deficient Ngn3⁺-progenitors resulted in differential cell proliferation alterations in different pancreatic endocrine cells. Importantly, *Men1*-disruption in either pancreatic endocrine or pan-pancreatic progenitors displayed tumors with impaired differentiation features. Moreover, distinct signaling pathways, including in particular ERK, were activated in a cell lineage-specific way among different islet cell tumors.

Conclusions: Targeting the *Men1* gene in pancreatic progenitors led to the development of heterogeneous islet tumors both at cellular and molecular levels. The data obtained from fine analyses of the models may afford useful insight into better understanding of tumor heterogeneity and finding new strategies of target therapy for islet tumors.

Keywords: neuroendocrine tumors, MEN, menin, proliferation, islet cells, ERK

INTRODUCTION

Pancreatic neuroendocrine tumors (panNETs) are seemingly simple but clinically complex tumors, with slow evolution but frequent metastasis at the time of diagnosis, because of their often silent development. The endocrine tumor suppressor *MEN1* gene, encoding menin protein, is involved in several sporadic panNETs and those in patients with hereditary Multiple Endocrine Neoplasia syndrome Type I bearing a germinal mutation on one allele of the gene. The second *MEN1* allele is almost always found inactivated by LOH in the tumors. In *MEN1* patients with panNETs, most frequent tumors are so-called non-functional, meaning that they are not characterized by the clinical symptoms due to hormone hypersecretion, even though they often produce pancreatic polypeptide (PP) or glucagon. Insulinomas represent the second most frequent panNETs in *MEN1* patients. On the contrary, it has been reported that the systematic analysis of whole pancreas from *MEN1* patients showed characteristic microadenomatosis, with a high frequency of glucagon expressing and multi-hormonal lesions (Anlauf, Schlenger et al. 2006). The cell and molecular bases of islet heterogeneity leading to non-functional and multi-hormonal tumor development are largely unknown, except cell transdifferentiation demonstrated in the context of alpha-cell specific *Men1*-inactivation (Lu, Herrera et al. 2010). The latter may explain at least partially some of the features of these tumors.

Pancreatic islet-cells biology is a widely investigated field nowadays, with most studies dedicated to the understanding of beta cell functions and pathology. In contrast, little is known about the biology of alpha, delta and PP cells. This is also true for *MEN1*-related panNETs affecting the latter cell-types, because the majority of basic research studies were devoted to the study of insulinoma biology and mechanisms involved, using mouse model with disruption of the *Men1* gene specifically in beta cells. The only exception would be heterozygous *Men1* knock-out (KO) model engineered by several laboratories that develops a

tumor spectrum mimicking the human disease (Crabtree, Scacheri et al. 2001, Bertolino, Tong et al. 2003, Loffler, Biondi et al. 2007). These mice develop whole range of panNETs including both insulinomas and glucagonomas. However, since panNETs develop randomly both in cell-type and timing in these mice, it renders this model difficult to work with, especially for dissecting the early event involved in tumor development following menin loss of expression.

To learn more about the complex nature of panNETs, especially concerning the occurrence of non-functional and multihormonal panNETs, it would be interesting to investigate the consequences of *MEN1* inactivation in pancreatic progenitors, combined with the comparison among different pancreatic endocrine lineages. Thus, we decided to generate and study new conditional Knock-Out (KO) mouse model where the *Men1* gene was disrupted in all pancreatic endocrine cells by targeting either the pan-pancreatic progenitors expressing *Ptf1a* or the pancreatic endocrine progenitors expressing *Ngn3*. These models allowed us to specifically study the effects of menin loss in pancreatic progenitors affecting simultaneously all pancreatic endocrine cells. Our results demonstrated that the mutant mice having *Men1*-deficient pancreatic progenitors displayed heterogeneous tumor phenotypes including islet tumors with dedifferentiation features. Our data also suggest a differential role of menin in different pancreatic endocrine cells in terms of cell proliferation and signaling pathways activated upon menin loss.

METHODS

-Animal study approval

All animal maintenance and experiments were performed in accordance with the animal care guidelines of the European Union and French laws and were validated by the local Animal Ethic Evaluation Committee (Protocol CLB 2010/016).

-Mouse studies

Men1^{F/F}-RIPCre⁺ (β *Men1* KO) mice {Bertolino, 2003 #45} and *Men1^{F/F}-GluCre⁺* (α *Men1* KO) mice were already described (Lu, Herrera et al. 2010). *Ngn3^{wt/tTA}* knock-in mice were generously provided by Dr Jan.N Jensen (Wang, Jensen et al. 2009). The *tetO-cre* mouse line (B6.Cg-Tg(tetO-cre)1Jaw/J MGI:2679524) was purchased from Charles River France. These two last mouse lines were crossed with *Men1^{F/F}* mice to generate *Men1^{F/F}-Ngn3^{tTA}-tetO-cre⁺* (*PancEndoMen1* KO) mice. For lineage tracing, *Rosa26^{eYFP}* mice {Srinivas, 2001 #44}, were crossed with previous mouse lines. Control mice used when comparing with *PancEndoMen1* KO mice were comprised of age-matched *Men1^{F/F}-Ngn3^{wt/tTA}*, *Men1^{+/+}-Ngn3^{wt/tTA}*, and *Men1^{+/+}-Ngn3^{wt/tTA}-tet-Ocre* mice. *PtflaCre* knock-in mice were generously provided by Dr Christopher V.E. Wright. All mouse lines were maintained in a mixed genetic background.

-Immunohistochemistry and immunofluorescence

Neonate and adult pancreases were fixed in Neutral buffered formalin (Lilly's fixative) for 2 hours at room-temperature or 18 hours at 4°C respectively, and then were embedded in paraffin. Serial section of 3µM were prepared, mounted on Superfrost Plus glass slides, and subjected to deparaffinization and rehydration. Endogenous peroxydases were quenched in 3% H₂O₂ solution for 30 minutes at room-temperature. Heat-induced epitope retrieval was performed by immersion in Antigen Unmasking Solution (H-3300, Vector) in a microwave oven. After blocking with antibody diluent (DAKO) sections were incubated overnight with a primary antibody. For immunofluorescence, stainings were revealed with Cy-3 or Cy-5 Tyramide amplification kit (PerkinElmer) according to the manufacturer's instructions with prior incubation with appropriate biotinylated secondary antibody, or incubated with appropriate Alexa488-555-or-647 coupled secondary antibodies (Life Technologies) for 1

hour. For immunohistochemistry (IHC), after 1st antibody incubation, biotinylated secondary antibody was applied for 1 hour and revealed using ABC-DAB system (Vector Lab).

-Antibodies.

Antibodies used in the study were, anti-glucagon (G2654, Sigma), anti-insulin (A056401, DAKO), anti-somatostatin (sc-7819, Santa Cruz), anti GFP recognizing eYFP (A-6455, Life Technologies), anti-Menin (A300-105A, A300-106A, Bethyl), anti-Ki-67 (sc-7846, Santa Cruz), and anti Erk1/2 phosphorylated on Thr202/Tyr204 (#4370, Cell Signaling). Donkey secondary antibodies coupled with either Alexa 488, 555, or 647 were used (Life technologies, Jackson Immunoresearch). For Immunohistochemistry or immunofluorescence with TSA, biotinylated secondary antibodies from horse or donkey were used (Vector Lab).

-Immunofluorescence analyses

Images were captured on a TCS-SP5 confocal microscope (Leica-Microsystems) or a Zeiss 780 confocal microscope. Cell counting was manually performed with ImageJ cell counter module (U. S. National Institutes of Health, Bethesda, Maryland, USA) on multiple channel pictures. Graphs and results of counting are represented as mean±S.E.M.

-Statistical analyses

Unpaired two-tailed Student's t test was applied to compare means.

RESULTS

Targeted *Men1*-disruption in Ngn3⁺-pancreatic endocrine progenitors.

The early effects of *Men1*-disruption in pancreatic endocrine cells has been only studied in beta cells. In order to study the effect of menin loss in Ngn3⁺-progenitors and simultaneously

in all pancreatic endocrine cell-types, we used a mouse model allowing targeted *Men1*-disruption in Ngn3^+ pancreatic endocrine progenitors. To generate this model, we crossed *Men1*^{F/F} mice with mice carrying a tetO-cre transgene and a recombined Ngn3 allele where the endogenous Ngn3 coding sequence was replaced by the tTA coding sequence (Figure 1A). *Men1*^{F/F}-*Ngn3*^{wt/tTA}-*tetO-cre*⁺ mice will be thereafter denominated as *PancEndoMen1* KO mice. We first ensured the specificity of the model by crossing *Ngn3*^{wt/tTA}-*tetO-cre*⁺ mice with *Rosa26*^{eYFP} mice allowing genetic lineage tracing of Cre recombinase activity. We looked at the expression pattern of eYFP at birth in the progeny of Ngn3 cells by immunofluorescence (IF) using an antibody directed against eYFP protein. As demonstrated in Figure 1B, eYFP was detected in islet like structures at birth, and colocalized with cells expressing either insulin, glucagon, or somatostatin. At birth, 25.9%±4.4 of insulin⁺ cells, 20.0%±6.0 of glucagon⁺ cells and 19.8%±4.3 of somatostatin⁺ cells expressed eYFP (Figure 1D). We next verified that the transgenic system allowed disrupting specifically the *Men1* gene in the pancreatic endocrine cells. To this end, menin expression was studied along with different pancreatic hormones in the pancreases of *PancEndoMen1* KO neonates. As shown in Figure 1C, menin expression was found lost in subsets of alpha, beta and delta cells. The quantifications revealed that 15.8%±3.3, 11.2%±3.0 and 13.2%±4.7 of insulin⁺, glucagon⁺ and somatostatin⁺ respectively had lost menin expression at birth (Figure 1E). Thus, the *PancEndoMen1* KO model allowed *Men1*-disruption in pancreatic endocrine cells with detectable menin loss at least at P0. We observed that the percentage of menin-negative cells in different pancreatic endocrine lineages is noticeably low compared with the percentage of eYFP-positive cells in the same cell populations (compare figure 1D and 1E).

After birth, in contrast to during embryogenesis, the formation of new endocrine cells from pancreatic endocrine progenitors Ngn3^+ no longer contributes significantly to islet mass, consistent with a rapid decrease in the number of Ngn3 expressing cells in the pancreas after

birth. However, pancreatic endocrine cells undergo a massive wave of postnatal proliferation, in order to generate a sufficient pool of endocrine cells necessary to the function of the endocrine pancreas in adult. Interestingly, at 6 weeks after birth, the proportion of menin deficient islet cells increased as compared to P0 (Figure 2). 67.4%±3.2 beta cells, 43%±2.1 alpha cells and 33.4±3.0 somatostatin cells displayed an absence of menin expression in 6-week-old *PancEndoMen1* KO mice (Figure 2B). Indeed, menin negative cell population over total islet cells increased by 4.2 times among beta cells, nearly 4 folds among alpha cells and by 2.5 fold among delta cells as compare to P0. The data demonstrated that in the absence of menin, the three major endocrine cell populations are able to expand during the period from P0 to 6 weeks after birth.

Pancreatic endocrine cell proliferation in the absence of menin.

Considering that neogenesis is not significant after birth, and that all endocrine cells of a given subtype (eg, beta (Brennand, Huangfu et al. 2007, Teta, Rankin et al. 2007), alpha or delta) are supposed to equally undergo cell proliferation to expand the endocrine cell mass after birth, the increased proportion of menin negative cells at 6 weeks of age suggests that menin deficient pancreatic endocrine cells may be already prone to a higher proliferation rate than their menin⁺ counterpart. Thus, we assessed the proliferation rate of menin⁺ and menin⁻ endocrine cells at P0 and at 6 weeks after birth, by performing co-staining of hormones, menin and Ki67, a proliferation marker. At P0, 9.4%±2.0 and 17.4%±4.9 of menin⁺ and menin⁻ beta cells respectively were stained for Ki67 (Figure 3 D). This twice increase was nevertheless not significant due to high heterogeneity between the mice. The proliferation of somatostatin⁺ cells at birth was null in most mice analyzed both in menin⁺ and menin⁻ cells. In contrast, menin⁻ alpha cells displayed a significant three-fold increased in Ki67⁺ as compared to menin⁺ alpha cells (Figure 3D; 18.1%±3.5 vs 5.8%±0.3).

At 6 weeks of age, however, the percentage of proliferative alpha and beta cells decreased in both $menin^+$ and $menin^-$ cell-populations as compared to P0 (Figure 3A, B, and E), consistent with the end of postnatal proliferation. We noticed that control mice displayed the same percentage of $Ki67^+$ staining between the alpha, beta and delta cell populations. Interestingly, $menin^-$ beta cells sustained a 4-fold higher proliferative rate as compared to $menin^+$ cells in the same mice and to control mice in which *menin* expression is not altered (Figure 3A and E). As at birth, $glucagon^+menin^-$ cells displayed a statistically significantly higher percentage of $Ki67^+$ cells (3 fold) as compared to their $menin^+$ counterparts in *PancEndoMen1* KO mice. However, there was no significant change when comparing with the percentage of alpha cells $Ki67^+$ in control mice (Figure 3B and 3D). In addition, mean percentage of proliferative alpha cells in the control mice ($1.9\% \pm 0.4$) was identical to that of combined $menin^+$ and $menin^-$ cells in *PancEndo Men1* KO mice ($1.5\% \pm 0.6$). Similarly, despite a tendency of *menin* deficient delta cell to be more proliferative, there was no significant change in delta cell proliferation in the absence of *menin* as compared to $menin^+$ delta cells from *PancEndoMen1* KO mice or delta cells in control mice.

Pancreatic endocrine tumor lesions in aged *PancEndoMen1* KO mice.

We next analyzed the pancreas of 12-month-old *PancEndo Men1* KO and control mice for tumor development. Control mice did not develop any islet lesions and staining for all four major hormones could be observed in islet by IHC as shown on Figure 4. On the contrary, *PancEndoMen1* KO mice developed numerous islet hyperplasia and tumor lesions that were negative for *menin* staining (see Figure 4 and Table 1). Of seven mice analyzed, all displayed islet adenomas. Consistent with the high percentage of *menin* deficient beta cells at 1 month and their increased proliferative rate as compared to control, most lesions in *PancEndoMen1* KO mice were $insulin^+$ (Table 1). Indeed, 78% of hyperplastic/dysplastic islets were $insulin^+$, whereas 15% were $glucagon^+$, 5% $somatosatin^+$ and only rare PP hyperplasia were observed

in *PancEndo Men1* KO mice pancreas at 12 months. Most adenomas were insulinomas and occurred in all mutant mice analyzed. Of 27 adenomas observed, only 3 were alpha cell tumors, and no mice developed delta or PP cell tumor. As shown in Figure 4, alpha cell lesions were often associated with beta cell lesions and thus formed mixed tumors.

Importantly, it is noted that beta cell lesions showed a strongly reduced insulin staining as compared to normal islet (Figure 4), consistent with previous reports (Bertolino, Tong et al. 2003). In addition, by 13 months, *PancEndoMen1* KO mice had insulin expressing tumors displaying a strong heterogeneous loss of insulin staining that was not positive for the other hormone tested (Figure 5A). Consistently, when observing early lesions developed by 4.5 month-old mutant mice with specific *Men1*-disruption in pancreatic *Ptf1a*⁺ progenitors (*PancMen1* KO mice), we could observe that insulin expression was markedly decreased as compared to their control littermates (Figure 5B).

Differential activation of proliferative pathways in postnatal menin deficient islet cells.

Postnatal islet cell proliferation is regulated by several pathways, including ERK signaling pathway (Chen, Gu et al. 2011). It was shown that ERK is essential for beta cell mass expansion after birth and that activation of ERK dramatically decrease after weaning and is mostly absent in adult islets. In addition, it has been described that menin represses ERK activation and activity in non-endocrine cell lines in vitro (Gallo, Cuozzo et al. 2002, Gao, Feng et al. 2011). Thus, we sought to determine if mechanisms controlling postnatal islet mass expansion could be reactivated in mouse *Men1* islet-tumors in our *PancEndoMen1* KO mice. To this end, we examined the activation of ERK signaling using antibodies directed against phosphorylated Thr202/Tyr204 ERK1/2 which reflect ERK activation. In control mice, most islets did not show any IF staining for phospho-ERK1/2, as shown on Figure 5. Interestingly, the majority of beta cell hyperplasia/dysplasia and beta cell tumors did not show

obvious ERK activation (Figure 5), except that some insulinomas displayed focally strong phospho-ERK staining, always in restricted areas in periphery of tumors. Remarkably, alpha cell lesions consistently showed activation of ERK that could be detected both in the cytoplasm and nucleus (Figure 5). Interestingly, by analyzing pancreas from $\beta Men1$ KO mice of the same age (12 months), tumoral cells never showed ERK activation, the latter being restricted to endothelial cells (Figure 5). We next sought to confirm if ERK pathway was already expressed in early alpha cell lesions that develop in $\alpha Men1$ KO mice. As shown on Figure 6, phospho-ERK was not detected in islets from 3.5 months old control mice, consistent with the previous report from Chen and colleagues. In contrast, alpha cells from 3.5-month-old $\alpha Men1$ KO islets displayed strong activation of ERK in the absence of menin (see Figure 6). Interestingly, some alpha cells that had not yet lost menin expression occasionally expressed phospho-ERK.

DISCUSSION

By targeting the *Men1* gene in $Ngn3^+$ -progenitors, we were able to study the biological consequences of *Men1*-disruption both in pancreatic endocrine progenitors and in all derived endocrine lineages. Our data demonstrate that *Men1*-disruption in $Ngn3^+$ -progenitors resulted in the development of a range of islet tumors having various features, recapitulating complex clinical tumor heterogeneity of panNETs. Importantly, different islet tumors displayed distinct altered molecular signaling pathways, which could be the molecular basis of tumor heterogeneity.

***Men1*-disruption in $Ngn3^+$ -progenitors gives rise to the development of a range of islet tumors**

PancEndoMen1 KO mice develop several types of pancreatic endocrine tumors, with the majority being insulinomas. We found that adenomas producing glucagon were quite rare as

compared to beta cell adenomas, whereas substantial alpha cell hyperplasias could be detected, a feature that has been described in pancreas from MEN1 patients (Perren, Anlauf et al. 2007). The difference in the proportion between alpha and beta cells in the pancreas and different proliferative rate may explain the low number of alpha cell tumors as compared to beta cell tumors. However, we described that alpha cell transdifferentiation upon menin loss can participate in the development of insulin expressing tumors in mice when alpha-cells are specifically targeted (Lu, Herrera et al. 2010). Such a mechanism may also occur in the present *PancEndo Men1* KO model, and may perhaps not be restricted to alpha cell, and potentially affect delta cells. Further analyses, including in particular co-immunostainings for somatostatin and insulin or glucagon and insulin, should be performed, in order to better appreciate the importance of such mechanisms in islet tumorigenesis in different contexts.

The most marked different tumor features observed in *PancEndo Men1* KO mice is the low and non-homogenous insulin-expression pattern of islet tumors, suggesting altered differentiation status of islet tumors. The mechanism involved has not been addressed in the current study. We recently demonstrated that MafB, normally absent from adult beta cells but necessary for beta cell differentiation during embryogenesis, is reexpressed in early *Men1*-deficient beta cells in *Men1^{F/F}-RipCre⁺* (β *Men1* KO) mice (Lu, Hamze et al. 2012). Thus, it seems that menin loss in pancreatic progenitors may possibly affect pancreatic endocrine cell differentiation, in particular the maturation of beta cells, due to altered expression of transcription factors involved in the differentiation and/or maturation of beta cells, such as MafB and or MafA. It is still not known if insulin down regulation in tumors is a direct consequence of *Men1* disruption in pancreatic endocrine progenitors or if it is indirectly due to tumor progression. More early detailed analysis and comparison with the β *Men1* KO mice may be helpful to decipher the exact role of menin in regulating the maintenance of the differentiation status of beta cells.

We did not observe tumors with more than 2 to 5% somatostatin expressing cells, which is in accordance with the rare occurrence of somatostatinomas or non functional tumors producing somatostatin in pancreas of MEN1 patients. This phenomenon may possibly be explained by a low proliferation capacity of delta cells upon menin loss. We noticed that this is not in accordance with what has been reported by a recent paper, describing which demonstrated that scarce delta cells in tumors developed in heterozygous *Men1*^{+/-} mice have a proliferative rate similar to tumoral beta cells within the same mutant mice (Walls, Reed et al. 2012).

***Men1*-disruption in Ngn3⁺-progenitors results in differential effects in postnatal cell proliferation among islets cell lineages**

Only limited studies addressed the early effects of *Men1* disruption in pancreatic endocrine cells. It has been demonstrated that *Men1* disruption in adult islet cells acutely enhance their proliferation at least 7 days after conditional ablation (Schnepp, Chen et al. 2006, Yang, Gurung et al. 2010). However, we still do not know what the consequences of an early loss of *Men1* during islet cell development would be. Our study demonstrates that pancreatic Ngn3⁺-progenitors are sensitive to tumor development upon menin loss. We studied more specifically the early effects of *Men1*-disruption by analyzing cell proliferation at birth during final maturation of islet cells and at the beginning of the normal massive wave of islet cell proliferation. Consistent with previous report (Zhang, Feng et al. 2006), we observed that nearly 9% of insulin⁺ menin⁺ cells stained for Ki67. However, in menin deficient beta cells, we only observed a non-statistically significant twice increase in Ki67 staining as compared to their menin⁺ counterpart. The absence of a significant increase in menin-deficient beta cell proliferation at birth could be explained by a differential role of menin in beta cells during development, postnatal period and in adult. Such differential role has been documented for other factors such as p27. Indeed, p27 null mice display a strong

proliferation of endocrine cells during development before birth, whereas p27 loss does not affect post-natal proliferation (Georgia and Bhushan 2006). *Men1* disruption thus, may not affect proliferation of beta cells during postnatal period, since proliferative mechanisms normally repressed by menin may have already been activated during this period.

In parallel, the fact that only alpha cells are more proliferative in the absence of menin may also be explained by the model used. Indeed, it has been shown that differentiation of the different pancreatic endocrine cell-types from Ngn3 progenitors relies on different sequential competence windows during development (Johansson, Dursun et al. 2007). Ngn3 expressing progenitors first give rise to alpha cells, whereas most beta cells develop later during development with delta cells being the last lineage appearing from Ngn3 progenitors during development. Thus, considering that after recombination of the *Men1* locus, the wild-type *Men1* mRNA and protein have to be completely degraded in order to affect the cell, alpha cells menin deficient are more likely to have lost menin expression for a longer time than other cell-types. In this case, the effect on the proliferation of beta and delta cells may appear later after P0. More precise conclusion may be drawn by analyzing mice older such as P7 or P15 or by using other models allowing earlier and more efficient *Men1* disruption in pancreatic progenitors using *Ptf1a-Cre* or *Pdx1-Cre* mice.

In contrast to what seen at P0, alpha and beta cells from both control and mutant 6-weeks-old aged mice had a strong decreased proliferation, whereas delta cell proliferation was increased (compare Figure 3D and 3E). It has to be noted that at this age, the three endocrine cell types analyzed (alpha beta and delta) had the same rate of proliferation in control mice, indicating that they have the same proliferative potential at least at 6 weeks. The fact that total alpha cell proliferation from *PancEndo Men1* KO mice (comprising menin deficient and menin expressing cells) is similar to that of control mice suggest that the mechanism of negative control may be activated to restrict the expansion of alpha cell mass. However,

menin deficient alpha cells, significantly more proliferative than menin-expressing alpha-cells in the same mice showed that the former is capable of escaping such a negative control, leading to alpha cell lesions in old mice.

The development of pancreatic endocrine cells is potentially impaired by *Men1*-disruption in Ngn3-progenitors

Our data showed that the proportion of menin-negative cells within each islet cell lineage is somehow low in the mutant mice at P0, when compared with the targeting efficiency shown by eYFP expression. Markedly, this proportion increased 2.5 – 4 folds at 1 month of age. Considering the increased cell proliferation rate observed in menin-deficient cells at P0, which is the most prominent in alpha-cells (3-fold increase), one would expect an increased, or at least equal proportion of menin-negative islet-cells when compared with targeting efficiency. The low proportion of menin-negative islet-cells seen in the mutant mice may reflect, therefore, a growth disadvantage of menin-deficient cells during the development. Indeed, we have previously reported that *Men1-null* cells had developmental defects. Although the current work addressed the effects of *Men1*-disruption in the rather later stages of development, it provides novel clues suggesting that menin is potentially required for the normal development of pancreatic endocrine cells from Ngn3-progenitors. However, much more detailed analysis of embryonic phenotype of PancEndoMen1 KO mice are required to exactly determine if menin loss could trigger differentiation defect from Ngn3+ progenitors or apoptosis, or decreased proliferation of differentiating endocrine cells.

Islet cell lineage-specific alteration of cell signaling pathways upon *Men1* inactivation

Many studies suggest that menin possesses various molecular and biological functions, including in particular its interaction with several transcriptional factors and regulators indicating its role in gene expression regulation. As a tumor suppressor, menin regulates the

proliferation of endocrine cells both in vitro and in vivo. However, the specific pathways and mechanism deregulated in affected cells in the absence of menin were mainly studied in beta cells, and remain poorly understood. Our results uncovered for the first time the activation of ERK1/2 in *Men1*-deficient alpha cells, whereas such activation did not occur in a model targeting specifically the *Men1* gene in beta cells (*βMen1 KO*). Interestingly, in the *PancEndo Men1 KO* model, even though most of insulin expressing tumors did not show ERK activation, both alpha-cell lesions and some clusters of insulin⁺ cells within beta cell tumors displayed increased phosphor-ERK1/2. The latter was also observed in some beta cell tumors from heterozygous *Men1*^{+/-} mice (data not shown). Such a differential activation between alpha cell and beta cell tumors suggest that distinct mechanism of proliferation/survival may be involved in the tumor development among different cell-types. It is still not known whether beta cell tumors with focal ERK activation are due to the earlier loss of menin during beta cell differentiation as compared to the *betaMen1 KO* model. Since alpha cell neoplastic lesions displayed ERK activation in *alphaMen1 KO* model, one could ask whether beta cell tumors displaying ERK activation may represent tumors that developed from transdifferentiation of alpha cells into beta cells. Further work is needed to clarify the issue.

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Table 1. Tumoral lesions developed in *PancEndoMen1* KO mice.

	Hyperplasia/Dysplasia	Adenoma	ADK	TOTAL
Insulin	160 (7/7)	24(7/7)	1(1/7)	185
Glucagon	32(7/7)	3 (2/7)	0 (0/7)	35
Somatostatin	11(3/7)	0 (0/7)	0 (0/7)	11
PP	3(1/7)	0 (0/7)	0 (0/7)	3
TOTAL	206	27	1	234

(Numbers in brackets represent the number of mice with lesions/total mice analyzed.

ADK: adenocarcinoma)

FIGURE LEGENDS

Figure 1. Targeted disruption of the *Men1* gene in pancreatic endocrine progenitors. (A) Schematic representation of the *Men1^{F/F}-Ngn3^{wt/tTA}-tetO-cre* model allowing recombination of the *Men1* floxed alleles specifically in Ngn3 expressing cells. (B) Representative images of IF analysis of eYFP expression for lineage tracing of Cre activity in wild-type *Men1 Ngn3^{wt/tTA}-tetO-cre-R26^{eYFP}* mice. Pancreas of neonate (P0) mice were analyzed. Triple IF were performed to analysis the colocalisation of eYFP and DAPI with either insulin, glucagon or somatostatin. Scale bars 25µM. (C) Representative images of IF analysis of menin expression in hormone expressing cells in the pancreas of control and *PancEndoMen1* KO neonate at birth. Scale bars 25µM. (D) Graph representing the average proportion ±S.E.M of eYFP expressing cells in the indicated hormone expressing cells from wild-type *Men1 Ngn3^{wt/tTA}-tetO-cre-R26^{eYFP}* mice. n ≥ 3 mice. (E) Graph representing the average proportion ±S.E.M of menin loss in the indicated hormone expressing cells in the pancreas from *PancEndoMen1* KO at P0. n ≥ 3 mice.

Figure 2. Increased proportion of menin deficient pancreatic endocrine cells in 6 week-old *PancEndoMen1* KO mice. (A) Representative images of IF analysis of menin expression in insulin and somatostatin expressing cells in the pancreas of 6-week-old control and *PancEndoMen1* KO mice. Middle and left panels represent a magnified view of the corresponding insets. (B) Representative images of IF analysis of menin expression in glucagon expressing cells in the pancreas of 6-week-old control and *PancEndoMen1* KO mice. Middle and left images represent a magnified view of the corresponding insets. (C) Graph representing the average proportion ±S.E.M of menin loss in the indicate hormone expressing cells in the pancreas from 6 week-old *PancEndoMen1* KO mice. n ≥ 3 mice.

Figure 3. Differential proliferation triggered by menin loss in alpha, beta and delta cells at birth and young adults. Representative images of IF analysis of menin and Ki67 with insulin (A), glucagon (B), or somatostatin (C), expression in the pancreas of 6-week-old control and *PancEndoMen1* KO mice. The three right hand panels images are a magnified view of the corresponding inset. Scale bars 25 μ M. (D) Graph representing the average proportion \pm S.E.M of Ki67⁺ cells among the indicated hormone⁺ menin⁺ or hormone⁺ menin⁻ cell populations in the pancreas from *PancEndoMen1* KO at P0. n \geq 3 mice. *, p \leq 0.05 using unpaired Student's t test. (E) Graph representing the average proportion \pm S.E.M of Ki67⁺ cells among the indicated hormone⁺ cell populations in controls and Ki67⁺ cells among the indicated hormone⁺ menin⁺ or hormone⁺ menin⁻ cell populations in the pancreas from *PancEndoMen1* KO at 6 weeks of age. n \geq 3 mice. *, p \leq 0.05; **, p \leq 0.01 using unpaired Student's t test.

Figure 4. Pancreatic endocrine lesions in aged *PancEndoMen1* KO mice. Representative IHC stainings for menin, insulin, glucagon, somatostatin and PP in the pancreas of 12-month-old control or *PancEndoMen1* KO mice. For *PancEndoMen1* KO mice, several different lesions are displayed.

Figure 5. (A) Representative IHC stainings for menin, insulin, and glucagon in the pancreas of 13-month-old control or *PancEndoMen1* KO mice. Inserted panels are a magnified view of insulin stainings showing decreased insulin expression in subsets of tumoral cells in *PancEndoMen1* KO mice. (B) Representative IHC stainings for menin, insulin, and glucagon in the pancreas of 4.5month-old control or *PancMen1* KO mice. Inserted panels are a magnified view of menin stainings. Scale bars 50 μ M.

Figure 6. Differential ERK activation in menin deficient tumoral lesions. Representative IF staining for glucagon, insulin and phospho-ERK1/2 in control (A), mixte alpha-beta cells tumor (B), and insulinoma (C), from 12-month-old *PancEndo Men1* KO mice, or insulinoma

from 12-month-old *βMen1* KO mice (D). Scale bars 25μM. (E) Representative IF staining for menin, glucagon, and phospho-ERK1/2 in pancreas from 3.5-month-old control or *αMen1* KO mice. Lower panels are the magnified view of the corresponding insets. pERK = ERK phosphorylated.

Figure 1

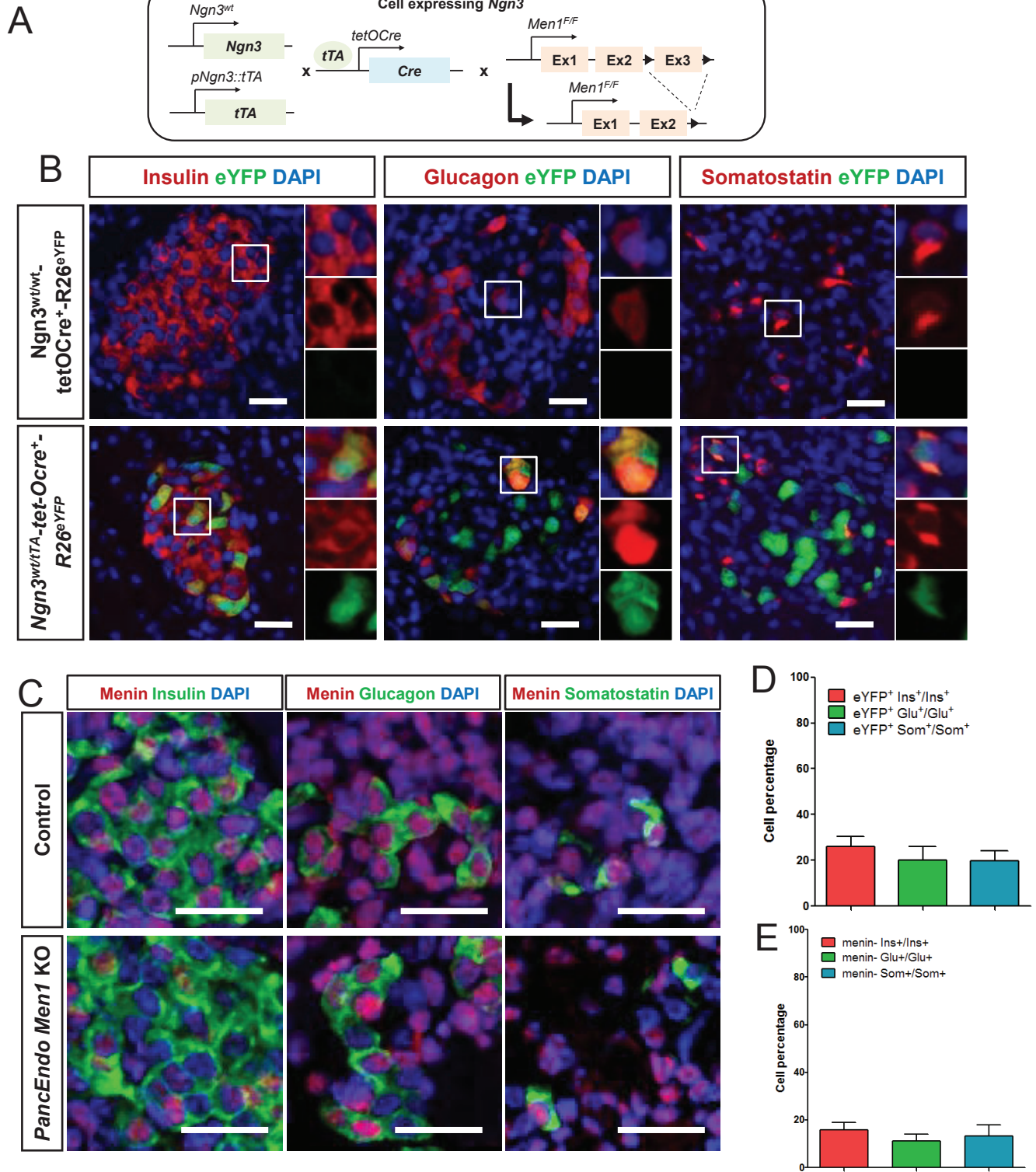
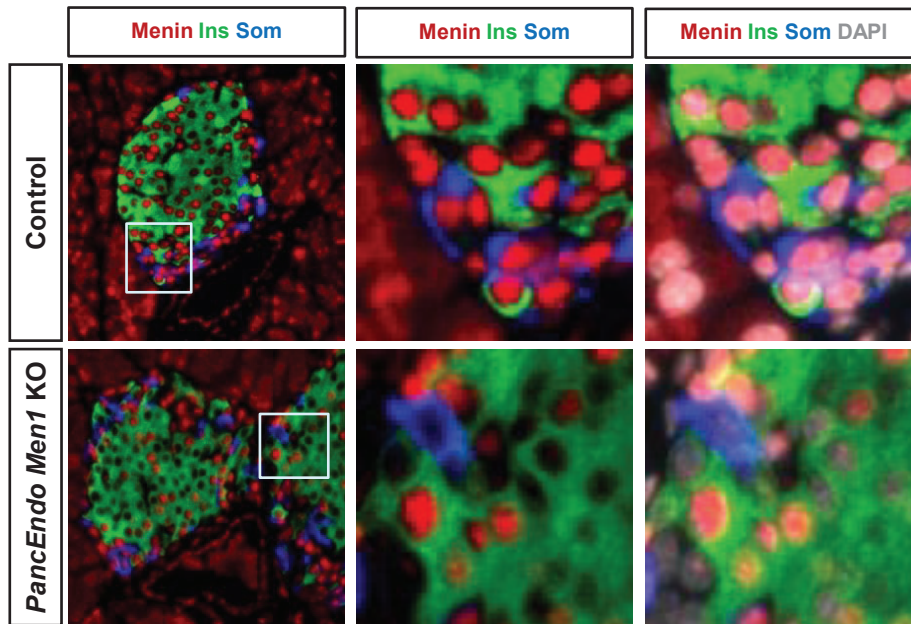
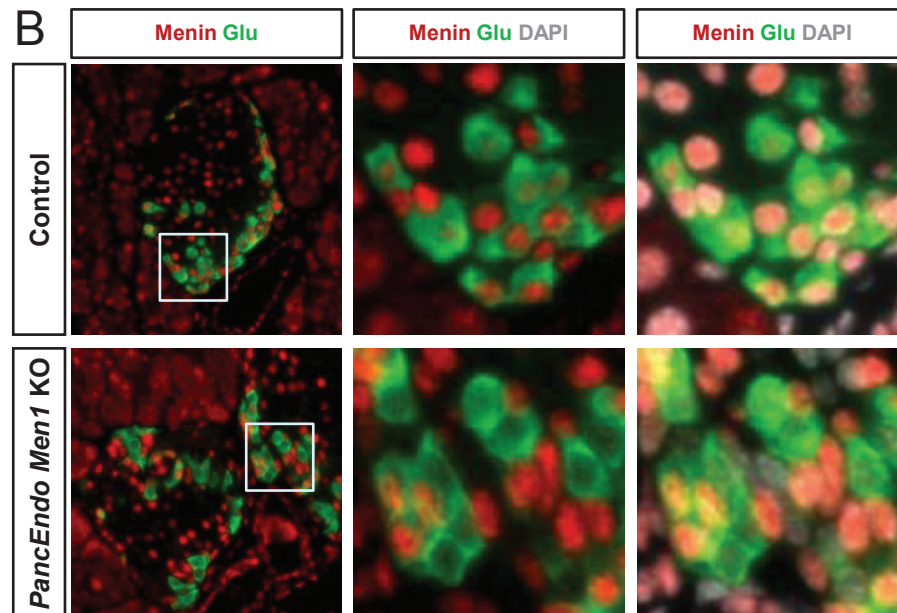


Figure 2

A



B



C

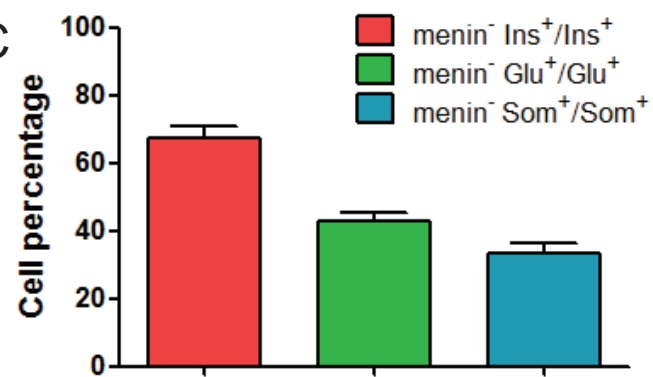


Figure 3

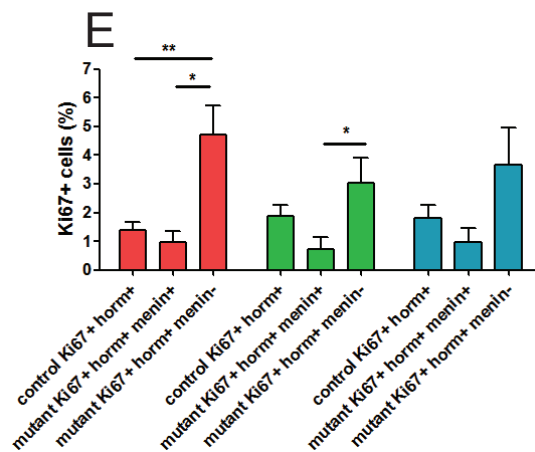
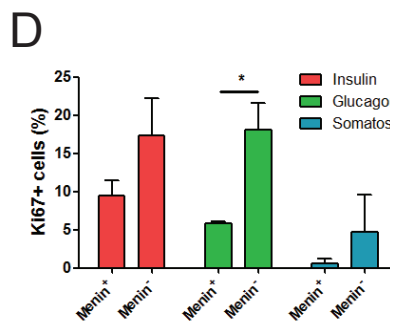
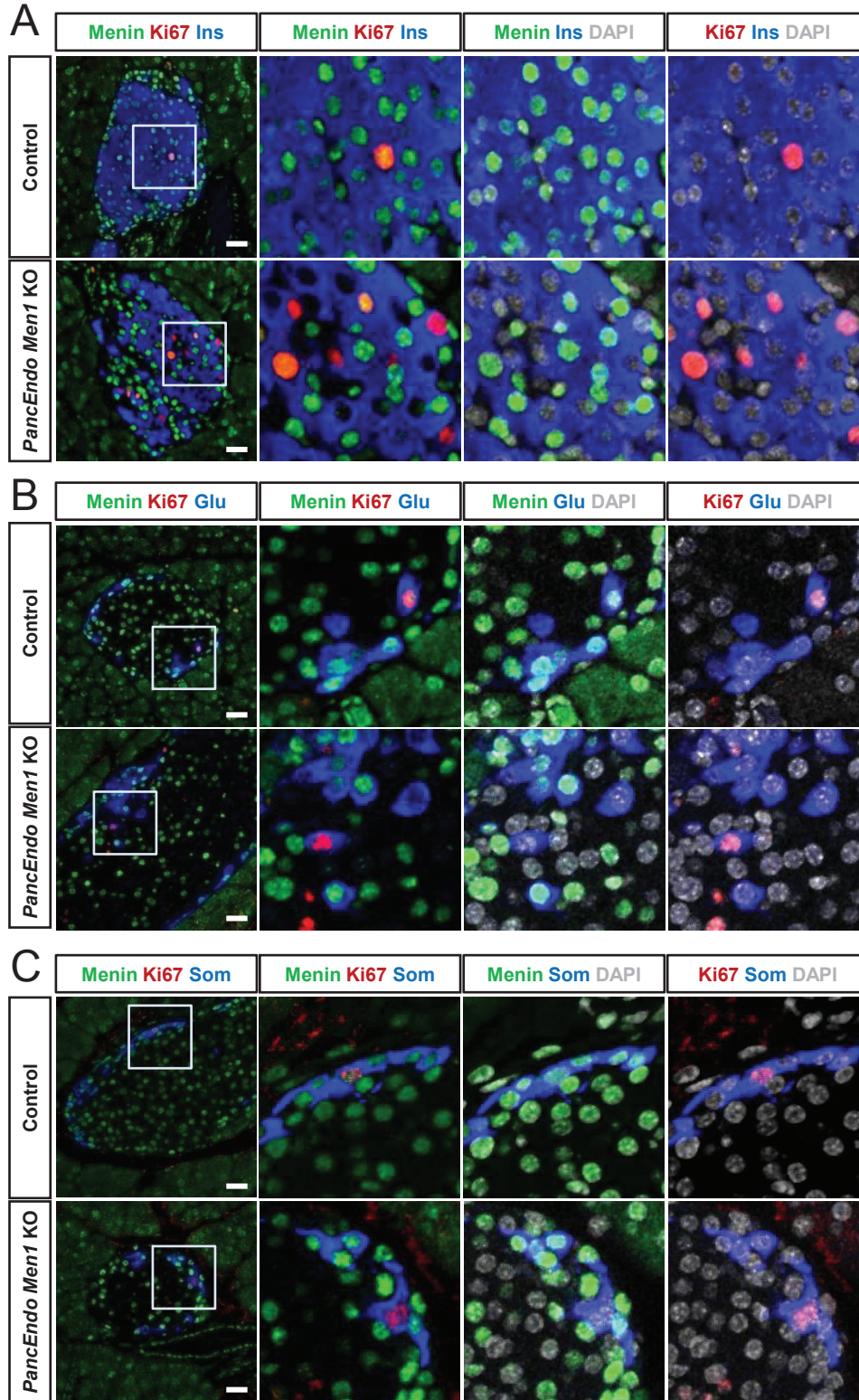


Figure 4

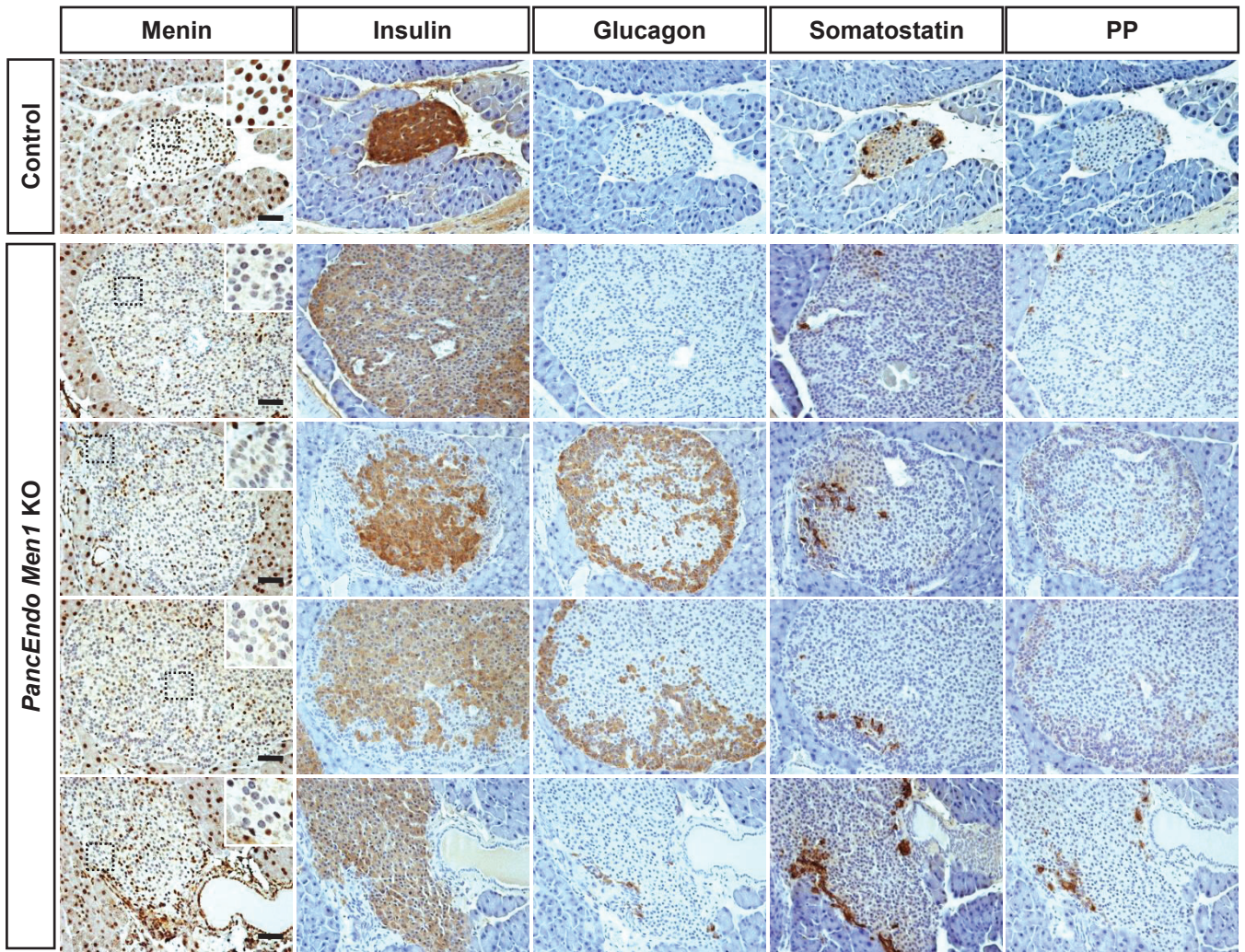


Figure 5

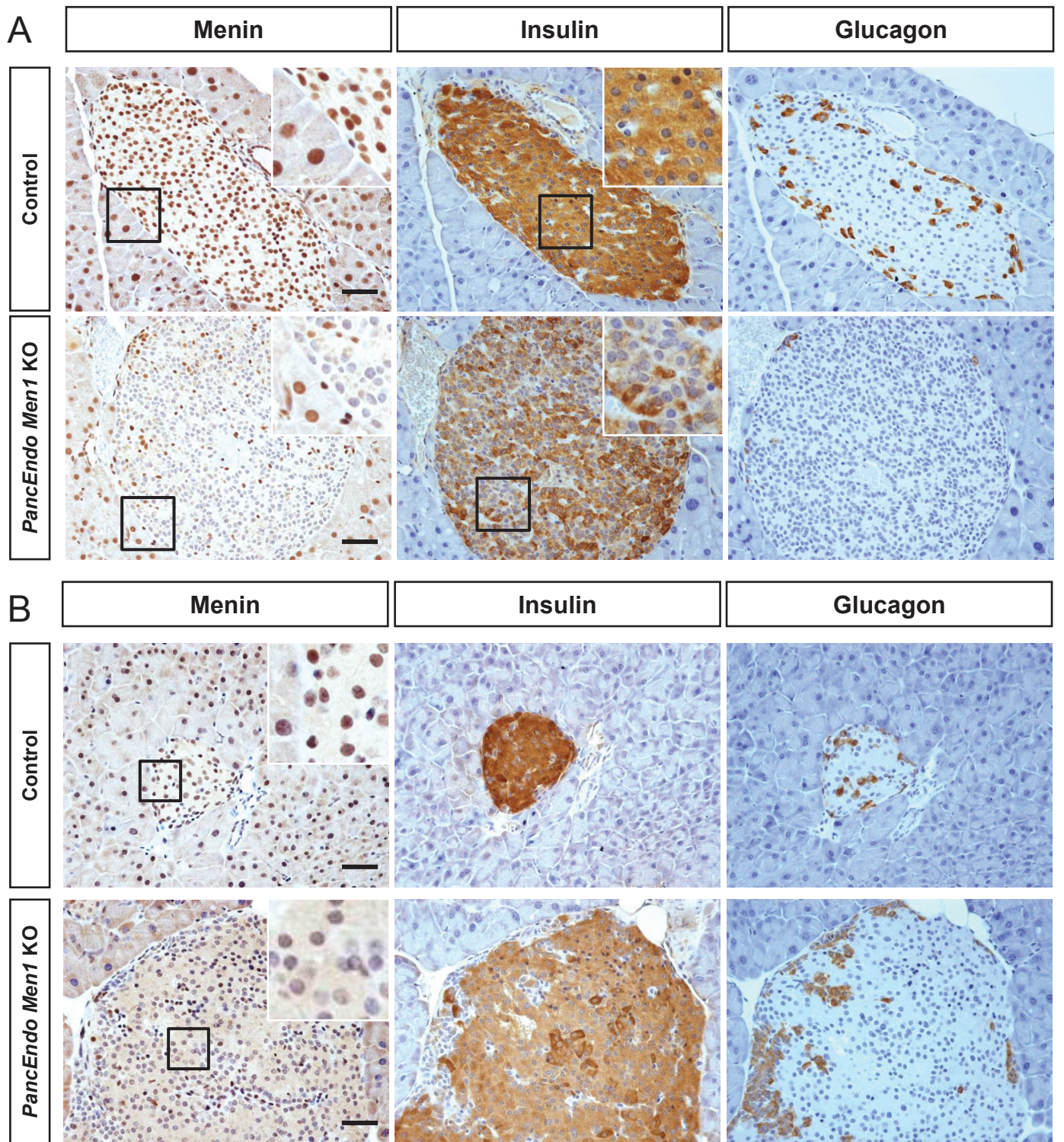
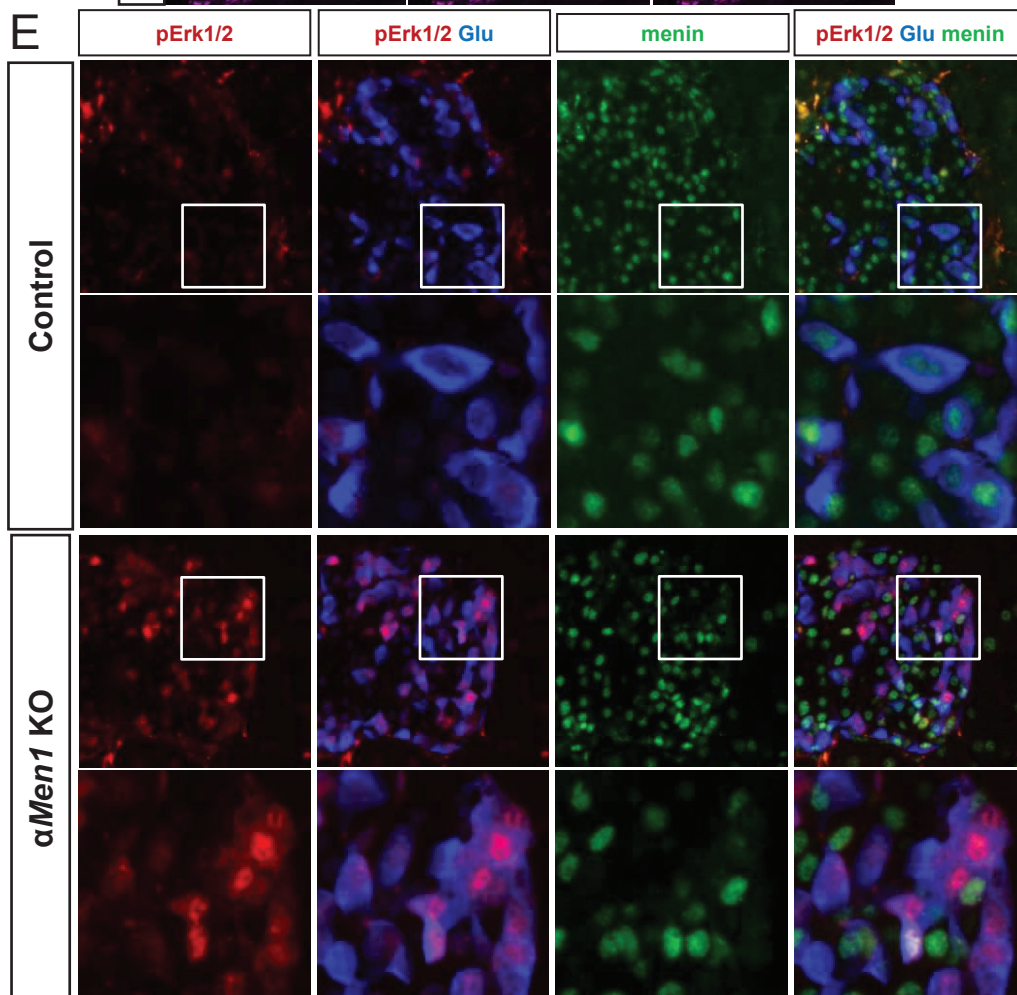
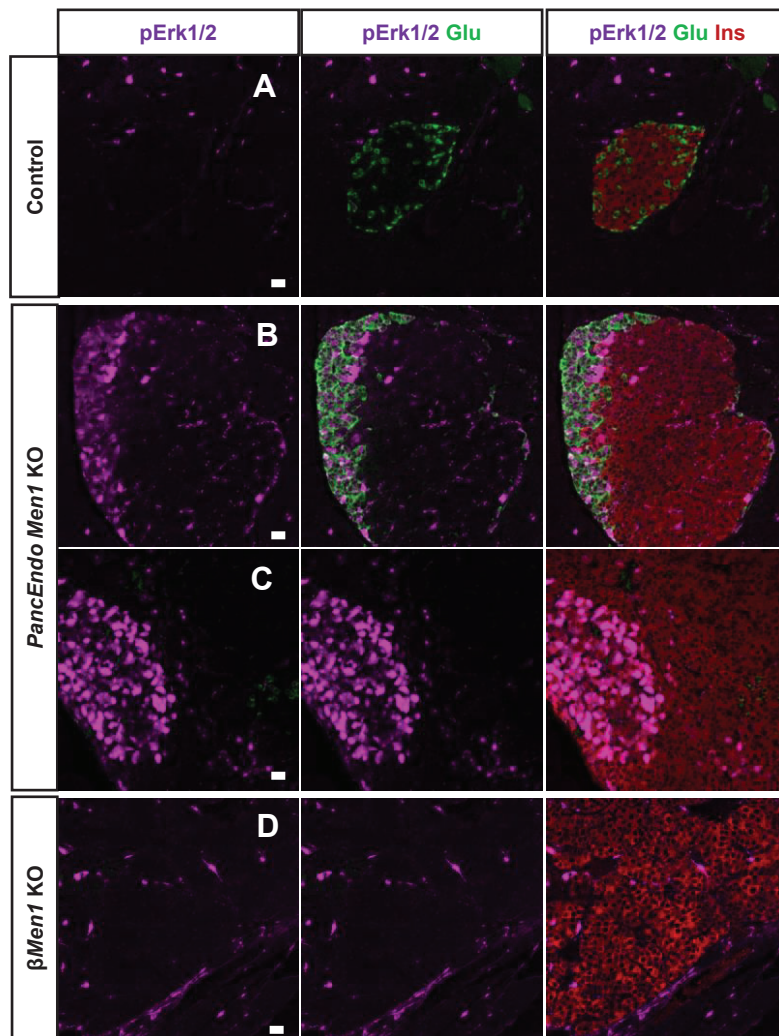


Figure 6



DISCUSSION

DISCUSSION.

Pancreatic gastrin expressing cells: where do they come and where they may go?: *Gastrin expression in the mouse developing pancreas*

3 DISCUSSION

3.1 PANCREATIC GASTRIN EXPRESSING CELLS: WHERE DO THEY COME AND WHERE THEY MAY GO?

3.1.1 GASTRIN EXPRESSION IN THE MOUSE DEVELOPING PANCREAS

During this thesis, we confirmed and further detailed the presence of gastrin expressing cells in the developing and neonate mouse pancreas, their existence having been already described in several mammalian species including human. We demonstrated that gastrin expressing cells are in fact subsets of both glucagon and insulin producing cells. This observation is reminiscent of what has been described for ghrelin expressing cells that mostly colocalize with glucagon expressing cells during development and postnatal period. However, it has also been demonstrated that a unique endocrine population called Epsilon cells exist in the pancreas, and these cells do not express common pancreatic endocrine hormones. Our current study did not allow determining whether mono-hormonal gastrin-expressing cells also exist in developing and neonate, besides the double gastrin/glucagon or gastrin/insulin expressing cell populations. This could be achieved by performing colocalization experiment with anti-gastrin antibodies and a cocktail of anti-4 majors pancreatic hormones (eg:insulin, glucagon, somatostatin, and PP).

Furthermore, the Epsilon cell population and ghrelin expression in alpha cells disappear shortly after birth in the mouse, just like what we and other demonstrated for gastrin. However, in human, it has been reported that ghrelin could keep on being expressed in alpha cells in adult pancreas (Raghu et al. 2013), even though different results were obtained in a previous study (Andralojc et al. 2009). The data from the literature and our lab failed to identify gastrin expression in normal post-weaning or adult human pancreas, though we cannot absolutely exclude the presence of a really rare scattered gastrin-expressing cell population that may persist during the adult life. However, we are not certain whether some alpha cells that express ghrelin during development and postnatal period could also express gastrin. Indeed, our analyses were hampered by the fact that our antibodies against gastrin and ghrelin were all raised in rabbit preventing us to perform co-immunostainings.

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In addition, we still don't know if all alpha cells express gastrin at one time or another during their differentiation or if only a subset of alpha cells is capable of such expression. Indeed, all alpha cells do not appear at the same time during pancreas development. Consequently, when studying alpha cells at birth for example, they don't all have the same "age" and may not be in the exactly same differentiation state. Answering this question may greatly enhance our understanding of alpha cell and specifically their differentiation. This is of crucial importance in the field of diabetes research as alpha cells emerge as possible source of beta cell by transdifferentiation mechanism. Such a question may not be applicable to the gastrin expressing beta cells, as only really few insulin⁺ cells co-express gastrin (at E14.5 (personal communication) and at P0). Thus, the double hormone⁺ beta cells are likely to represent an extremely discrete subpopulation of beta cells during development and postnatal period. Considering the rarity of these cells, one may even ask if these cells are truly programmed to exist or if they are only the result of mere coincidence resulting from differential threshold of transcription factors expression or other mechanisms, such as differential exposition to growth factors.

The fact that the majority of gastrin expressing cells and ghrelin expressing cells coexpress glucagon suggests that alpha cells are a metastable and heterogeneous endocrine cell population before their final differentiation. In addition, it has been shown, using lineage tracing with Cre recombinase inserted in the coding sequence of the *Ghrelin* locus, that nearly 15% of adult alpha cells expressed the genetic reporter demonstrating that cells that have expressed Ghrelin are contributing to adult alpha cell mass. Thus it would be interesting to demonstrate if pancreatic gastrin expressing cells contribute to alpha and beta cell population in adult. If yes, are they, once become gastrin⁻ cells, entirely the same as other alpha and beta cells, or do they keep some "gastrin cell memory" with them? More direct evidence is still needed to clarify the issue. This could be done by generating mice expressing Cre recombinase under the control of the endogenous *Gastrin* gene locus, combined with subpopulation isolation and fine molecular analyses. Indeed, the data from our tumor analyses in the first paper favor the hypothesis that gastrinomas are derived from embryonic and/or neonatal gastrin⁺ cells (coexpressing either insulin or glucagon), and they remain at such a "immature" state during tumorigenesis. However, we cannot totally exclude the possibility

DISCUSSION.

Pancreatic gastrin expressing cells: where do they come and where they may go?: *Mechanisms of pancreatic gastrin expression*

that they can come from alpha and/or beta-cells having “gastrin cell memory” because of *Men1*-disruption.

3.1.2 MECHANISMS OF PANCREATIC GASTRIN EXPRESSION

The mechanisms controlling gastrin expression in the pancreas during development and after birth are completely elusive. Many transcription factors necessary for islet cell development and maturation are also common to gastric and duodenal endocrine cells development. Indeed, transcription factors such as Nkx2.2, Pax4 and Pdx1 are necessary for duodenal gastrin⁺ cells development (Desai et al. 2008, Wang et al. 2009, Beucher et al. 2012). In addition to *Gastrin* transcriptional expression, it is well known that *Gastrin* mRNA have to be translated and further processed by different cleavages including the enzyme activity of both PC1/3 and PC2 (Rehfeld 2006, Rehfeld et al. 2008). Interestingly, it has been shown that in adult islets, PC1/3 expression is restricted to beta cells, whereas PC2 is expressed in both alpha and beta cell populations (Neerman-Arbez et al. 1994, Kilimnik et al. 2010). PC1/3 and PC2 are necessary for the maturation of Proinsulin to active C-peptide, whereas Proglucagon is cleaved by PC2 to release glucagon in alpha cells, or by PC1/3 to release peptide such as GLP-1 which is expressed in the intestinal L cells. Interestingly, it was shown that during development and more specifically in neonates, glucagon expressing cells also express PC1/3 protein (Kilimnik et al. 2010). In this case, Progastrin may be fully processed to its bioactive form. This processing is important as most antibodies available in diagnostic only recognize fully processed gastrin and are well far too specific (Rehfeld et al. 2012), probably leading to unappreciated expression of Progastrin peptide in different organs. Development of antibodies recognizing Progastrin and not only fully processed gastrin are emerging as an urgent need, because the putative role of Progastrin (and not forcedly fully processed amidated Gastrin-17) in the proliferation of colorectal cells has been described (Wang et al. 1996). It would be, therefore, interesting to assess the expression of Progastrin by immunohistochemistry in adult human and murine pancreas to ensure that islet cells do not effectively express gastrin precursor protein. Interestingly, it has been demonstrated that some subsets of alpha cells in normal human adult islets coexpress Glucagon and PC1/3 leading to the production of GLP-1 peptides in the latter (Marchetti et al. 2012).

DISCUSSION.

Pancreatic gastrin expressing cells: where do they come and where they may go?: *Biological role of normal pancreatic Gastrin expressing cells*

At this time, it is difficult to know what molecular mechanism is responsible for the progressive loss of gastrin expression in the pancreas after birth. As most gastrin expressing cells are coexpressing glucagon at least in the mouse, does the loss of PC1/3 expression in these cells combined with a decrease in Progastrin expression account for the loss of gastrin in the pancreas after birth? It has been shown that Progastrin level is decreased after birth in rat (Bardram et al. 1990), suggesting that at least a decrease in Progastrin expression may explain the progressive loss. On the contrary few data are available on *Progastrin* mRNA expression in islets and more specifically in the different types of islet-cells. *In situ* hybridization performed on adult rat pancreas failed to reveal *Progastrin* mRNA expression in islets (Shimizu et al. 1999). However, considering the great differences that exist between ghrelin expression in human and rat pancreas (Raghay et al. 2013), it would be urgent to perform better analyses on isolated human islets or isolated alpha and beta cells, using more sensitive techniques such as PCR.

3.1.3 BIOLOGICAL ROLE OF NORMAL PANCREATIC GASTRIN EXPRESSING CELLS

Several hypotheses have been put forward concerning the biological significance of the cells expressing gastrin in developing and neonate pancreas. *Gastrin null* mice present with slight fasting hypoglycemia and improved glucose tolerance in oral and intraperitoneal challenge at three months of age, which could be explained by a decreased glucagon secretion after glucose stimulation (Boushey et al. 2003). In contrast, another group reported that *Gastrin null* mice develop insulin resistance and increased fasting glycemia at 5 months of age (Cowey et al. 2005). These data are not informative as to whether the defects observed result from eventual islet development defects or due to the absence of gastrin in adult mice. Indeed, Boushey and colleagues performed glucagon and insulin pancreas content analysis in newborn *Gastrin null* mice and did not notice any difference. Regretfully, they did not perform histological analysis to study if there were some defects in endocrine cell differentiation. Interestingly it has been shown that Gastrin receptor (CCK-BR, CCKR2) is expressed in developing and adult human alpha cells (Saillan-Barreau et al. 1999). Thus, Gastrin may possibly affect the proliferation, differentiation or physiology of alpha cells. In addition, CCK can also signal through the gastrin receptor, thus it still has to be demonstrated if compensation by CCK expression may occur in *Gastrin null* mice. However *Cck-BR null*

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Gastrinomas in mice: what we may learn?: *Origin of pancreatic gastrinomas*

mice do not show particular phenotype in the pancreas (Langhans et al. 1997), but analyses on the development and function of pancreatic endocrine cells in these mice need to be assessed.

In addition, it may remain possible that gastrin expression in the pancreas may not have any biological role. Indeed it may just be the result of the differentiation process of pancreatic endocrine cells during which the combination of protein expression and chromatin aperture would result in non-specific gastrin expression.

3.2 GASTRINOMAS IN MICE: WHAT WE MAY LEARN?

3.2.1 ORIGIN OF PANCREATIC GASTRINOMAS

The current works provide compelling evidence showing the crucial role played by pancreatic endocrine cells themselves in the development of pancreatic gastrin expressing tumors, following *Men1* gene disruption. Our data demonstrate that subsets of alpha and beta cells that express gastrin during the development or the postnatal period should represent the cells of origin of pancreatic gastrinomas. Our work also further confirmed the role of the *Men1* gene in the pathogenesis of pancreatic gastrinomas in the mouse, that our team and others previously described in heterozygous *Men1* mutant mice (Bertolino et al. 2003, Loffler et al. 2007).

However, several points remain to be elucidated. Our analyses, did not address the functionality of gastrin producing tumors in our different mouse models. The analyses of Gastrin levels in the serum withdrawn from mutant mice and more precocious histological analysis of gastric mucosa may tell us more about the functionality of such tumors. Indeed, if gastrin producing tumors effectively secrete high amount of gastrin, this should lead to pseudo ZES in mice, with hyperplasia of ECL cells in the gastric corpus, fundus or antrum. In addition, it remains surprising that gastrin producing tumors that we reported also express insulin even at lower levels than other insulin expressing tumors. However, all human pancreatic gastrinomas do not systematically express insulin. It has, nevertheless already been indeed reported the case of a sporadic pancreatic tumor leading to clinical manifestation of both gastrinoma and insulinoma (Lodish et al. 2008). More importantly, this tumor displayed a coexpression of both insulin and gastrin inside same cells (Lodish et al. 2008). Interestingly, data from our lab demonstrated a substantial number of human glucagonomas containing dispersed gastrin-expressing cells (unpublished data). We suspect that many of the case could

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Gastrinomas in mice: what we may learn?: *Origin of pancreatic gastrinomas*

be neglected because of the lack of clinical symptom and specific antibodies. In addition, it has already been reported the development of gastrinoma subsequently to an insulinoma in some patients (Mizuno et al. 2001). Thus, one may ask if pancreatic gastrinomas could originate from insulin expressing lesions that would loss insulin expression. Indeed, gastrinomas are often lately detected during the tumor development which could explain why generally no insulin is found in pancreatic gastrinomas. In addition, a high proportion of pancreatic endocrine tumors are non-functional, which could explain why pancreatic gastrinomas are generally not associated or do not occurs after an initial diagnostic of insulinoma in patients.

In this thesis, we suggested that the origin of pancreatic gastrinomas may possibly in part reside in the pre and neonatal gastrin expressing alpha and/or beta cells. However, we could not directly address this issue because mice expressing Cre recombinase specifically in gastrin expressing cells have not been engineered so far. Such genetic tool may be used to disrupt the *Men1* gene specifically in pancreatic gastrin expressing cells during development to address the precise contribution of these cells to pancreatic gastrinoma development. Intriguingly, mice expressing the SV40 large T antigen under the control of the human gastrin promoter develop insulin expressing tumors that surprisingly do not express gastrin, demonstrating a potential role of pancreatic gastrin expressing cells in tumor development (Montag et al. 1993). Unfortunately, the precise pattern of transgene expression has not been assessed to validate that the human promoter used can effectively and only target gastrin expressing cells. The fact that this model does not develop gastrinomas may suggest that SV40 T antigen is not sufficient to drive gastrinoma development and that other events are needed to trigger gastrinoma development. In our model, we were neither able to detect cluster of gastrin expressing cells at early time points (i.e. at 1 and 2 months of age, personal communication), nor to trace what happens right after *Men1*-disruption in gastrin expressing cells. It would be possible that these cells may lose gastrin expression as their wild-type counterpart but are able to reactivate this expression later in life, leading to gastrin producing tumors. Another explanation may reside in a transdifferentiation mechanism that may arise randomly from insulin expressing tumors leading to spontaneous development of gastrin expressing tumors. Considering the low frequency of gastrin expressing tumors arising in our different mouse models, and there is no increased frequency of gastrin-expressing tumors with age, this is unlikely to be the case, but we are aware that it is worth considering. Remarkably,

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Menin and pancreatic endocrine progenitors: an issue remains open?: *Duodenal gastrinomas in mice*

the low number of gastrinomas is in total accordance with both the low proportion of pancreatic gastrinomas in MEN1 patients, and the low proportion of gastrin expressing cells in the developing pancreas.

3.2.1 DUODENAL GASTRINOMAS IN MICE

Works from my hosting team described the occurrence of gastrinomas in the duodenum and the glandular stomach of aged *Men1*^{+/-} mice (16%) (Bertolino et al. 2003). However, the frequency seems to be much lower than what reported in *MEN1* patients. It seems that mice, on the contrary to human, are not prone to duodenal gastrinoma development. Indeed, the models that we generated allow specific *Men1*-disruption in endocrine Ngn3⁺ progenitors, also target the duodenal endocrine progenitors expressing Ngn3. Our analyses of the duodenum of 12 month-old mutant mice did not evidence any development of tumors. This is in agreement with a recently published study where the *Men1* gene was disrupted in the entire intestinal epithelium (including the duodenum) but did not trigger gastrinoma development (Veniaminova et al. 2012). Interestingly, it was demonstrated that a high proportion of gastrin expressing cells still kept menin expression at protein level. This suggests that in these cells, menin protein half-life is higher than the epithelial renewing or that in the mouse, menin is important for gastrin cell development leading to a specific positive selection of gastrin expressing cells that have not recombined *Men1* locus. The absence of duodenal gastrinoma development in several *Men1* models is reminiscent of the phenotype observed in mice with *Apc* mutations that do not develop colon tumors as opposed to humans with such mutations. The differences between epithelial turnover between species, or difference in microenvironnement in the mouse and human along the intestinal tract may explain the lack of duodenal gastrinomas in the mouse as recently suggested for colorectal cancer (Leedham et al. 2013). The microenvironnement may indeed play an important role and may also explain the nearly absence of gastrinomas development in human stomach whereas more than 95% of gastrin expressing cells are located in the stomach.

3.3 MENIN AND PANCREATIC ENDOCRINE PROGENITORS: AN ISSUE REMAINS OPEN?

3.3.1 ROLE OF MENIN IN ENDOCRINE PANCREAS DEVELOPMENT

DISCUSSION.

Menin and pancreatic endocrine progenitors: an issue remains open?: *Role of menin in endocrine pancreas development*

Menin's role in development and more particularly in the endocrine pancreas development had been previously highlighted by several studies on *Men1* KO embryos (Bertolino et al. 2003, Fontaniere et al. 2008, Lemos et al. 2009). Pancreatic explants culture and chimeric mice formed from both Wild-type ES cells and *Men1* KO ES cells demonstrated that menin was required for pancreatic islet cells differentiation (Fontaniere et al. 2008). One group reported the generation of *Pdx1-Cre-Men1^{F/F}* mice allowing the disruption of the *Men1* gene in pancreatic progenitors expressing Pdx1 but also in beta cells (Shen et al. 2009). The mice seemed to develop normally and developed pancreatic endocrine tumors, though only insulinomas were found in this model. However, the authors did not check if menin expression was lost during development and if any changes in islet cell composition were observable at birth. This is of great importance as it was described that some *Pdx1-Cre* transgenic mice are active only in beta cells and not in pancreatic progenitors (Heiser et al. 2006). Thus, the exact role of menin in pancreatic endocrine cells development remained unaddressed. One aspect of our works with the *PancEndoMen1* KO model was to further dissect the potential role of *Men1* in the differentiation of Ngn3⁺ pancreatic endocrine progenitors. Interestingly we could not directly evidence obvious developmental defects occurring after *Men1* disruption in Ngn3⁺ progenitors. However, if menin loss would have been detrimental for islet cells development, we should be able to find only a relatively small percentage of cells with absence of menin expression at P0. The percentage of menin loss in alpha, beta and delta cells at birth were slightly lower, when compared with the number of eYFP expressing cells in lineage traced control mice *Ngn3^{wt/TA}-tet-Ocre R26^{eYFP}* mice. Nevertheless, the lower presence of menin-negative cells may also be explained by other factors, such as relatively long lasting stability of menin protein or decreased proliferation of menin-deficient endocrine cells during late embryogenesis. More detailed analysis, in particular those during embryonic stage, are needed to confirm the observation.

Moreover, using different models targeting *Men1* in maturing endocrine cells, we do not observe any impact of *Men1* inactivation on their development. Considering that menin play important role in epigenetic regulation, we could speculate that menin absence may have earlier effects, such as inducing nuclear reprogramming in endoderm progenitors for example, preventing the differentiation specifically of pancreatic endocrine cells. This remains to be investigated using strategies allowing earlier disruption of *Men1*.

DISCUSSION.

Biological effects of menin loss in pancreatic endocrine cells: finding the common mechanisms from the diversity?: *Menin and pancreatic endocrine cell proliferation during development*

3.4 BIOLOGICAL EFFECTS OF MENIN LOSS IN PANCREATIC ENDOCRINE CELLS: FINDING THE COMMON MECHANISMS FROM THE DIVERSITY?

3.4.1 MENIN AND PANCREATIC ENDOCRINE CELL PROLIFERATION DURING DEVELOPMENT

One major enigma in the study of menin's biological role is to decipher the consequences of menin loss at different stages during pancreatic development, as compared with its loss in adult differentiated endocrine cells. Indeed, in human MEN1 patients and *Men1*^{+/-} mice, pancreatic endocrine tumorigenesis is dependent on the loss of function of the remaining wild-type functional allele of *Men1*, mainly by LOH. However, LOH is susceptible to happen at any time during the life, including during embryogenesis. When analyzing the frequency of menin loss and proliferation in different endocrine cell-types in *PancEndoMen1* KO mice, we were surprised to find that the percentage of *Men1* disrupted cells was at the most equivalent to the percentage of cells expressing the genetic reporter in control *Ngn3*^{wt/tTA}-*tetOCre*⁺-*R26*^{eYFP} mice. This observation seems to indicate that loss of menin during embryogenesis did not trigger their proliferation. Indeed, it would have been expected that the percentage of menin negative cells would have been superior to the percentage of cells labeled with the genetic reporter in *Ngn3*^{wt/tTA}-*tetOCre*⁺-*R26*^{eYFP} if menin deficient cells had proliferated. However, this is only an indirect deduction and we need to study the proliferation of menin deficient cells in *PancEndoMen1* KO mice during embryogenesis in order to get further insight. In the case where we could confirm such absence of effect of menin loss on the proliferation of endocrine cells during embryogenesis, it would be essential to understand the molecular basis of such absence, as it may help in elaboration of strategies to inhibit the development and progression of endocrine tumors in MEN1 patients.

Even if we consider that the effect of menin loss in the pancreatic progenitors is unsettled by the current study, one may ask if such loss may trigger different tumor development as compared with menin loss in fully differentiated cells. The histological analyze of aged *PancEndoMen1* KO mice at 13 months demonstrated a generalized but uneven decrease in insulin expression. Such phenomenon was not observable in the β *Men1* KO mice. In addition, 4.5-month-old *Ptf1aCre*⁺-*Men1*^{F/F} mice pancreases also displayed such generalized diminished insulin expression in the lesions. We may wonder if this is the result of menin loss during embryogenesis leading to an alteration of beta cell maturation or if it is

DISCUSSION.

Biological effects of menin loss in pancreatic endocrine cells: finding the common mechanisms from the diversity?: *Differential effect of menin loss in different islet-cell types*

indirectly related to factors secreted by other types of menin-deficient islet-cells which are not present in the β *Men1* KO model. Our data strongly suggest that *Men1*-disruption in pancreatic progenitors may lead to the development of less differentiated and functional tumors. Thus, this may indicate that the heterogeneity of islet tumors observed in MEN1 syndrome may be at least partially due to the cells of origin of these tumors. Cohorts of 18 month-old *PancEndoMen1* KO mice and 12 months old *PtflaCre⁺-Men1^{F/F}* mice will soon be thoroughly analyzed both on histological and biological levels in order to better investigate the evolution and outcome of derived islet tumors.

3.4.1 DIFFERENTIAL EFFECT OF MENIN LOSS IN DIFFERENT ISLET-CELL TYPES

Anatomopathological analyses of aged *PancEndoMen1* KO mice confirmed the essential role of menin in repressing alpha and beta cell proliferation, even when disrupted before and/or during their differentiation. This is in contrast with other previous models of *Men1* inactivation, which mainly studied the role of menin loss in differentiated islet cells, or models that did not allow to discriminate between effect of menin loss in adult differentiated or early developing differentiated islet cells. Analyses of the proliferation of menin deficient cells at 6 weeks, and the relative low number of alpha cell lesions in aged *PancEndoMen1* KO mice confirmed that *Men1* disrupted beta cells have an increased proliferation as compared to menin-deficient alpha cells, as it has been described in tumors from *Men1^{+/-}* mice (Walls et al. 2012). This, combined with the fact that beta cells are more numerous than alpha cells in normal pancreas, may explain why macrotumors secreting insulin are more frequent than those secreting glucagon in MEN1 patients. However, our group also suggested that transdifferentiation of alpha cells into insulin secreting cells may be a mechanism of insulinoma development following menin loss in alpha cells (Lu et al. 2010). However, to date, we could not evaluate in detail the contribution of such mechanism in *PancEndoMen1* KO mice. Lineage tracing of alpha cells would be of help, but it needs a Flip Recombinase system to target alpha cells, which is still not available.

In contrast to alpha or beta cells lesions, somatostatin or PP expressing lesions were largely rarer in 12-month-old mice. Results obtained for somatostatin are not surprising considering that 1) at 6 weeks, menin deficient delta cells only demonstrate a tendency for an increased proliferation as compared to normal delta cells; and 2) that somatostatin expressing

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tumors are rare in MEN1 patients. However, analyses of the proliferation of delta cells in mixed tumors from *Men1*^{+/-} mice have clearly demonstrated that these delta cells have proliferation rate at least equivalent to tumoral beta cells (Walls et al. 2012). Thus, we still need to perform proliferation analyses in different pancreatic endocrine cell-types of aged *PancEndoMen1* KO mice to refine these analyses. In addition, somatostatin analogs and therefore somatostatin, have proven their effects in decreasing proliferation of gastroenteropancreatic endocrine tumor cells in human (Strosberg and Kvols 2010). Autocrine action of somatostatin may act as an intrinsic inhibitor of delta cell tumor development. Conversely, other tumorigenic cells such as menin-deficient alpha and beta cells may directly or indirectly repress delta cell amplification, in order to avoid such repressive action of somatostatin on alpha and beta cell tumorigenic growth. As discussed in the manuscript n°2, the absence of somatostatin expressing tumors may also reflect a potential transdifferentiation or dedifferentiation-redifferentiation mechanisms converting menin-deficient into insulin secreting cells. This process could be even more prevalent than alpha-to-beta transdifferentiation as beta cells share very common differentiation program with delta cells.

We did not observe the appearance of PP expressing tumors in *PancEndoMen1* KO mice at 12 months of age. This is reminiscent of the nearly absence of such tumors in heterozygous *Men1*^{+/-} mice (Crabtree et al. 2001, Bertolino et al. 2003, Loffler et al. 2007, Harding et al. 2009). These observations highly contrast with the tumor types found in MEN1 patients, which have frequently non-functional prelesions and tumors expressing PP. Interestingly, Thakker's group reported that PP⁺ cells in mouse *Men1*^{+/-} tumors had high proliferation rate comparable to tumoral beta cells and significantly increased by more than 10 fold as compared to control PP cells (Walls et al. 2012). Unfortunately, we were not able to assess the proliferation rate of menin-deficient PP cells in our model due to the inadequacy of the antibody used. Herrera's group already described that several antibodies against PP cross-react with alpha cells (Desgraz and Herrera 2009). We used a different antibody than theirs, but all these observations raise the possibility that indeed, alpha cells may potentially also express PP. Validations of PP antibodies are currently ongoing in the lab, and more analyses on the role of menin in the regulation of PP cell proliferation and or biology may be further addressed.

DISCUSSION.

Biological effects of menin loss in pancreatic endocrine cells: finding the common mechanisms from the diversity?: *Cell signaling pathways heterogeneity in MEN1 tumorigenesis*

3.4.2 CELL SIGNALING PATHWAYS HETEROGENEITY IN MEN1 TUMORIGENESIS

When trying to understand if postnatal proliferation pathways were sustained after menin loss in islet-cells, we were surprised to detect an increased ERK1/2 activation principally in alpha cells lesions. We could not find any insulin⁺ tumors that showed a homogeneous ERK activation in contrast to what is observed in alpha cells. Indeed, only subsets of beta cells tumors had clustered areas with phosphor-ERK1/2 staining that comprised only 5 to 10 percent of the tumor area in *PancEndoMen1* KO mice but also in *Men1*^{+/-} mice. Tumors and lesions of age matched *βMen1* KO mice did not have such activation. Considering the known positive role of ERK signalization on tumorigenesis in several cancers (Chappell et al. 2011), we suspected that such activation may contribute to the proliferation of menin deficient alpha cells and subsets of beta cells. This is the first observation, to our knowledge, demonstrating differential mechanism of proliferation between different endocrine cell types and perhaps even between different subtypes of beta cells in *Men1*-related tumorigenesis. However, we need to further explore the potential role of ERK signaling in the proliferation of pancreatic endocrine cells.

In addition, it would be relevant to analyze the activation of AKT/mTOR pathway in the different types of lesions developed in *PancEndoMen1* KO mice, in order to decipher if this pathway may be differentially activated. Several studies of human pancreatic endocrine tumors showed that this pathway is frequently altered (Shida et al. 2010, Jiao et al. 2011), and it was demonstrated that AKT was activated in mouse *Men1*^{+/-} pancreatic endocrine adenomas (Wang et al. 2011). Moreover, substantial progress has been achieved in treating these tumors with the inhibitors of this pathway, in particular, those of mTOR (Yao et al. 2011). We believe that further studying the role of AKT/mTOR pathway in MEN1-related tumors would be of help for refine the therapeutic strategy and finding the new targets. Indeed, our preliminary study using cultured cells found distinct effects in mTOR activation triggered by MEN1 inactivation, suggesting the potential importance of cells of origin of individual tumors in response to targeted therapies.

CONCLUSIONS
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4 CONCLUSIONS AND PERSPECTIVES

The aim of this work was to investigate the biological and oncosuppressive role of *Men1* in pancreatic endocrine progenitors. The overall project was articulated on the study of a new generated model allowing specific *Men1* inactivation in Ngn3⁺ pancreatic endocrine progenitors and the comparison with other models disrupting *Men1* in pan-pancreatic progenitors and more differentiated endocrine cells.

We first addressed the potential role of *Men1* disruption in pancreatic endocrine progenitors in the development of pancreatic gastrinomas or gastrin expressing pancreatic tumors. The results obtained demonstrated that specific menin loss in Ngn3⁺ progenitors, leading to the loss of menin in perinatal pancreatic-gastrin expressing cells, triggered the development of tumors expressing gastrin. Our data also showed that subsets of alpha and beta cells during development and postnatal period express gastrin. Consistently, the disruption of *Men1* in either alpha or beta cells also triggered the development of a low frequency of gastrin expressing tumors. Altogether our results suggest the important role played by perinatal pancreatic gastrin expressing endocrine cells in the pathogenesis of pancreatic gastrinomas. Data obtained may be further investigated to better characterize these gastrin expressing lesions and compare them with human pancreatic gastrinomas. In addition, our observations may be of help to better understand this pathology and improve the treatment of these tumors that are often malignant.

In the second part, we investigated the early effects and tumorigenic potential of *Men1* disruption in pan-pancreatic progenitors and pancreatic endocrine progenitors during embryogenesis. The analyses demonstrated a differential proliferative effect of menin loss depending on the stage and endocrine-cell type. It seems that *Men1* disruption may not affect proliferation or even slightly decrease the proliferation of pancreatic endocrine cells at the end of embryogenesis, in contrast with the postnatal period. In addition, *Men1* disruption before the commitment of pancreatic beta cells, may affect the maturation and or maintenance of the differentiation status of beta cells. The current work also highlighted that proproliferative signaling pathway, ERK, was differentially activated in menin deficient pancreatic tumors depending on the lineage affected. Thus, our results may suggest that signaling pathways involved in pro-proliferative and/or pro-survival roles in islet tumors may vary depending on the cell of origin of a given pancreatic endocrine tumor. The validation of these results in

human pancreatic endocrine tumors is crucially needed. But if confirmed, they may shed light onto the development of better targeted-therapeutic strategies. This particularly necessary in the field of pancreatic endocrine tumor where different kinds of tumors (gastrinoma/insulinoma/glucagonoma/non-functional, etc..) are often not separately considered in research studies due to their rarity. An aspect of the project that was not addressed in the current thesis was the role played by menin in the intestinal and more particularly duodenal-endocrine progenitors. Indeed, the *Ngn3^{wt/tTA}-tet-Ocre* mouse model used in the studies conducted during this thesis also targets the gastric and intestinal *Ngn3⁺* endocrine progenitors. This part of the project was unfortunately not studied in detail, mainly because of the lack of macroscopic tumor development in the duodenum of aged *Men1^{F/F}-Ngn3^{wt/tTA}-tet-Ocre* mice. Consequently, we preferred to focus our analyses on the pancreatic lesions developed by these mice. However, sporadic duodenal gastrinomas or those developing in MEN1 patients can be of small size but already metastatic at diagnosis. Thus, small gastrin lesions may possibly develop in *Men1^{F/F}-Ngn3^{wt/tTA}-tet-Ocre* mice and better investigations are required to better appreciate the role played by menin in gastro-entero endocrine progenitors and differentiated enteroendocrine cells.

Finally, we are persuaded that more detailed analysis of events that follow menin disruption in developing pancreas may help to better understand the biology of islet cells and the mechanisms of tumor development of islet-cells.

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ANNEXES

Annexe 1 :

Both PAX4 and MAFA are expressed in a substantial proportion of normal human pancreatic alpha cells and deregulated in patients with type 2 diabetes

Bonnavion R[#], Jaafar R[#], Kerr-Conte J, Assade F, van Stralen E, Leteurtre E, Pouponnot C, Gargani S, Pattou F, Bertolino P, Cordier-Bussat M, Lu J and Zhang CX

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PLoS ONE 8(8): e72194. doi:10.1371/journal.pone.0072194

Annexe 2 :

High incidence of mammary intraepithelial neoplasia development in Men1-disrupted murine mammary glands.

Seigne C, Auret M, Treilleux I, **Bonnavion R**, Assade F, Carreira C, Goddard-Léon S, Lavergne E, Chabaud S, Garcia A, Mazoyer S, Lu J, Bachelot T, Frappart L, Zhang CX. J Pathol. 2013 Mar;229(4):546-58. doi: 10.1002/path.4146.

Annexe 3 :

Reexpression of oncoprotein MafB in proliferative β -cells and Men1 insulinomas in mouse.

Lu J, Hamze Z, **Bonnavion R**, Herath N, Pouponnot C, Assade F, Fontanière S, Bertolino P, Cordier-Bussat M, Zhang CX.

Oncogene. 2012 Aug 2;31(31):3647-54. doi: 10.1038/onc.2011.538. Epub 2011 Nov 28.

Annexe 4 :

Alpha cell-specific Men1 ablation triggers the transdifferentiation of glucagon-expressing cells and insulinoma development.

Lu J, Herrera PL, Carreira C, **Bonnavion R**, Seigne C, Calender A, Bertolino P, Zhang CX. Gastroenterology. 2010 May;138(5):1954-65. doi: 10.1053/j.gastro.2010.01.046. Epub 2010 Feb 2.

Both PAX4 and MAFA Are Expressed in a Substantial Proportion of Normal Human Pancreatic Alpha Cells and Deregulated in Patients with Type 2 Diabetes

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Abstract

Pax4 and MafA (v-maf musculoaponeurotic fibrosarcoma oncogene homolog A) are two transcription factors crucial for normal functions of islet beta cells in the mouse. Intriguingly, recent studies indicate the existence of notable difference between human and rodent islet in terms of gene expression and functions. To better understand the biological role of human PAX4 and MAFA, we investigated their expression in normal and diseased human islets, using validated antibodies. PAX4 was detected in $43.0 \pm 5.0\%$ and $39.1 \pm 4.0\%$ of normal human alpha and beta cells respectively. We found that MAFA, detected in $88.3 \pm 6.3\%$ insulin⁺ cells as in the mouse, turned out to be also expressed in $61.2 \pm 6.4\%$ of human glucagon⁺ cells with less intensity than in insulin⁺ cells, whereas MAFB expression was found not only in the majority of glucagon⁺ cells ($67.2 \pm 7.6\%$), but also in $53.6 \pm 10.5\%$ of human insulin⁺ cells. Interestingly, MAFA nuclear expression in both alpha and beta cells, and the percentage of alpha cells expressing PAX4 were found altered in a substantial proportion of patients with type 2 diabetes. Both MAFA and PAX4 display, therefore, a distinct expression pattern in human islet cells, suggesting more potential plasticity of human islets as compared with rodent islets.

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Introduction

The development and functions of different types of islet cells are controlled, to a large extent, by essential cell-lineage-specific transcription factors. Among them, the transcriptional regulators Pax4 and Maf are of crucial importance for both cell differentiation and normal functions of islet beta cells in the mouse [1,2]. Pax4 is mandatory for the development and maturation of mouse beta cells [1]. The evidence from the recent studies suggest that PAX4 is also crucial for mature beta cell expansion and survival, and that PAX4 mutations or polymorphisms are associated with both type 1 and type 2 diabetes [3]. Importantly, Pax4 overexpression in mouse pancreatic progenitors resulted in beta cell conversion [4]. MafB is involved in embryonic development of both mouse pancreatic alpha and beta cells [5,6], whereas MafA participates mainly in the maturation of mouse beta cells [2]. During adult life in the mouse, MafB is specifically expressed in alpha cells [6] and MafA in beta cells [2]. Interestingly, we and

others have recently demonstrated that MafB expression can be reactivated in mouse beta cells undergoing adaptive proliferation [7,8] and during tumorigenesis [7], indicating that the dynamic modulation of Maf expression could be involved in the control of beta cell proliferation and their endocrine functions. Based largely on the knowledge acquired from the above mentioned studies in the mouse, several strategies to cure diabetes are aimed at replenishing the pool of beta cells, by overexpressing beta cell specific transcription factors, including Pax4, MafA and Pdx1 in different cell-types.

Although the expression pattern and biological functions of these islet transcription factors have been substantially explored in rodent models and cell lines, little was known in human islets until recently. PAX4 mRNA was undetectable [9] and PAX4 protein expression has been barely studied in normal adult human islets, mainly because of the lack of specific tools. Intriguingly, Dorrell *et al.* recently reported that MAFB was detected at equal levels in

Table 1. Human patient samples.

Patient#	Sex	Age (year)	BMI (kg/m ²)	HbA1c (%)	Clinical features
1	M	64	39.2	10.3%	T2D
2	M	58	32.8	7.0%	T2D
3	M	62	32.7	7.4%	T2D
4	M	49	29.9	7.7%	T2D
5	M	57	36.5	8.2%	T2D
6	F	55	22.4	7.7%	T2D
7	M	59	37.7	7.0%	T2D
Mean ± S.E.M T2D	–	57.7±1.8	33.0±2.2	7.9±0.4%	–
8	M	66	36.9	5.3%	Control obese
9	F	50	33.3	6.2%	Control obese
10	F	46	33	5.7%	Control obese
11	M	55	39	5.8%	Control obese
Mean ± S.E.M Control obese	–	54.3±4.3	35.6±1.5	5.8±0.2%	–
12	M	18	21.6	5.6%	Control
13	M	17	23.7	5.6%	Control
14	M	18	23.3	ND	Control
15	M	41	22.2	5.5%	Control
16	M	36	24.2	ND	Control
17	M	43	22.5	5.6%	Control
18	F	47	25.7	ND	Control
19	F	47	20.8	5.7%	Control
20	H	48	24.7	ND	Control
Mean ± S.E.M Control	–	35.0±4.5	23.2±0.5	–	–

BMI: Body Mass Index; HbA1c: Glycated Hemoglobin; ND: Not Determined.
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FACS purified adult human alpha and beta cells by RT-qPCR analysis, suggesting that MAFB expression in human islets may be different from that in mouse [9]. Consistent with this work, Dai *et al.* subsequently reported MAFB protein expression by immunohistochemistry in a rather small proportion of beta cells in normal adult human islets [10]. Considering the marked difference reported between MAFB expression in human and murine islets, the goal of this study was to find out detailed protein expression patterns of PAX4 and MAFA in human adult alpha and beta cells.

Materials and Methods

Ethics statement

Human paraffin embedded pancreatic tissues from the collection (CHRU Lille, France) conserved for traceability in the context of our clinical islet transplant program [11] were obtained from healthy subjects (n = 9), Type 2 Diabetics (n = 7), and non-diabetic subjects with obesity (n = 4, Table 1). Verbal consents from the donor were obtained for use of the samples in research. The study was conducted in accordance with the guidelines in the Declaration of Helsinki. Ethical review board (« Comité d’Ethique du Centre Hospitalier Régional et Universitaire de Lille ») approved the use of human pancreatic cells in vitro, and their associated paraffin sections. Authorization to access research grade pancreases is granted by Agence de la BioMedecine, France. The Agence de la BioMedecine has standardized procedures for Coordinators to assure that the informed consent procedure is identical throughout France.

Cell transfection and western-blotting

αTC1-9 were transfected with the empty vector pCI-neo, pCI-mPax4 or pCI-hPAX4 expressing mouse or human PAX4 respectively and were analysed as previously described [7]. MEF cells were transfected with pcDNA-MAFA and pcDNA-MAFB expressing human MAFA and MAFB, respectively, and analysed as previously described [7].

Pax4 vectors cloning

Mouse Pax4 expressing vector was constructed by subcloning of a PCR fragment obtained from βTC3-cell line cDNA into the pCI-neo mammalian expression vector and was verified by sequencing. The following primers containing SalI and NotI restriction sites were used: 5'-ATAAGTCGACATGCAGCAGGACGGACTCAGC-3' and 5'-ATAAGCGGCCGCTTATGGCCAGTTTGAGCAATG-3'. Human PAX4 expressing vector was constructed by subcloning of a PCR fragment obtained from a human insulinoma cDNA into the pCI-neo mammalian expression vector and was verified by sequencing. The following primers containing SalI and NotI restriction sites were used: 5'-ATAAGTCGACATGCATCAGGACGGGATCAGC-3' and 5'-ATAAGCGGCCGCTTAAGGCCAGTGTGAGAAGTG-3'.

Antibodies used

The anti-MAFA antibody (ab26405, Abcam) and a panel of anti-MAFB antibodies were subjected to different tests to assess their specificity toward MAFA and MAFB proteins both in mouse and in human tissues. The anti-MAFA (ab26405, Abcam) and the

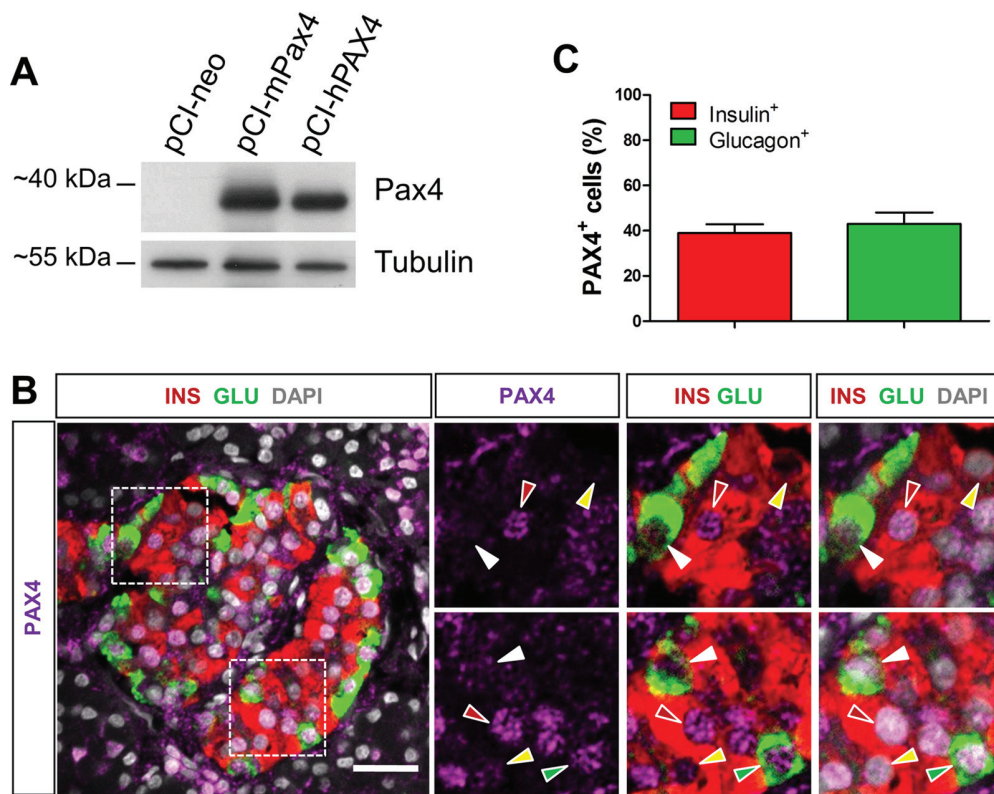


Figure 1. PAX4 is detected in both human pancreatic alpha and beta cells. (A) Detection of ectopically expressed mouse and human PAX4 by western blotting. The capacity of the anti-Pax4 antibody to recognize human PAX4 was assessed using α TC1-9 cells transfected respectively with the pCI-neo empty vector or constructs expressing mouse Pax4 (mPax4) or human PAX4 (hPAX4) protein by western-blotting analysis. Protein extracts from transfected cells were used for the detection, using antibody against Pax4. (B) Representative images of triple IF staining with antibodies against PAX4 together with glucagon and insulin. Right panels are the amplified view of the insets in the left panel. Red arrowheads, PAX4⁺ INS⁺ cells. Yellow arrowheads, PAX4⁺ INS⁻ cells, Green arrowheads, PAX4⁺ GLU⁺. White arrowheads, PAX4⁻ GLU⁺ cells. (C) Percentages of insulin⁺ cells and glucagon⁺ cells expressing PAX4 represented as the averaged counting results \pm S.E.M from n=5 control individuals (A total of 164 GLU⁺ cells and 904 INS⁺ cells were analyzed). Scale bars = 25 μ m. doi:10.1371/journal.pone.0072194.g001

noncommercial anti-MAFB2 (mouse monoclonal antibodies, clone 1F4 [12]) antibodies were validated and used for the current study. Anti-glucagon (G2654, Sigma), the anti-insulin (A056401, DAKO), the anti-PDX1 (ab47383, Abcam) and anti-ARX (noncommercial, [13]), mMaFB1 (anti mouse MaFB, IHC-00351, Bethyl), and hMAFB3 (Anti human MAFB, HPA005653, Sigma) were also used in the study.

Immunofluorescence analyses

Briefly, serial sections (4 μ m) mounted on glass slides were de-waxed and rehydrated through a series of ethanol dilutions then distilled H₂O. Heat-induced epitope retrieval was performed by immersion in Antigen Unmasking Solution (H-3300, Vector) in a microwave oven. After blocking with antibody diluent (DAKO) sections were incubated with a primary antibody, and treated with Cy-3 or Cy-5 Tyramide amplification kit (PerkinElmer) according to the manufacturer's instructions or incubated with appropriate Alexa488-555- or -647 coupled secondary antibodies (Life Technologies). For PAX4 detection in human pancreas, heat-induced epitope retrieval was not performed but sections were incubated in PBS-Triton X100 0.2% for 5 min at room-temperature. However this prevented us from performing ARX and PAX4 co-expression because ARX could not be detected with this protocol. All nuclear co-localization experiments were performed and controlled to

ensure that no non-specific crossreaction occurred. Images were captured and analyzed on a TCS-SP5 confocal microscope (Leica-Microsystems) or a Zeiss 780 confocal microscope. Cell counting was manually performed with ImageJ cell counter module (U. S. National Institutes of Health, Bethesda, Maryland, USA) on multiple channel pictures. Graphs and results of counting are represented as mean \pm S.E.M.

Results

Endogenous PAX4 nuclear expression can be detected both in human pancreatic alpha and beta cells

First, we sought to validate the capacity of a widely used anti-Pax4 antibody [4,14,15] to recognize human PAX4 (Figure 1A) expressed in α TC1-9 cells, since the antibody has not been reportedly used in human pancreatic tissues. As illustrated in Figure 1A, the antibody recognized properly ectopically expressed human PAX4 and its mouse counterpart. Using triple IF staining of PAX4, glucagon, and insulin, we found that surprisingly, PAX4 was expressed with an equivalent intensity in 43.0 \pm 5.0% and 39.1 \pm 4.0% of normal human alpha and beta cells respectively (Figure 1B, C). The relative expression intensity was much less in human islets than in mouse islet beta cells (data not shown). Importantly, this unexpected result is consistent with reported

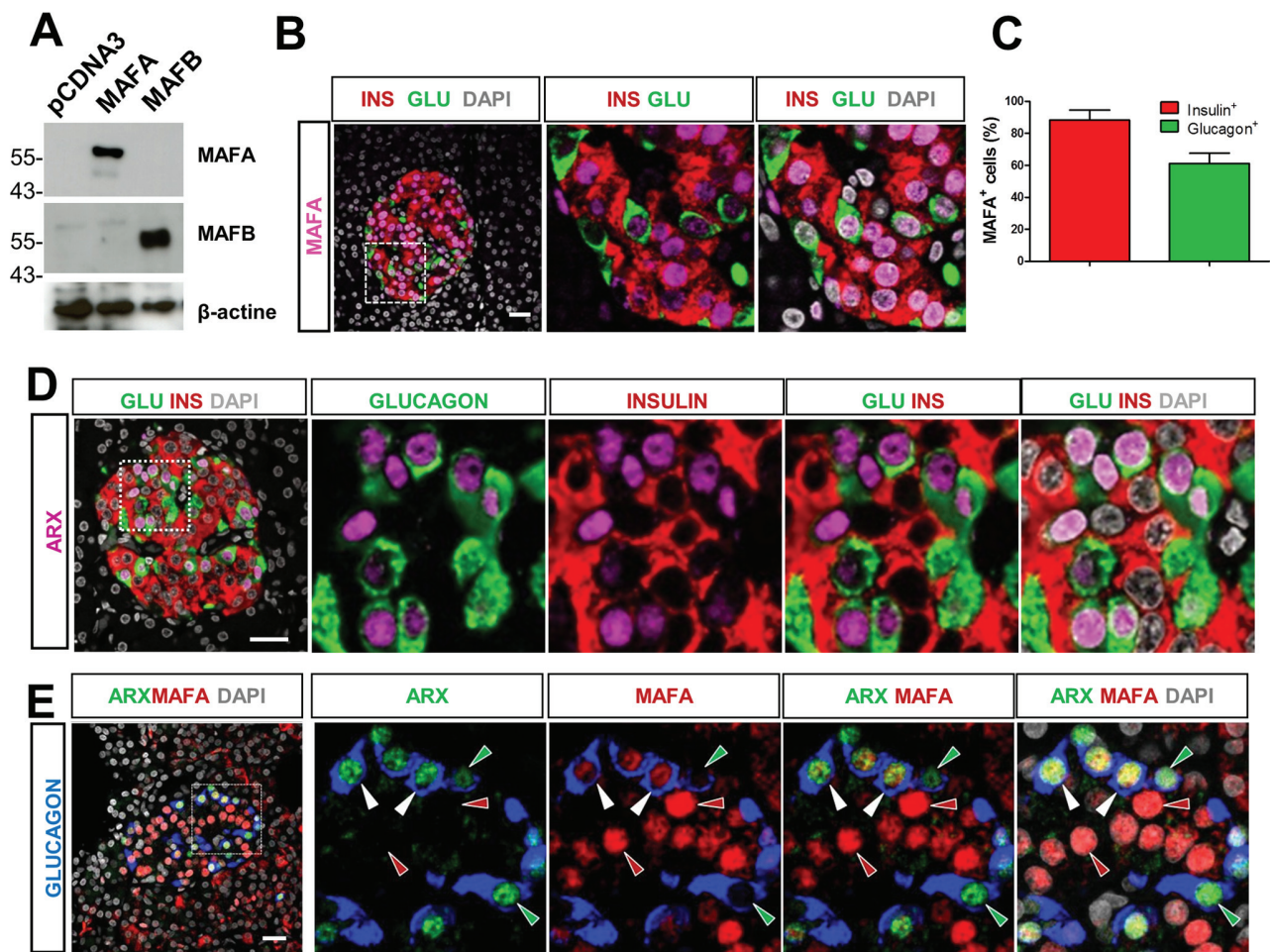


Figure 2. MAFA is detected in both human pancreatic alpha and beta cells. (A) Detection of ectopically expressed human MAFA and MAFB by western blotting. The specificity of selected antibodies against human MAFA and MAFB were evaluated using mouse embryonic fibroblasts (MEF), which do not express endogenous murine MafA and MafB proteins, transfected respectively with constructs expressing human MAFA or MAFB protein by western-blotting analysis. Protein extracts from MEF transfected with empty pcDNA and pcDNA expressing respectively human MAFA and MAFB were used for the detection, using antibodies against MAFA (Abcam) or MAFB (anti hMAFB2, mouse monoclonal antibodies, clone 1F4). Note that the anti-MAFA antibody and the noncommercial anti-hMAFB2 reacted specifically without cross-reaction, whereas the other tested commercially available anti-MAFB antibodies failed (data not shown) (B) Triple immunofluorescent (IF) staining showing MAFA expression in human islets from healthy donors. (C) The percentages of cells expressing MAFA were $88.3 \pm 6.3\%$ MAFA⁺ beta cells and $61.2 \pm 6.4\%$ MAFA⁺ alpha cells. Results are the averaged expression \pm S.E.M of counting results from n=4 control individuals (1058 INS⁺ cells and 345 GLU⁺ cells were counted in total). (D) ARX expression was detected in human pancreatic alpha cells but not human pancreatic beta cells. Representative images of triple IF-staining showing ARX expression in human islets from healthy donors. ARX was detected only in nuclei of alpha cells. (E) Co-localisation of MAFA with ARX in human islets. Right panels are the amplified view of the inset in the left panel. Red arrowheads, MAFA⁺ARX⁻GLU⁻ cells. Green arrowheads, MAFA⁺ARX⁺GLU⁺ cells. Scale bar = 25 μ m. doi:10.1371/journal.pone.0072194.g002

weak *PAX4* mRNA expression found in both human adult alpha and beta cells [9].

Expression patterns of MAFA and MAFB are largely distinct in human endocrine pancreas than those in the mouse

Then, to better investigate endogenous expression of MAFA and MAFB in human islets, we validated the specificity of anti-MAFA and MAFB antibodies used in the current study. We found that the anti-MAFA antibody (Abcam) and the non-commercial anti-hMAFB2 (mouse monoclonal antibodies, clone 1F4 [12]) reacted specifically without cross-reaction both to their corresponding human counterpart ectopically expressed in mouse embryonic fibroblasts (Figure 2A) and their corresponding protein

in mouse pancreas (Figure 3A). These antibodies were then further validated for the detection of endogenous human MAFA and MAFB expression in fixed human pancreatic sections by IHC (data not shown).

As expected, the validated antibodies recognized human MAFA in the nucleus of most insulin⁺ cells ($88.3 \pm 6.3\%$) on sections of adult human pancreas from healthy individuals (Figure 2B and C). Surprisingly, as opposed to what was observed in mouse alpha cells, MAFA was also detected in more than half of human glucagon⁺ cells ($61.2 \pm 6.4\%$, Figure 2B and C). MAFA expression in alpha cells was confirmed by colocalisation with an alpha cell-specific transcription factor, ARX (Figure 2D and 2E). In parallel, using an antibody recognizing specifically both mouse and human MAFB (Figure 2A and 3A), we found that $67.2 \pm 7.6\%$ of human

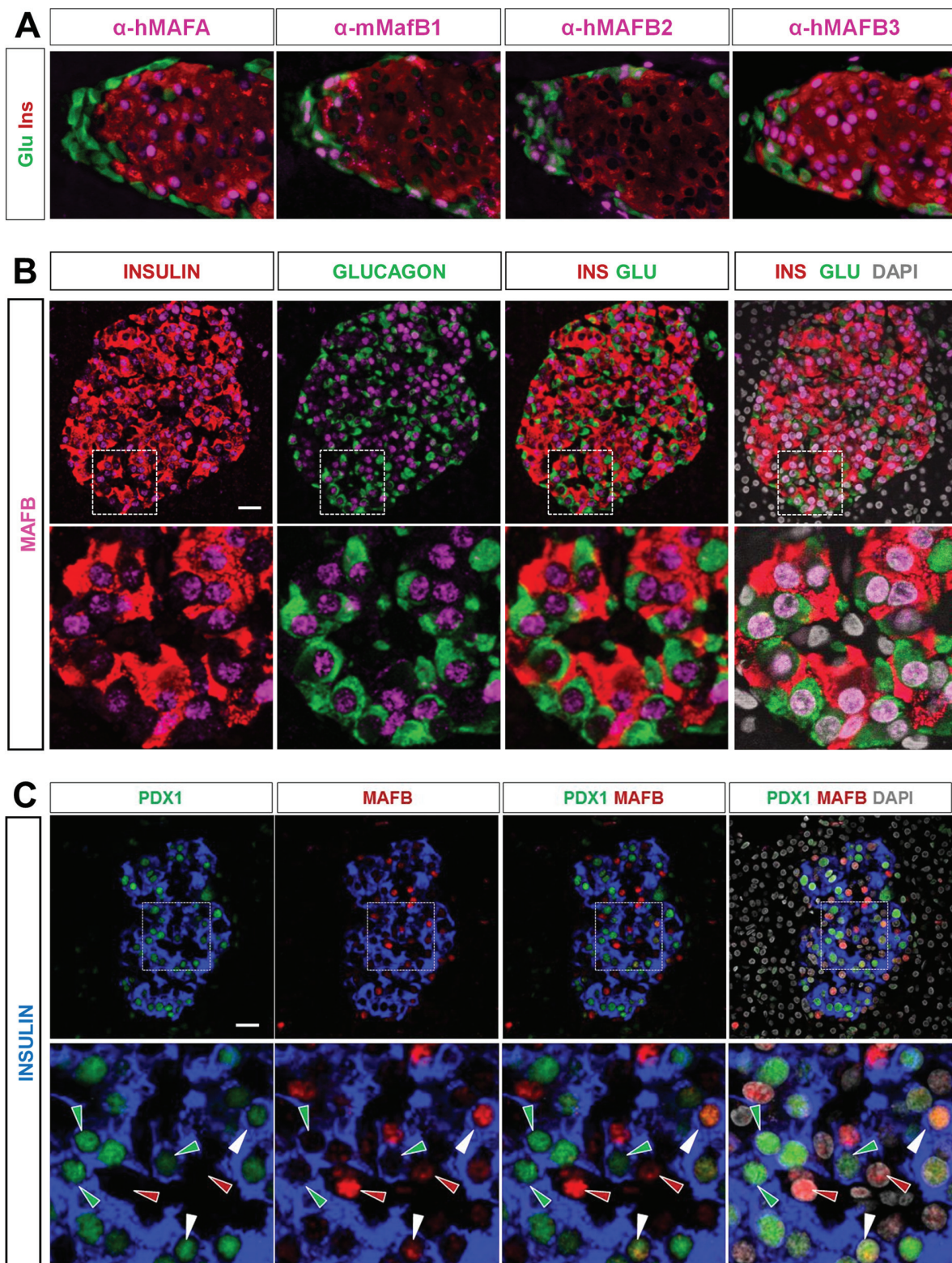


Figure 3. MAFB is expressed in both human alpha and beta cells. (A) Different antibodies were tested for their specificity toward mouse MafB by triple immunofluorescent (IF) staining of mouse pancreatic sections. Representative images of triple IF staining with anti-glucagon, insulin, and hMAFA, mMafB1, hMAFB2 and hMAFB3 antibodies. Note that hMAFB3 did not show an alpha cell specific pattern as did mMafB1 and hMAFB2 antibodies. (B) MAFB expression was detected in both human pancreatic alpha and beta cells. Representative images of triple IF-staining showing MAFB expression in human islets from healthy individuals. The percentages of positive cells for MAFB in glucagon⁺ (green) and insulin⁺ (red) cells were $67.2 \pm 7.6\%$ MAFB⁺ alpha cells and $53.6 \pm 10.5\%$ MAFB⁺ beta cells. Results are the averaged expression \pm S.E.M of counting results from $n=4$

control individuals (463 GLU⁺ cells and 797 INS⁺ cells were counted in total). Scale bar = 25 μM. (C) MAFB expression in insulin⁺ PDX1⁺ cells. The percentage of double insulin⁺ PDX1⁺ cells positive for MAFB was 30.2±5.8% (858 INS⁺ PDX1⁺ counted cells in total). The above percentages of expression are the averaged counting results ±S.E.M from n = 3 control individuals. Scale bar = 25 μM.
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glucagon⁺ cells displayed an overt MAFB staining, yet MAFB was also detected in a large number of insulin⁺ cells (53.6±10.5%, Figure 3B), and double insulin⁺ PDX1⁺ cells (30.2±5.8%, Figure 3C), similar to what was reported in a recent study but to a larger extent [16].

MAFA and PAX4 expression is found deregulated in patients with type 2 diabetes

Recently, Butler and colleagues reported that nuclear localization of MAFA was abolished in beta cells of type 2 diabetes patients [17]. We analysed MAFA expression in pancreatic sections from 9 control nonobese, nondiabetic individuals, 4 obese non-diabetic individuals and 7 type 2 diabetic (T2D) patients. We found that MAFA expression was unaltered in islets from subjects

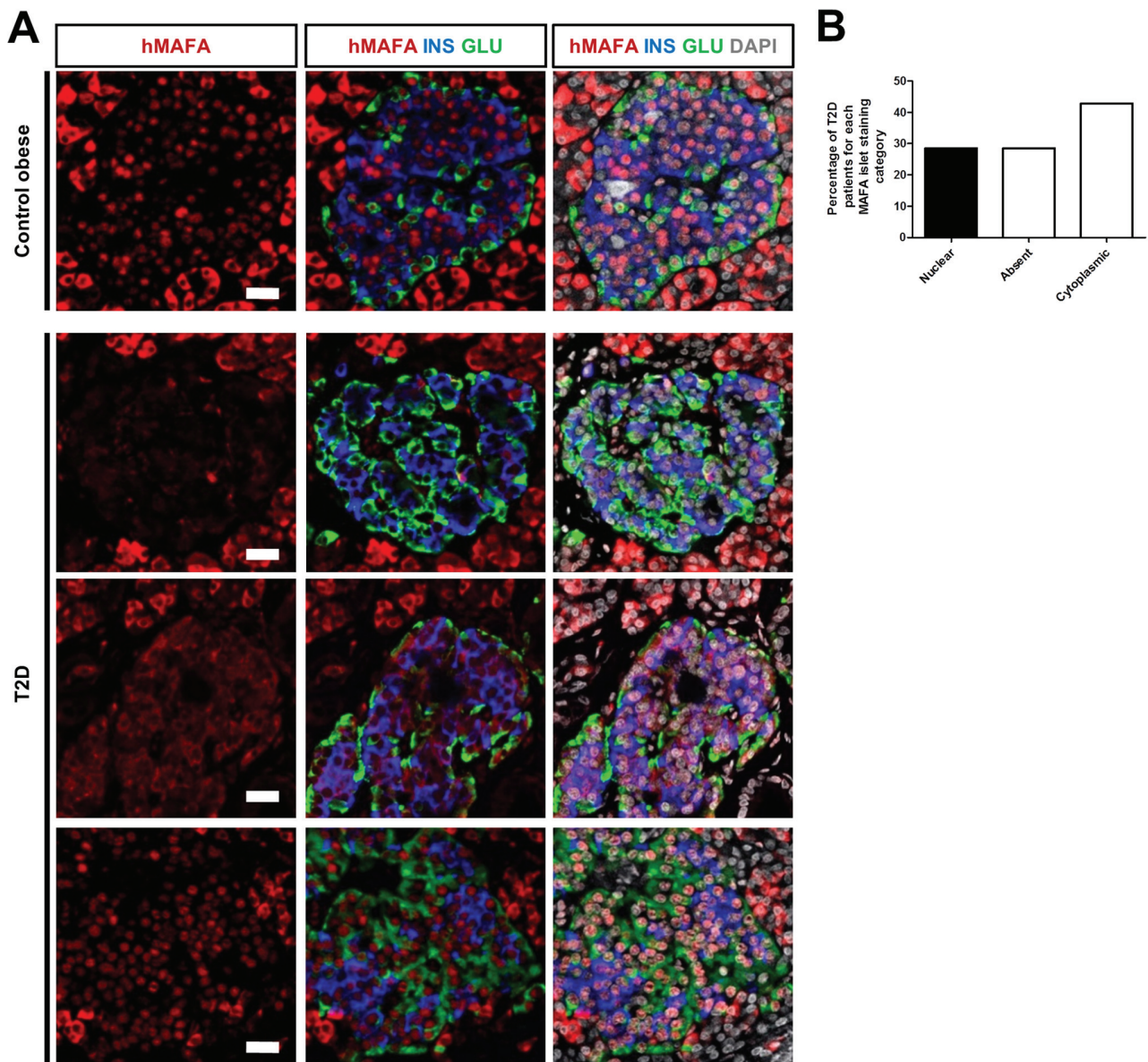


Figure 4. MAFA expression in islets from subjects with obesity or T2D diabetes. (A) Representative images of triple IF staining of MAFA together with insulin and glucagon in subjects with obesity and T2D patients showing a MAFA-negative (upper panels), a MAFA-cytoplasmic (middle) and a nuclear MAFA staining (lower panels) were respectively shown. (B) Percentage of T2D patients displaying each category of prominent MAFA expression in islets. Scale bars = 25 μm.
doi:10.1371/journal.pone.0072194.g004

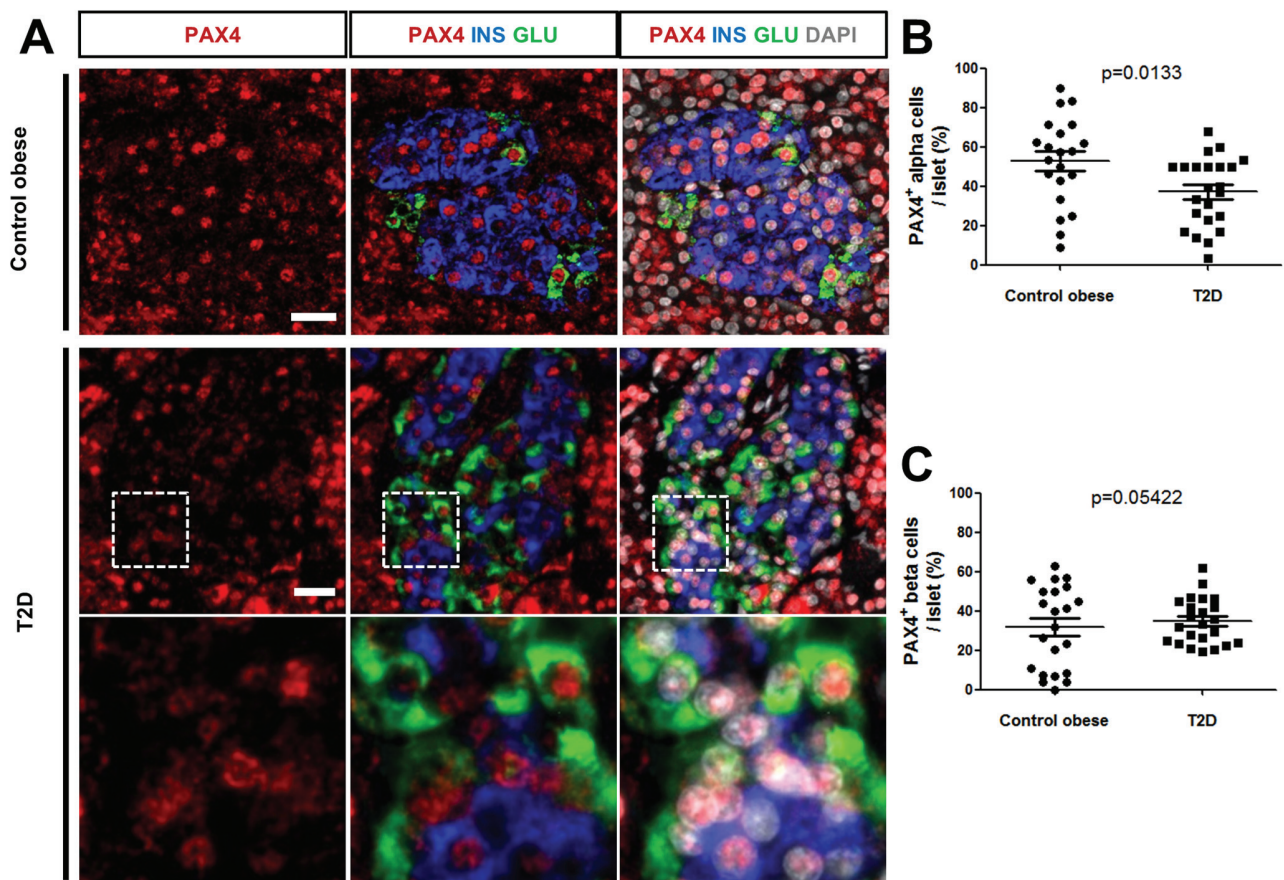


Figure 5. PAX4 expression in islets from subjects with obesity or T2D diabetes. (A) Representative triple IF of PAX4 together with insulin and glucagon showing PAX4-positive islet from control subject with obesity and T2D patients. The lower panel represents a magnified view of the insets in the upper panel. Scale bars = 25 μ m. (B) Overall distribution of PAX4⁺GLU⁺ cells per islet from control subjects with obesity vs 37.2 \pm 3.7% with n = total of 23 islets from 5 T2D patients, p=0.0133 using unpaired two-tailed Student's *t* test. (C) Overall distribution of PAX4⁺INS⁺ cells per islet from control subjects with obesity and T2D patients (31.7 \pm 4.5%, n = total of 22 islets from 4 control subjects with obesity vs 34.8 \pm 2.5% with n = total of 23 islets from 5 T2D patients). Differences in average expression were analyzed using unpaired two-tailed Student's *t* test, and p values were represented on the graphs. doi:10.1371/journal.pone.0072194.g005

with obesity as compared to normal individuals. On the contrary, among tested T2D patients, MAFA expression was completely lost in 2 patients, and shifted to a predominant cytoplasmic localisation in 3 patients (Figure 4A and B). More importantly, this deregulated MAFA nuclear expression was not only detected in beta cells, consistent with what has been reported by Butler *et al* [17], but also concurrently observed in alpha cells in the same samples. It was noticed that islet MAFA expression was not altered in the remaining two T2D patients, suggesting heterogeneity in molecular pathway alterations among the patients. We did not detect any significant change in PAX4 nuclear expression in alpha or beta cell from T2D patients, when compared with the subjects with obesity or control individuals (Figure 5A). However when analyzing the overall distribution of PAX4 expression, we observed a statistically significant decrease in the percentage of PAX4⁺ alpha cells per islet from the T2D cohort compared to individuals with obesity (respectively 37.2 \pm 3.7% vs 52.8 \pm 4.8%) (Figure 5B), whereas there was no significant change in the percentage of beta cells expressing PAX4 per islet in the same patients (Figure 5C).

Discussion

Our present study revealed significantly different PAX4 and MAFA protein expression patterns in human islets compared to what was previously established in rodents. For the first time, our data uncover the expression of PAX4 and MAFA in approximately half of human adult alpha cells, while the expression of both factors is known to be exclusively present in beta cells during adult life in the mouse. Consequently, in humans, one might conclude that PAX4 and MAFA proteins are apparently neither specific markers nor specific transcriptional factors in beta cells.

It is noted that our data are consistent with the previous results from transcriptomic analysis of purified human islets, which indicate that PAX4 and MAFA expression at low transcription levels was indeed perceptible in alpha cell population [9]. The weak expression of both transcription factors in alpha cells and the lack of specific and sensitive tools for protein expression detection may explain at least partially the failure to detect them so far in human alpha cells. The current work confirmed the recent publication by Dai *et al* [10], who used a different anti-human MAFB antibody. Furthermore, by examining nuclear co-localization of PDX1/MAFB, we showed that MAFB is expressed in a

fairly large population of PDX1-expressing cells. The discrepancy between the MAFB positivity in insulin⁺ cells found in our work (42% being the average of percentages of INS⁺MAFB⁺ and PDX1⁺MAFB⁺ staining combined) and that (9%) reported by Dai *et al.* [10] may mainly result from detection and counting methods. In particular, co-nuclear IF immunostaining used in the current study may facilitate a more accurate evaluation. How MAFB works and interacts with other transcriptional factors, MAFA in particular, within beta cells to exert its biological functions remains to be shown.

The data obtained from our study provide compelling evidence demonstrating the existence of subpopulations, in terms of protein expression, among human alpha and beta cells. It would, therefore, be relevant to investigate whether GLU⁺PAX4⁺ cells represent a functionally, and even developmentally, different population than the GLU⁺PAX4⁻ cells that are similar to alpha cells in rodent islets. Similarly, the same question could be applied to GLU⁺MAFA⁺ cells versus GLU⁺MAFA⁻ cells. It would be worth mentioning that, a recent study in rat islets indicated that functionally distinct beta cell sub-populations do exist [18]. Our data, therefore, may provide further molecular basis supporting the presence of these subpopulations and unknown cell plasticity in both alpha and beta cells of the adult human endocrine pancreas. The current finding may also urge further investigations on the capacity of these distinct subpopulations to transdifferentiate into insulin-secreting cells, a phenomenon recently discovered in rodents [15,19]. To this end, it would be critical in the future, material permitting, to isolate and characterize these subpopulations.

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High incidence of mammary intraepithelial neoplasia development in *Men1*-disrupted murine mammary glands

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Abstract

Mutations of the *MEN1* tumour suppressor gene predispose patients to the development of multiple endocrine neoplasia type 1 (MEN1) syndrome, which is characterized by multiple endocrine tumours, including prolactinomas. The recent findings of the interaction between menin, encoded by the *MEN1* gene, and the oestrogen receptor, as well as the observation of rare cases of mammary carcinomas in our heterozygous *Men1* mutant mice, led us to investigate a putative tumour suppressor function of the *Men1* gene in mouse mammary cells by disrupting the gene in luminal epithelial cells. A significantly higher incidence of mammary intraepithelial neoplasia (MIN) was observed in mutant *WapCre-Men1^{F/F}* mice (51.5%) than in *WapCre-Men1^{+/+}* (0%) or *Men1^{F/F}* (7.1%) control mice. The majority of MIN observed in the mutant mice displayed complete menin inactivation. Because of the leakage of *WapCre* transgene expression, prolactinomas were observed in 83.3% of mutant mice, leading to premature death. As there was no correlation between MIN development and elevated serum prolactin levels, and phospho-STAT5 expression was decreased in mammary lesions, the increased incidence of MIN lesions was most likely due to *Men1* disruption rather than to prolactinoma development. Interestingly, in MIN lesions, we found a decrease in membrane-associated E-cadherin and beta-catenin expression, the latter of which is a menin partner. Finally, reduced menin expression was found in a large proportion of two independent cohorts of patients with breast carcinomas. Taken together, the current work indicates a role of *Men1* inactivation in the development of mammary pre-cancerous lesions in mice and a potential role in human mammary cancer.

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Keywords: *Men1*; MIN; mammary neoplasia; adenosquamous carcinoma; prolactinoma; *WapCre* leakage; breast cancer

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No conflicts of interest were declared.

Introduction

Breast cancer is the most common malignancy worldwide and remains the leading cause of cancer-related mortality in women [1]. Only a relatively small proportion of breast cancers (1–2%) arise in individuals who inherit mutations in breast cancer susceptibility genes, such as the *BRCA1* and *BRCA2* genes [2]. Mouse modelling of human breast cancer mimics many essential features of the various types of human breast disease [3]. Over the past 10 years, such modelling has advanced rapidly and become a useful tool for identifying new genes involved in

tumour susceptibility. The *MEN1* gene is a tumour suppressor in a wide range of endocrine and non-endocrine tissues. *MEN1* mutations predispose patients to multiple endocrine neoplasia type 1 (MEN1) syndrome, characterized by the occurrence of multiple endocrine tumours mainly affecting the parathyroid glands, endocrine pancreas, and anterior pituitary gland [4]. Shortly after the identification of the *MEN1* gene, several non-endocrine tumours such as lipomas and angiomas were also reported in MEN1 patients [5,6]. The *MEN1* gene encodes menin, a protein that is primarily nuclear localized and has been shown to interact with a variety of other proteins [7]. In particular,

its interaction with several transcriptional factors and co-factors suggests that menin may act as an adaptor protein that is involved in the regulation of gene expression. Indeed, menin has recently been shown to physically interact with beta-catenin [8,9] and to be required for Wnt/beta-catenin signalling in islet tumour cells [8]. Additionally, Dreijerink *et al.* reported a physical and functional interaction between menin and oestrogen receptor alpha (ER α), the former acting as a co-activator of ER-mediated transcriptional activity through recruitment of a histone methyltransferase complex on ER α target genes [10]. The existence of a link between the *Men1* gene and hormone-dependent tissues was further suggested by a study by Crabtree *et al.* in which one prostate cancer developed in their heterozygous *Men1* mouse cohort [11]. Similarly, three cases of mammary carcinomas and six prostate cancer cases have been observed in our previously analysed cohort of heterozygous *Men1* mutant mice [12,13].

In the present study, we conditionally disrupted the *Men1* gene in luminal mammary epithelial cells in mice to study the effects of menin inactivation on mammary tissues. We found that a significantly higher proportion of mutant mice developed mammary intraepithelial neoplasia (MIN), which suggests that *Men1* disruption has a role in the predisposition of mice to pre-cancerous mammary lesions. Interestingly, a decrease in menin expression was also observed in a substantial proportion of breast cancers in two independent cohorts of patients.

Materials and methods

Transgenic mice

Heterozygous *Men1* mutant mice and mice carrying two floxed alleles of the *Men1* gene (*Men1*^{F/F}) were generated in a mixed C57BL6-129/Sv genetic background as previously described [14]. *WapCre* transgenic mice were obtained from the Human Cancers Consortium Mouse Repository [B6.Cg-Tg(*WapCre*)11738Mam, strain number 01XA8] [15] and they expressed Cre under the control of the whey acidic protein promoter. Mice were maintained on a 12-h light/12-h dark cycle with free access to water and standard mouse chow and housed in pathogen-free facilities. Mammary samples from *Men1*^{loxP/loxP}/*Rip-Cre*⁺ female mice [16] were harvested by M Auret from Dr Nick Hayward's team at the Queensland Institute of Medical Research, Australia and examined histologically by our pathologist. Genotyping and recombination analysis are described in the Supplementary materials and methods.

All animal experiments were approved by the Regional Animal Experiments Committee of Centre National de la Recherche Scientifique (CNRS) and were conducted in accordance with the ethical guidelines.

Histology and serum prolactin dosage

Female mice were exclusively used in the experiments. Dissected organs were fixed in 4% neutral buffered formalin for at least 24 h at room temperature and were then embedded in paraffin and sectioned at 3 μ m. They were stained with H&E and microscopic histological analysis was performed by a pathologist (L Frappart), including the samples from *Men1*^{loxP/loxP}/*Rip-Cre*⁺ female mice. Histopathological analyses were based on the criteria described by the Annapolis Pathology Panel [3]. The classification of the World Health Organization was also taken into account. Immunohistochemical (IHC) staining and 5-bromo-2'-deoxyuridine incorporation detection were performed as described in the Supplementary materials and methods. Blood was collected from control and mutant females by intracardiac puncture immediately after cervical dislocation, and serum was isolated. The circulating prolactin level was measured using a mouse/rat Prolactin ELISA kit (Calbiotech, Inc. San Diego, California, USA) following the manufacturer's instructions.

Western blot analysis of human breast cancers

A panel of 26 specimens of primary human breast tumours, collected before therapy from patients who were diagnosed between 1992 and 1999 and underwent surgery at the Centre Léon Bérard (Lyon, France), was obtained from the Biological Resources Department of Centre Léon Bérard (French agreement number DC-2008-99).

TMA analysis of human breast cancers

Breast cancers were obtained from 121 patients (M0) who underwent surgery at the Centre Léon Bérard (CLB) in 1998 and 1999. The corresponding formalin-fixed, paraffin-embedded tumours were inserted as triplicates (600 μ m each) into four tissue micro-array (TMA) blocks. Menin expression was assessed using IHC; the intensity of nuclear staining was graded as either 1 (low) or 2 (normal). Distribution of clinical parameters was compared between patients showing low or normal menin nuclear staining. Overall survival (OS), defined as the time from diagnosis to death or date of last follow-up, and progression-free survival (PFS), defined as the time from diagnosis to death or progression or to date of last follow-up (for censored patients), were studied. Survival distributions were estimated using the Kaplan–Meier method and compared between menin level groups using the log-rank test. Details are available in the Supplementary materials and methods.

Results

Menin expression in adult mammary glands

To investigate the possible role of the *Men1* gene in mammary glands, we first used immunohistochemistry to analyse menin expression during the postnatal

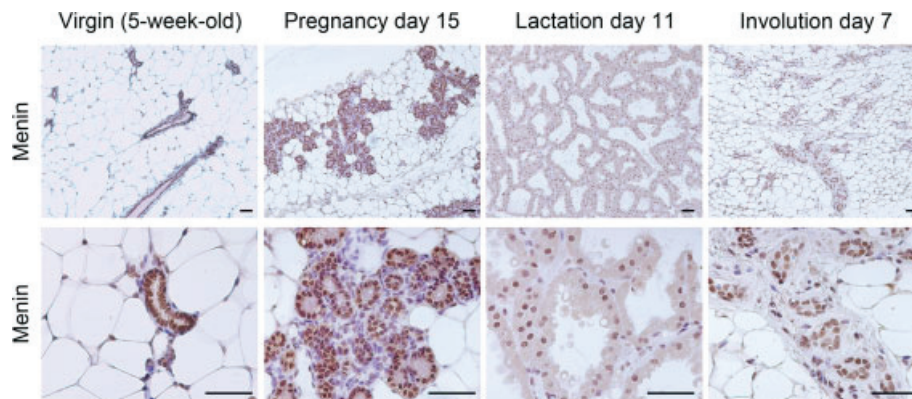


Figure 1. Menin is expressed in mammary epithelial cells during different stages of postnatal mammary gland development. Immunohistochemical detection of menin expression was performed in 5-week-old virgin, 15-day pregnant, 11-day lactating, and 7-day involuting mammary glands from *Men1* wild-type female mice. Predominantly nuclear expression of menin was detected both in luminal and in myoepithelial basal cells in the mammary epithelium, as well as in adipocytes, but not in the surrounding stromal cells. The lower panel is a four-fold magnification of the upper panel. Scale bars = 50 μ m.

development of mammary glands in wild-type female mice (Figure 1). Menin expression was found both in the luminal and in the basal (myoepithelial) cell layer of the mammary epithelium, showing predominantly nuclear staining, in 5-week-old virgin mice as well as in mice during pregnancy, lactation, and involution of the mammary glands.

Breast cancers found in heterozygous *Men1* mutant mice

Three cases of breast cancers were previously detected among 36 heterozygous *Men1* mutant mice older than 19 months of age (8.3%) [12]. Menin expression was completely lost in two of these cancers (Supplementary Figure 1A), whereas a mammary cancer cell line that has been successfully established from the third mammary gland cancer, named TS1, displayed loss of the remaining wild-type *Men1* allele and of menin expression (Supplementary Figures 1B and 1C).

Mammary-specific *Men1* disruption

To further study the role played by menin in mammary cells, we decided to disrupt the *Men1* gene specifically in mammary epithelial cells. For this purpose, mice carrying floxed *Men1* alleles (*Men1*^{F/F}) [14] were crossed with *WapCre* transgenic mice expressing Cre recombinase under the control of the whey acidic protein (*Wap*) promoter, known to be expressed specifically in luminal mammary epithelial cells during pregnancy and lactation (Figure 2A) [15]. To check the mammary specificity of *WapCre*-mediated *Men1* disruption, we collected mammary glands from 3-month-old virgins and from parous female mice at lactation day 15 after their first, second or third pregnancy. *WapCre*-mediated *Men1* disruption was validated by the detection of PCR fragment shift, which was specific for the *Men1* deleted allele (Δ), resulting from the excision of the floxed exon 3 (Figure 2B). As expected, *Men1* disruption occurred in the mammary

glands of the *WapCre-Men1*^{+F} mice having undergone pregnancies, as illustrated by the increased intensity of the Δ -specific PCR product. Of the three virgin *WapCre-Men1*^{+F} mice analysed, one presented with unexpected, but weak Δ -specific PCR product, indicating the *WapCre* transgene leakage in the mammary glands. As previously described [15], the leakage of this transgene was observed in the brains in all tested mice. Surprisingly, we also noted previously undocumented *WapCre* leakage in the pituitary glands of two of the three tested mice (Figure 2B). *Men1* disruption was not detected in any of the other tested organs (data not shown). *Men1* disruption in mammary cells was further assessed in *WapCre-Men1*^{F/F} mice by menin detection with IHC. Loss of menin expression was seen in a subset of mammary luminal epithelial cells in virgin mice (10–20%, data not shown), in approximately half of luminal cells during the first pregnancy, and in nearly 90% of luminal cells after the third pregnancy (Figure 2C).

Normal mammary gland development in *WapCre-Men1*^{F/F} mice

To investigate whether *Men1* disruption had an impact on the physiology of the mammary glands, we performed morphological analysis of both those glands from the *Men1*^{F/F} and *WapCre-Men1*^{F/F} virgin female littermates at 3 months of age, at day 15 of pregnancy (P15), and 7 days after weaning (involution day 7, I7) for up to three pregnancies (G1, G2, G3). Although menin inactivation occurred in a substantial proportion of the cells (see above) in the mutant mice, we did not notice any macroscopic or microscopic morphological differences between control and mutant mice (Figure 3A). Consistent with these observations, *WapCre-Men1*^{F/F} female mice were able to breastfeed their pups and all pups showed normal survival and growth (data not shown). To assess whether *Men1* disruption resulted in epithelial proliferation modifications, we evaluated the incorporation of BrdU into

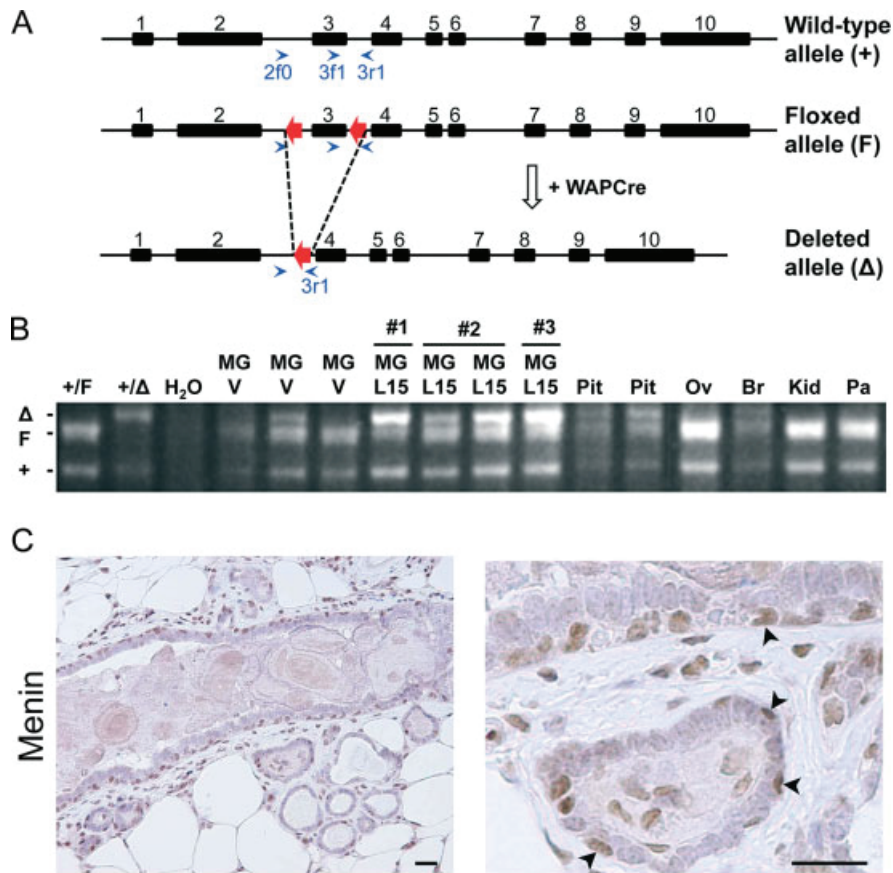


Figure 2. *Men1* conditional ablation in mouse mammary luminal cells. (A) Structure of the wild-type (+), floxed (F), and deleted (Δ) murine *Men1* alleles. Exons are indicated by black boxes with corresponding numbers. Red arrows represent the *loxP* sequences. Blue arrowheads indicate the position of the primers used for PCR genotyping and recombination analyses (3f1, 3r1, 2f0). (B) PCR amplification of wild-type (+, 215 bp), floxed (F, 280 bp), and deleted (Δ , 300 bp) *Men1* allele fragments with genomic DNA extracted from different organs collected from *WapCre-Men1^{+F}* female mice. Mammary glands were obtained from three different virgin mice (MG V) and 15-day lactating mice during their first (#1 MG L15), second (#2 MG L15) or third (#3 MG L15) pregnancy. The *Men1* deleted (Δ) allele is detected as expected in mammary glands but also in the brains and pituitary glands of these mice. Genomic DNA extracted from the tails of *Men1^{+F}* (+/F) and *Men1^{+Δ}* (+/Δ) mice was used as a control. MG = mammary gland; Pit = pituitary gland; Ov = ovary; Br = brain; Kid = kidney; Pa = pancreas. (C) Representative images of menin IHC in the mammary gland from a 12-month-old *WapCre-Men1^{F/F}* female mouse (after three rounds of pregnancy). The panel on the right is a four-fold magnification of the left panel and shows that only basal myoepithelial cells remain positive for menin expression (arrowheads). Scale bar = 100 μ m.

the mammary glands of age-matched controls and mutant littermate mice during the first and the third pregnancy (Figures 3B and 3C). No significant difference in the number of BrdU-positive epithelial cells between *WapCre-Men1^{F/F}* and *Men1^{F/F}* female mice was detected. Taken together, *WapCre-Men1^{F/F}* female mice did not show any detectable consequence of *Men1* disruption on morphological development, physiology, or mammary gland proliferation during pre- or post-pubertal development.

High frequency of mammary intraepithelial neoplasia in *WapCre-Men1^{F/F}* mice

The effect of *Men1* inactivation in mammary gland tumorigenesis was studied in a cohort of 120 multiparous female mice. To induce *WapCre* transgene expression, 66 *WapCre-Men1^{F/F}* female mice, as well as 28 *Men1^{F/F}* and 26 *WapCre-Men1^{+/+}* female mice as the two control groups, underwent three rounds

of pregnancy. Females were then monitored over a 20-month period and necropsied at different age ranges, specifically 9–12, 12–15 and > 15 months of age. As summarized in Table 1, control *WapCre-Men1^{+/+}* mice developed no mammary lesions at any age. Additionally, control *Men1^{F/F}* mice showed a low but constant incidence of mammary intraepithelial neoplasia (MIN), with one case in each of the two older age groups (10% and 8.3%, respectively), but none in the youngest age group. On the other hand, MIN lesions were found in 38.5% of mutant mice in the 9–12 months age group. The incidence increased with age and reached 48.6% and 66.7%, respectively, in both the 12–15 and the > 15 months age groups, with 4.8-fold ($p = 0.0548$, non-significant) and 8-fold ($p = 0.0057$) increases compared with the corresponding age groups of the control *Men1^{F/F}* mice. Starting at 12 months of age, *WapCre-Men1^{F/F}* mutant mice had an extremely high mortality rate, due to pituitary tumour development (see below). Only two mutant mice survived beyond 18 months

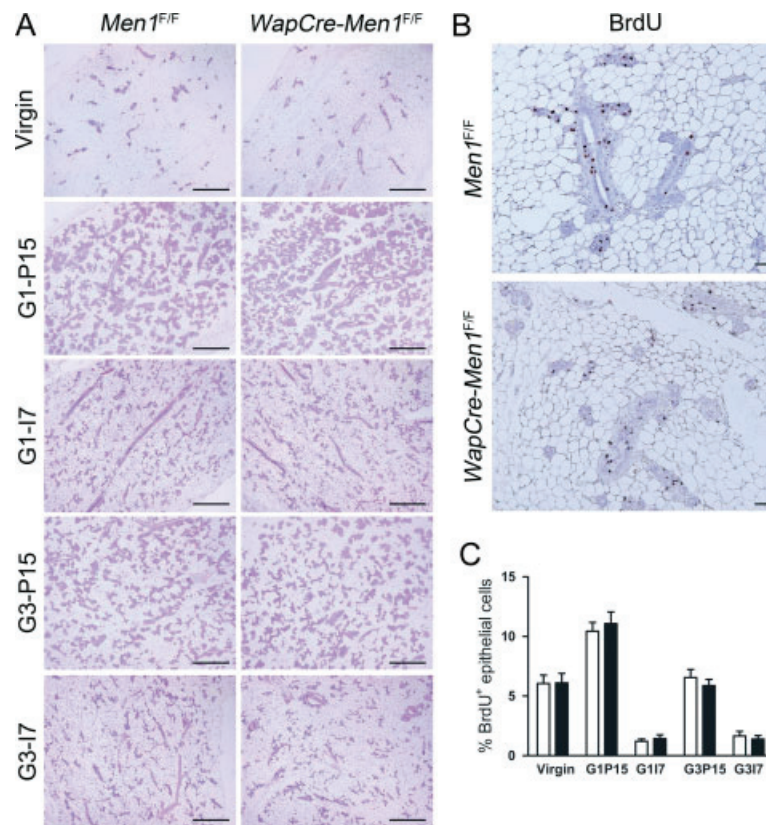


Figure 3. Normal mammary gland development and proliferation in young *WapCre-Men1^{F/F}* female mice. (A) H&E-stained sections of mammary glands collected from 3-month-old virgin control *Men1^{F/F}* and mutant *WapCre-Men1^{F/F}* age-matched female mice, as well as those during their first (G1) and third (G3) pregnancies, 15 days after observation of the vaginal plug (P15) and 7 days after pup weaning (I7). Note that control and mutant mice present a similar mammary gland structure at all stages of postnatal development. Scale bar = 200 μ m. (B) Representative images of BrdU incorporation in mammary glands harvested from control *Men1^{F/F}* and mutant *WapCre-Men1^{F/F}* age-matched virgin female mice. Scale bar = 50 μ m. (C) The percentage of BrdU-positive epithelial cells was calculated as described in the Materials and methods section. Bars represent the mean percentage \pm SEM for two mice of each genotype. The percentage of BrdU-positive epithelial cells was not significantly different ($p=0.9866$, two-way ANOVA) between *Men1^{F/F}* (white bars) and *WapCre-Men1^{F/F}* (black bars) mice, neither in virgin mice (6.062 ± 0.699 versus 6.103 ± 0.809) nor during their first and third pregnancies (G1P15: 10.44 ± 0.736 versus 11.075 ± 0.98 ; G3P15: 6.561 ± 0.666 versus 5.884 ± 0.528) or involution (G1I7: 1.206 ± 0.182 versus 1.437 ± 0.317 ; G3I7: 1.656 ± 0.384 versus 1.395 ± 0.294).

Table 1. Age-related incidence of mammary lesions in *WapCre-Men1^{F/F}* females

Genotype	Histology	9–12 months	12–15 months	> 15 months	Overall incidence
<i>WapCre-Men1^{+/+}</i>	Normal	9/9 (100%)	8/8 (100%)	9/9 (100%)	26/26 (100%)
	MIN	0/9 (0%)	0/8 (0%)	0/9 (0%)	0/26 (0%)
	AdsC	0/9 (0%)	0/8 (0%)	0/9 (0%)	0/26 (0%)
<i>Men1^{F/F}</i>	Normal	6/6 (100%)	8/10 (80%)	10/12 (83.4%)	24/28 (85.7%)
	MIN	0/6 (0%)	1/10 (10%)	1/12 (8.3%)	2/28 (7.1%)
	AdsC	0/6 (0%)	1/10 (10%)	0/12 (0%)	1/28 (3.6%)
	PdC	0/6 (0%)	0/10 (0%)	1/12 (8.3%)	1/28 (3.6%)
<i>WapCre-Men1^{F/F}</i>	Normal	8/13 (61.5%)	16/35 (45.7%)	6/18 (33.3%)	30/66 (45.5%)
	MIN	5/13 (38.5%)	17/35 (48.6%) [*]	12/18 (66.7%) [†]	34/66 (51.5%) [†]
	AdsC	0/13 (0%)	2/35 (5.7%)	0/18 (0%)	2/66 (3%)

Mammary lesions were classified according to the recommendations of the Annapolis Pathology Panel [2]. MIN = mammary intraepithelial neoplasia; AdsC = adenocarcinoma; PdC = poorly differentiated carcinoma. The number of mice of each histological category over the total number of the mice examined is indicated and the corresponding percentage is shown in parentheses.

p values (chi-square test *WapCre-Men1^{F/F}* versus *Men1^{F/F}*): *0.0548; [†]0.0057; [‡]< 0.0001.

of age and both developed MIN lesions. The overall incidence of MIN lesions in *WapCre-Men1^{F/F}* mutant mice was found to be significantly (7.2-fold) higher than that in *Men1^{F/F}* control mice ($p < 0.0001$), with rates of 51.5% and 7.1%, respectively. The histological examination also revealed the occurrence of

adenocarcinoma in one *Men1^{F/F}* and two *WapCre-Men1^{F/F}* mice (non-significant difference). A poorly differentiated infiltrating carcinoma was also detected in a *Men1^{F/F}* mouse.

MIN lesions, known to be potential precursor lesions of mammary cancer, are focal or multifocal areas

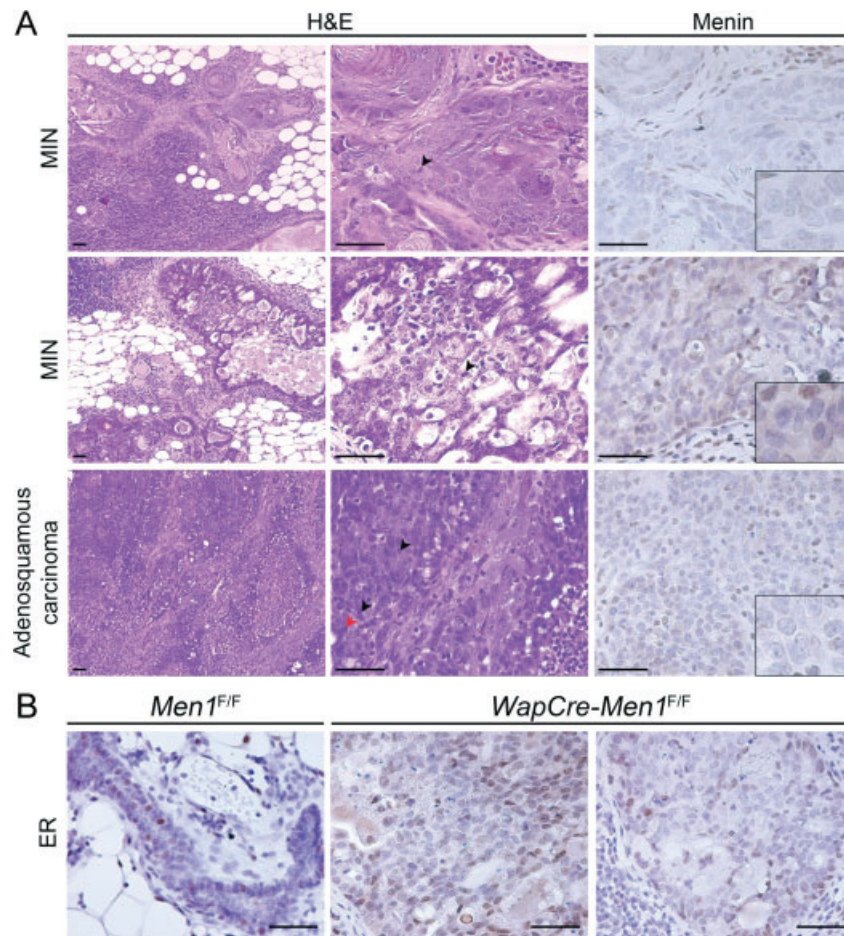


Figure 4. *WapCre-Men1^{F/F}* female mice develop mammary neoplasia showing menin inactivation and reduced ER expression. (A) Representative histological sections (H&E staining, left and middle columns) and IHC detection of menin (right column) for two mammary intraepithelial neoplasia (MIN) lesions and an adenosquamous carcinoma found in *WapCre-Men1^{F/F}* mice (18-, 14-, and 13-month-old mice, respectively). MIN lesions display intraluminal proliferation of cells with nuclear atypia (nuclear enlargement and chromatin clumping) and prevalent mitoses (black arrowheads). Adenosquamous carcinoma is characterized by the association of glandular tumour tissue with clusters of squamous metaplasia and signs of invasion into the surrounding stroma. The red arrowhead indicates a tripolar atypical mitosis. The middle and right columns are four-fold magnification of the first column. Insets show an amplified view of a portion of menin-immunostained mammary lesion illustrating the complete loss of menin expression in neoplastic cells, while basal myoepithelial cells remain menin-positive. Scale bar = 50 μ m. (B) In normal mammary glands from multiparous 12-month-old *Men1^{F/F}* control mice, ER was detected in approximately 20–30% of epithelial cells but was decreased in lesions detected in *WapCre-Men1^{F/F}* mice ($n = 7$). Representative images of two MIN from multiparous *WapCre-Men1^{F/F}* mice (14- and 17-month-old mice, respectively) are presented. Scale bar = 50 μ m.

of intraluminal epithelial proliferation with significant nuclear atypia (large pleomorphic nuclei with hyperchromasia) and frequent mitosis, but they do not show evidence of invasion [3] (Figure 4A, top and middle panels). Taken together, we can conclude that the development of MIN lesions frequently occurred in the mutant mice, whereas mammary carcinomas appeared to be rare and non-significant events in these mice.

Menin expression was absent in 10/13 MIN lesions and in the two adenosquamous carcinomas that developed in the *WapCre-Men1^{F/F}* mice (Figure 4A, right column). All three tested MIN lesions and the two carcinomas found in the *Men1^{F/F}* mice demonstrated menin expression (data not shown). Overall, our data indicate that *Men1* ablation in mammary epithelial cells significantly exacerbated the development of

pre-cancerous MIN lesions, whereas *Men1^{F/F}* female mice are only prone to low-incidence spontaneous MIN and carcinoma lesions. By IHC analysis of ER α , both the intensity and the number of ER α -positive cells were clearly reduced (Figure 4B) in MIN lesions of mutant mice compared with normal mammary glands.

Prolactinoma development in *WapCre-Men1^{F/F}* female mice secondary to pituitary leakage of the *WapCre* transgene

WapCre-Men1^{F/F} mice were found to die significantly earlier (mean survival: 14 months) than the other two genotypes (mean survival: 21 months) (Figure 5A). Necropsies identified that the cause of death was highly haemorrhagic pituitary adenomas, consistent with the detected *WapCre* leakage in the pituitary.

Pituitary tumour development started at approximately 9 months of age in the *WapCre-Men1^{F/F}* mice, reaching an incidence of 61.5% at 9–12 months, 94.3% at 12–15 months, and 77.8% at > 15 months (Table 2). The pituitary tumours were shown to originate from the pars distalis by histological examination (Figure 5B). Immunohistochemistry of menin expression confirmed that the *Men1* gene was completely inactivated. The expression of growth hormone was almost completely absent in these tumours, while the number of prolactin-secreting cells was largely increased, indicating prolactinomas. The serum prolactin level in *WapCre-Men1^{F/F}* mice was two-fold higher than that of control mice (Figure 5C). Our results showed that menin inactivation, caused by pituitary leakage of the *WapCre* transgene, triggers prolactinoma development in *WapCre-Men1^{F/F}* mice, which is associated with hyperprolactinaemia and leads to the premature death of these mice.

Increased incidence of MIN in *WapCre-Men1^{F/F}* mice is not completely explained by pituitary tumour development

Prolactin is a well-known stimulating signal for rodent mammary epithelial cell proliferation [17], whereas its importance in the development of human breast cancer remains controversial. A number of mouse models have described a potential oncogenic role of constitutively activated prolactin signalling in mammary glands [18]. To assess the potential effect of increased serum prolactin levels on the incidence of MIN observed in our mutant mice, we compared the mean levels of serum prolactin between *WapCre-Men1^{F/F}* mice without any detected mammary lesion and *WapCre-Men1^{F/F}* mice that developed mammary lesions (Figure 5D). We did not observe a significantly higher level of prolactin in mice with mammary lesions, suggesting the absence of a correlation between the occurrence of mammary lesions and hyperprolactinaemia. Furthermore, when excluding the mutant mice with hyperprolactinaemia from the analysis of MIN incidence (Supplementary Table 1), the incidence of MIN lesions in mutant mice remained virtually the same. The activation of the prolactin signalling pathway is usually assessed by the tyrosine residue phosphorylation of the transcription factor STAT5 (signal transducer and activator of transcription 5) [19]. Therefore, we analysed phospho-STAT5 expression by immunohistochemistry in the MIN lesions that had developed in *WapCre-Men1^{F/F}* mice (Figure 5E). When compared with the intensity of staining in normal mammary glands from *Men1^{F/F}* mice, we found that the level of phospho-STAT5 expression in the MIN lesions from *WapCre-Men1^{F/F}* mice was significantly decreased rather than increased (Figure 5F).

To confirm that the increase in MIN lesions observed in our *WapCre-Men1^{F/F}* mice could not be simply considered a consequence of the hyperprolactinaemia

detected in these mice, we analysed mammary glands from another *Men1* mutant mouse model that developed prolactinomas [16]. In the *Men1^{loxP/loxP}/Rip-Cre⁺* mice, the authors observed a pituitary leakage of the *Rip-Cre* transgene (rat insulin promoter), which also led to the development of prolactinomas. In that model, the expression of menin protein was intact in the mammary glands. Histopathological examination of the mammary glands was performed in a group of 20 *Men1^{loxP/loxP}/Rip-Cre⁺* female mice 9–16 months of age and six *Men1^{loxP/loxP}* control mice (age: 11–12 months). Among the 20 *Men1^{loxP/loxP}/Rip-Cre⁺* female mice, four developed MIN lesions (20%, Table 3 and Supplementary Figures 2A–2F), which was not statistically significant when compared with control mice ($p = 0.38$). Hence, our *WapCre-Men1^{F/F}* female mice developed 2.5 times more MIN lesions than the *Men1^{loxP/loxP}/Rip-Cre⁺* mice, and the difference in the rates was statistically significant ($p = 0.0107$). Taken together, the above data suggest that the increased incidence of MIN lesions in *WapCre-Men1^{F/F}* mice is likely to be independent of prolactinoma development, thereby evoking a more direct role of *Men1* inactivation in the development of these lesions.

Altered membrane expression of both beta-catenin and E-cadherin in MIN and carcinomas that developed in *WapCre-Men1^{F/F}* mice

The inactivation of cell–cell adhesion factors and the activation of the Wnt/beta-catenin signalling pathway have both been described to promote mammary tumorigenesis, the latter being particularly related to the development of mammary squamous metaplasia [20,21]. Therefore, we investigated whether E-cadherin expression and beta-catenin expression were altered in the observed MIN lesions. In contrast to the prominent cell membrane expression of both beta-catenin (Figure 6A) and E-cadherin (Figure 6G) in the mammary glands of control mice, cell membrane expression of both beta-catenin and E-cadherin was nearly absent in the mammary lesions of *WapCre-Men1^{F/F}* mice, with only residual cytoplasmic expression (Figures 6D–6F, 6H, and 6I). Decreased cell membrane expression of both beta-catenin and E-cadherin was also observed in lesions from *Men1^{F/F}* control mice, often accompanied by the nuclear activation of beta-catenin in peripheral cells (Figures 6B and 6C). We further analysed beta-catenin and E-cadherin expression upon menin down-regulation by *Si-MEN1* in MCF7 cells using western blot. Menin partial inactivation resulted in decreased E-cadherin expression in MCF7 cells (Figure 6J), whereas no altered beta-catenin expression was detected (data not shown). Interestingly, neither E-cadherin (Figure 6J) nor beta-catenin (data not shown) was detected in the TS1 cell line derived from the mouse *Men1* breast cancer.

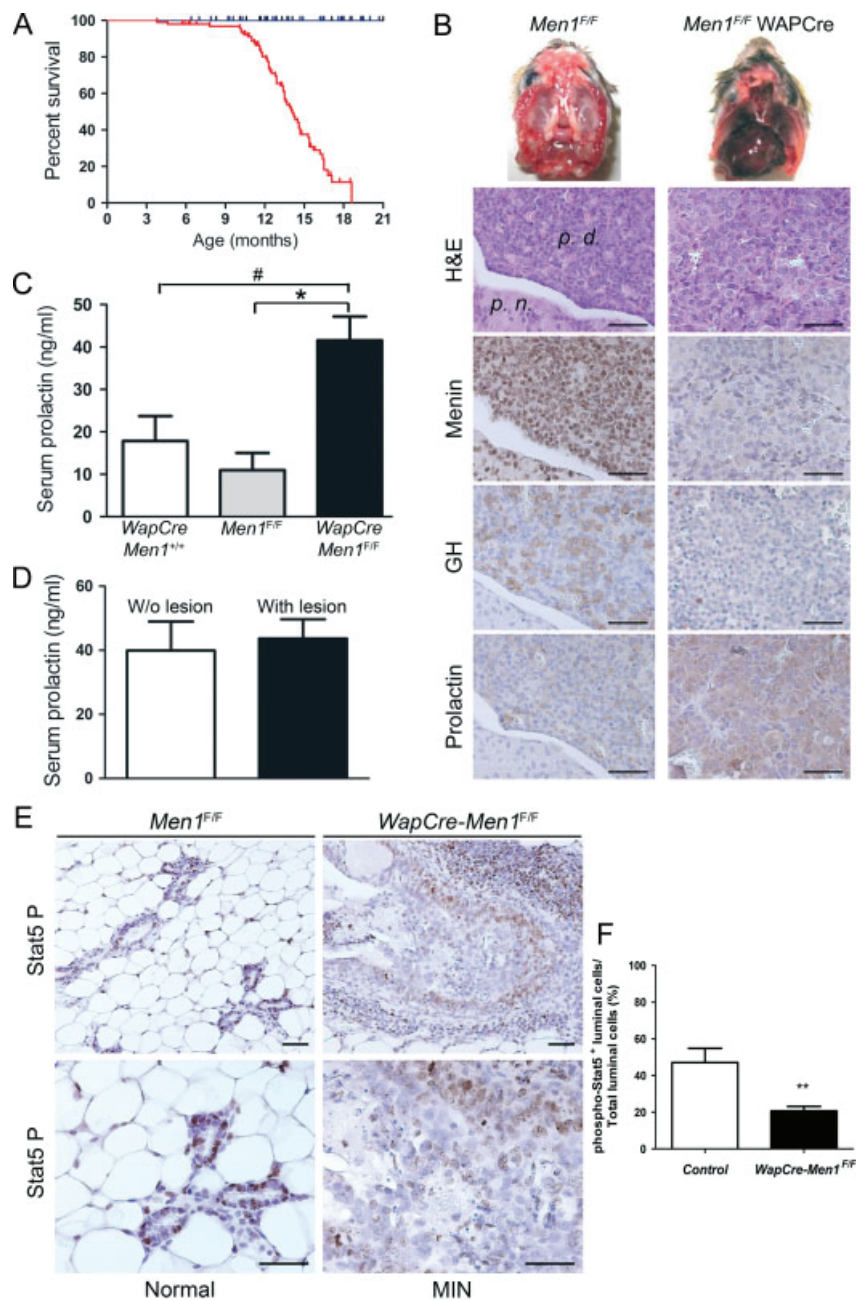


Figure 5. Pituitary leakage of the *WapCre* transgene leads to the development of prolactinomas in *WapCre-Men1^{F/F}* female mice, which does not completely explain the increased incidence of MIN lesions. (A) Kaplan–Meier analysis determining the survival ratio of female *Men1^{F/F}* (blue line, $n = 34$), *WapCre-Men1^{+/+}* (black line, $n = 27$), and *WapCre-Men1^{F/F}* mice (red line, $n = 75$) according to age. Ill and distressed mice that were killed or died prematurely were counted as a death event. Mice sacrificed at the pre-determined time points were considered to be alive. *WapCre-Men1^{F/F}* mice were found to die significantly earlier due to pituitary tumours, with a mean survival of approximately 14 months, compared with approximately 21 months for the other two genotypes (log-rank test, $p < 0.0001$). (B) The pituitary adenomas that developed in *WapCre-Men1^{F/F}* mice were prolactinomas. H&E staining and immunohistochemical detection of menin, growth hormone (GH), and prolactin expression were performed using a normal pituitary gland from a control 19-month-old *Men1^{F/F}* mouse (upper panel) and a pituitary adenoma found in a 12-month-old *WapCre-Men1^{F/F}* mouse (lower panel). p.d. = pars distalis; p.n. = pars nervosa. Scale bar = 50 μ m. (C) Mean serum prolactin concentration was significantly increased in *WapCre-Men1^{F/F}* (41.55 ng/ml \pm 5.653, $n = 32$) compared with *Men1^{F/F}* (11.00 ng/ml \pm 4.008, $n = 10$) and *WapCre-Men1^{+/+}* mice (17.84 ng/ml \pm 5.885, $n = 10$). # $p = 0.0323$; * $p = 0.0056$ (unpaired two-tailed t -test). (D) Elevated prolactin levels do not correlate with mammary lesion development in *WapCre-Men1^{F/F}* mice. Mean serum prolactin concentrations in *WapCre-Men1^{F/F}* mice over 9 months of age either without (w/o) mammary lesions (39.91 ng/ml \pm 9.045, $n = 18$) or with mammary lesions (43.66 ng/ml \pm 6, $n = 14$) were not significantly different ($p = 0.7478$, unpaired two-tailed t -test). (E) IHC detection of phospho-STAT5. Representative images of normal mammary glands and a MIN lesion from a 13-month-old control mouse and a 14-month-old *WapCre-Men1^{F/F}* mouse, respectively, are shown. Scale bar = 50 μ m. (F) Quantitation of luminal cells expressing phospho-STAT5 in control *WapCre-Men1^{+/+}* mice (47.01% \pm 7.809, $N = 5$) and *WapCre-Men1^{F/F}* mutant mice (20.69 \pm 2.444, $N = 6$). At least 4900 luminal cells were counted for each group. Data are represented as the mean percentage \pm SEM (** $p = 0.0069$, unpaired two-tailed t -test).

Table 2. Age-related incidence of pituitary adenomas in *WapCre-Men1^{F/F}* females

Genotype	Pituitary histology	9–12 months	12–15 months	> 15 months	Overall incidence
<i>WapCre-Men1^{+/+}</i>	Normal	0/9 (0%)	0/8 (0%)	0/9 (0%)	0/26 (0%)
<i>Men1^{F/F}</i>	Normal	0/6 (0%)	0/10 (0%)	0/12 (0%)	0/28 (0%)
<i>WapCre-Men1^{F/F}</i>	Normal	5/13 (38.5%)	2/35 (5.7%)	4/18 (22.2%)	11/66 (16.7%)
	Adenoma	8/13 (61.5%)	33/35 (94.3%)	14/18 (77.8%)	55/66 (83.3%)

The number of mice of each histological category over the total number of the mice examined is indicated and the corresponding percentage is shown in parentheses.

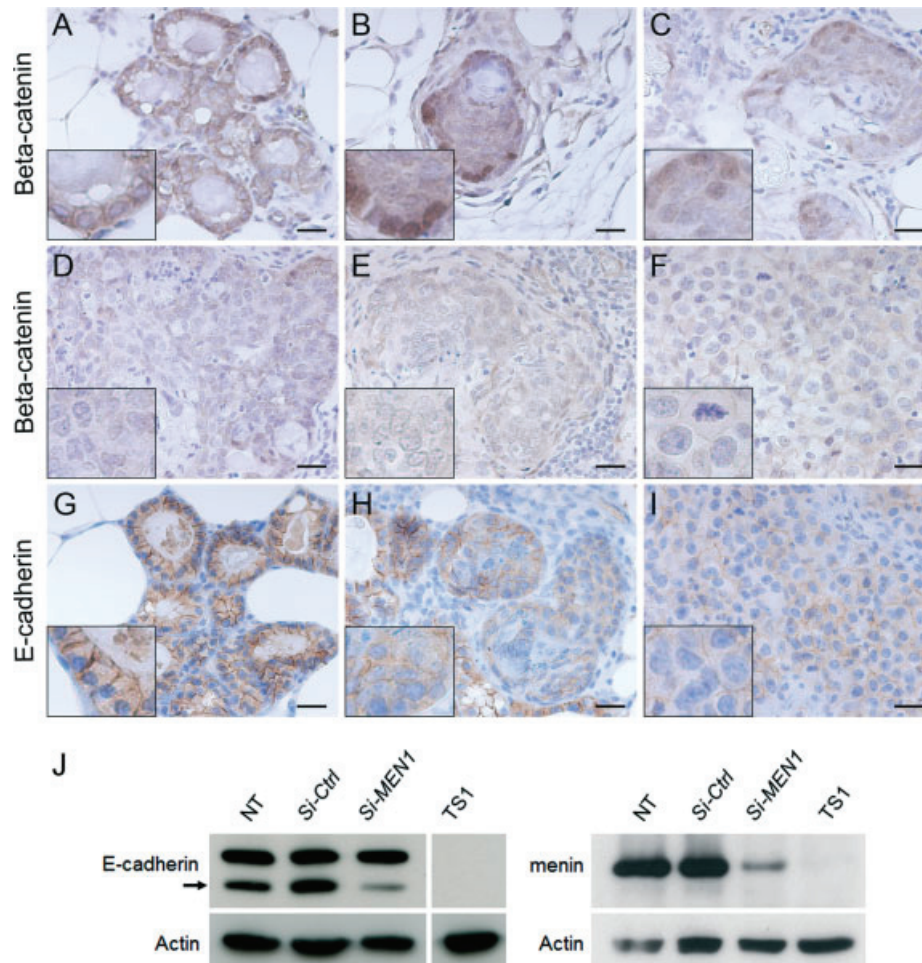


Figure 6. Decreased membrane expression of beta-catenin and E-cadherin in MIN lesions and adenosquamous carcinomas in *WapCre-Men1^{F/F}* mice. (A–F) Beta-catenin staining was mainly cell membrane-localized with some cytoplasmic localization in normal mammary glands from control mice (A, an 18-month-old *Men1^{F/F}* mouse), whereas nuclear beta-catenin staining could be detected in MIN lesions that developed in *Men1^{F/F}* mice (B, 8-month-old mouse). In contrast, membrane and cytoplasmic beta-catenin expression was decreased in MIN lesions and adenosquamous carcinomas that developed in *WapCre-Men1^{F/F}* mice. Representative photographs show beta-catenin staining in three MIN lesions (C, D, and E are from an 18-month-old, a 17-month-old, and a 12-month-old mouse, respectively) and an adenosquamous carcinoma (F, 13-month-old mouse) from the mutant mice. (G–I) E-cadherin staining was localized to the cell membrane of normal mammary glands from control mice (G, 12-month-old *Men1^{F/F}* mouse). Membrane expression of E-cadherin was decreased in MIN lesions (H, 12-month-old mouse) and adenosquamous carcinomas (I, 13-month-old mouse) that developed in *WapCre-Men1^{F/F}* mice. The insets show an amplified view of a section of the mammary lesions. Scale bar = 100 μ m. (J) Western blot analysis of E-cadherin expression in MCF7 cells transfected by either non-targeting siRNA (Si-Ctrl) or anti-MEN1 siRNA (Si-MEN1). NT = not transfected. Cytoplasmic protein extracts were used for E-cadherin detection and mixed cytoplasmic and nuclear protein extracts were used for menin detection. The mature E-cadherin form is indicated by the arrow. Protein loading was monitored by actin. The analysis was repeated twice.

Reduced menin expression in human breast cancers

The increased incidence of MIN lesions in *WapCre-Men1^{F/F}* mice prompted us to investigate menin expression in human sporadic breast cancers. First, we determined menin expression levels by IHC and found that 95 of 121 tumours (78.5%) displayed low menin expression (Figures 7A–7C). We also

performed western blotting to analyse menin expression in another independent panel of 26 sporadic breast cancers. Compared with menin expression levels in normal human breast tissue, four of the tested cancer samples (15%) showed a sharp decrease in menin expression (Figure 7D). Statistical analyses of the TMA data showed that neither age, menopausal

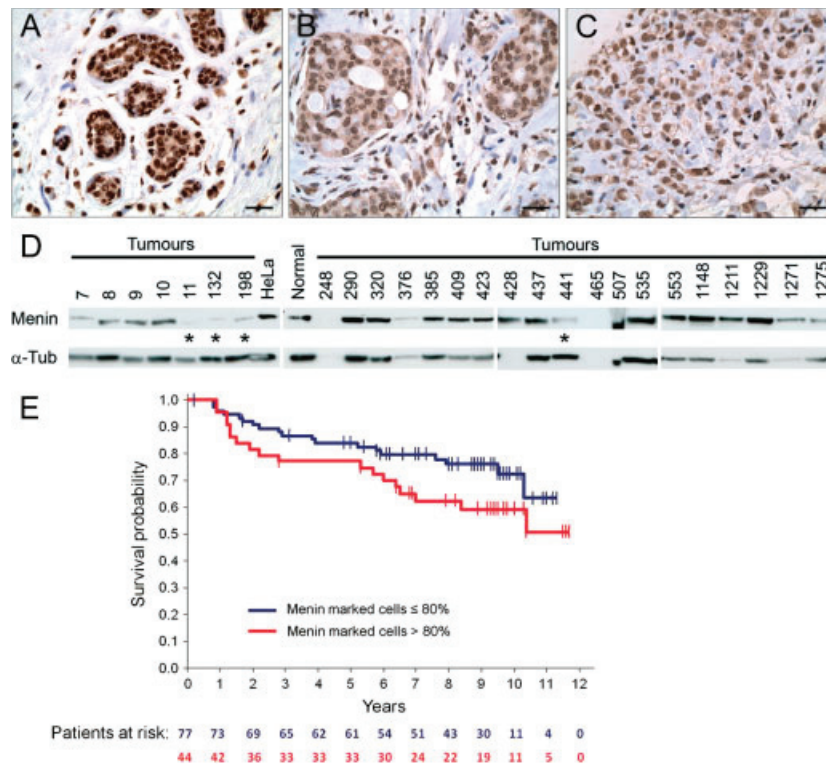


Figure 7. Menin expression is reduced in a large proportion of human breast cancers. (A–C) Representative images of menin IHC in normal human mammary gland (A: staining intensity 2) and of reduced menin expression in patients with breast cancer (B, C: staining intensity 1). (D) Menin detection by western blot in a panel of 26 ER-positive tamoxifen-treated breast cancer patients. * marks the samples with clearly reduced menin expression. (E) Comparison of Kaplan–Meier estimates for overall survival in breast cancer patients included in the TMA analysis showing the survival distribution curves between tumours showing normal expression of menin (staining intensity 2, red curve) and low menin nuclear staining (intensity 1, blue curve). Corresponding numbers of patients at risk are indicated below the curves.

Table 3. Incidence of mammary lesions in *Men1^{loxP/loxP}/Rip-Cre⁺* female mice developing prolactinomas

Normal	16/20 (80%)
MIN	4/20 (20%)
Carcinoma	0/20 (0%)

Mammary lesions were classified according to the recommendations of the Annapolis Pathology Panel [2]. MIN = mammary intraepithelial neoplasia. The number of mice of each histological category over the total number of the mice examined is indicated and the corresponding percentage is shown in parentheses. Analysed females were aged from 9 to 16 months.

status, histological grade, lymph node involvement, hormonal status nor cancer subtype was significantly correlated with low menin expression (Table 4). Furthermore, neither overall survival (OS, log-rank test $p = 0.5181$) nor progression-free survival (PFS, log-rank test $p = 0.3702$) was significantly different between low (intensity grade 1) or normal (intensity grade 2) menin-staining tumour groups (Figures 7E and 7F). Together, these results suggest that menin expression is not a prognostic biomarker for patient outcome in this cohort of breast cancer patients.

Discussion

By disrupting the *Men1* gene specifically in mammary luminal cells, the present study assessed the potential role of *Men1* inactivation in mammary tumourigenesis. *Men1* ablation in mammary luminal cells did

not result in any obvious defects in the postnatal development of mammary glands, whereas it did predispose mice to the development of pre-cancerous lesions. Our data therefore provide a strong indication of an important and hitherto unknown role of the *Men1* gene in mammary cells. We noticed a low incidence of similar pre-cancerous lesions in *Men1^{F/F}* control mice. The fact that *WapCre-Men1^{+/+}* mouse littermates did not show a similar phenotype does not support the view that the mouse genetic background could be the factor causing the predisposition to MIN lesions as previously reported [22–24]. Rather, although the floxed *Men1* allele has previously been proven to be both functional and non-hypomorphic [12,14], we cannot exclude the possibility that some unknown disturbance of the floxed *Men1* allele could interfere with menin's function in mammary cells. Importantly, the process of mammary lesion development appeared to be different in mutant mice compared with *Men1^{F/F}* control mice, as evidenced by its early appearance and increasing incidence over time in the former. At the molecular level, the pattern of phosphorylated STAT5 and beta-catenin expression were substantially different in MIN lesions found in the mutant mice compared with those rarely observed in *Men1^{F/F}* control mice, suggesting that the few MIN lesions found in *Men1^{F/F}* control mice may be caused by different molecular mechanisms.

Table 4. Comparison of the distribution of clinical parameters between breast cancer patients (TMA cohort) whose tumours show either low (intensity 1) or normal (intensity 2) menin nuclear staining

	Intensity of menin nuclear staining			Test
	1 N = 95	2 N = 26	All N = 121	
Age at diagnosis (years)				
Mean (std)	58.33 (12.73)	60.20 (11.47)	58.74 (12.44)	Wilcoxon $p = 0.458$
Median (min–max)	56.00 (37.60–87.40)	61.30 (41.60–77.90)	56.30 (37.60–87.40)	
Q1–Q3	48.60–66.20	50.80–70.60	48.80–68.00	
Menopausal status				
No	31 (32.6%)	6 (23.1%)	37 (30.6%)	Chi ² $p = 0.349$
Yes	64 (67.4%)	20 (76.9%)	84 (69.4%)	
Tumour size				
< 30 mm	60 (63.2%)	16 (61.5%)	76 (62.8%)	Chi ² $p = 0.880$
≥ 30 mm	35 (36.8%)	10 (38.5%)	45 (37.2%)	
Histological type				
Lobular	10 (10.5%)	0 (0.0%)	10 (8.3%)	Fisher exact $p = 0.231$
Ductal	76 (80.0%)	24 (92.3%)	100 (82.6%)	
Others	9 (9.5%)	2 (7.7%)	11 (9.1%)	
Histological grade (SBR)				
1	20 (21.1%)	3 (11.5%)	23 (19.0%)	Fisher exact $p = 0.620$
2	41 (43.2%)	12 (46.2%)	53 (43.8%)	
3	34 (35.8%)	11 (42.3%)	45 (37.2%)	
Lymph node involvement				
NO	42 (44.2%)	10 (38.5%)	52 (43.0%)	Fisher exact $p = 0.703$
Micro-metastases	15 (15.8%)	6 (23.1%)	21 (17.4%)	
Macro-metastases	38 (40.0%)	10 (38.5%)	48 (39.7%)	
ER: % marked cells				
< 10%	12 (12.6%)	5 (19.2%)	17 (14.0%)	Fisher exact $p = 0.698$
10–50%	7 (7.4%)	1 (3.8%)	8 (6.6%)	
> 50%	76 (80.0%)	20 (76.9%)	96 (79.3%)	
PR: % marked cells				
< 10%	24 (25.3%)	5 (19.2%)	29 (24.0%)	Fisher exact $p = 0.504$
10–50%	22 (23.2%)	4 (15.4%)	26 (21.5%)	
> 50%	49 (51.6%)	17 (65.4%)	66 (54.5%)	
HER2 status				
Missing	2	0	2	Fisher exact $p = 1.000$
0/+	83 (89.2%)	24 (92.3%)	107 (89.9%)	
++/+++	10 (10.8%)	2 (7.7%)	12 (10.1%)	
Cancer subtype				
Luminal A	57 (60.0%)	13 (50.0%)	70 (57.9%)	Fisher exact $p = 0.611$
Luminal B	26 (27.4%)	8 (30.8%)	34 (28.1%)	
HER2	2 (2.1%)	1 (3.8%)	3 (2.5%)	
Triple negative	10 (10.5%)	4 (15.4%)	14 (11.6%)	

ER = oestrogen receptor; PR = progesterone receptor.

The possible interplay between the occurrence of mammary lesions and pituitary tumour development in the mutant mice is a complex issue. Importantly, our data indicate that (1) we found no correlation between the serum prolactin level and MIN development; (2) the expression of phospho-STAT5 was significantly diminished in the MIN lesions of the mutant mice; (3) in another *Men1* mouse model that developed prolactinomas but with intact mammary expression of menin [16], the incidence of MIN was not significantly increased compared with control mice. Altogether, our data support that *Men1* disruption in mammary cells played a role in MIN development.

Mammary glands are not known to be affected in patients with MEN1 syndrome. However, several observations have provided meaningful clues that suggest a possible role of *MEN1* in mammary tissues. An epidemiological study revealed that 7% of patients with sporadic breast cancer were also diagnosed with hyperparathyroidism [25]. Because *MEN1* mutations

account for approximately 30% of sporadic parathyroid adenomas, one could speculate that a minor proportion of breast cancer cases could also be related to a *MEN1* mutation. Consistent with this clinical observation, we and others previously detected several cases of breast and prostate cancer in older heterozygous *Men1* mutant mice [11–13]. Taking into account the above-mentioned observations and the interaction between menin and ER α , the high incidence of MIN development in *Men1* mammary-specific mutant mice is, after all, not an unexpected phenotype. Instead, the long latent period before MIN onset and the incomplete penetrance of this mutation suggest that *Men1* inactivation itself may not be a major breast cancer-inducing event. However, *Men1* inactivation appears to create a cancer-prone context that requires other participating factors that lead to tumourigenesis.

Cell–cell adhesion is part of the basic machinery involved in the organization of epithelial tissues. Multiple lines of evidence suggest that its deregulation

plays a role in breast cancer initiation and progression [20,21,26–30]. Interestingly, we observed clearly reduced membrane expression of beta-catenin and E-cadherin in MIN lesions from mutant mice. Previous work, including ours, provided evidence that E-cadherin and beta-catenin membrane expression was reduced in mice with *Men1* inactivation-induced islet lesions [8,14]. Our current data suggest that menin's role in regulating cell–cell adhesion may also be involved in the control of normal mammary cell growth and proliferation, although the underlying mechanism needs further investigation.

Interestingly, our analyses of two series of human breast cancers demonstrated that a large proportion of breast cancers displayed reduced menin expression. This is consistent with a previous report by Imachi *et al.* [31]. It is noted that these authors described a correlation between menin expression and a worse prognosis in affected patients, whereas we could not find any similar correlation in our samples. The discrepancy between our data and those of Imachi *et al.* could be explained by different populations of breast cancer patients used in the studies. Further analyses using larger clinical cohorts are needed to better evaluate the role of menin expression in breast cancer occurrence and progression.

Taken together, by specifically disrupting the *Men1* gene in mammary gland cells, we have provided compelling evidence that menin inactivation in mice results in a high incidence of pre-cancerous mammary lesions. The discovery of this unknown role of menin may shed light on the complex molecular mechanisms of mammary tumourigenesis. In this regard, mice in which *Men1* was disrupted specifically in the mammary glands are a tool to identify and study additional factors that are important for the initiation and progression of breast cancer.

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Author contribution statement

CS conducted most of the experiments, analysed data, and prepared figures and the manuscript. MA performed experiments and analysed data of the *Men1^{loxP/loxP}/Rip-Cre⁺* mice. IT and SG conducted histological analysis of the first panel of human breast cancers. AG and SM performed the western blot analyses of the second panel of human breast cancers. CC, JL, and RB performed mouse model maintenance and histological analysis of mouse models. RB prepared the revised figures and manuscript. FA performed the analyses using MCF7 cells. EL and SC conducted statistical analyses. TB supervised human breast cancer treatment and collections. LF analysed histological data on transgenic mice and supervised the project. CXZ conceived the study, supervised the overall project, and wrote the manuscript.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary materials and methods.

Figure S1. Menin inactivation in mammary gland carcinomas detected in heterozygous *Men1* mutant mice.

Figure S2. Histological sections of mammary lesions found in *Men1^{loxP/loxP}/Rip-Cre⁺* mice presenting with prolactinoma development.

Table S1. Incidence of MIN lesions in *WapCre-Men1^{F/F}* females without hyperprolactinaemia.

SHORT COMMUNICATION

Reexpression of oncoprotein MafB in proliferative β -cells and *Men1* insulinomas in mouse

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MafB, a member of the large Maf transcription factor family, is essential for the embryonic and terminal differentiation of pancreatic α - and β -cells. However, the role of MafB in the control of adult islet-cell proliferation remains unknown. Considering its oncogenic potential in several other tissues, we investigated the possible alteration of its expression in adult mouse β -cells under different conditions of proliferation. We found that MafB, in general silenced in these cells, was reexpressed in ~30% of adaptive β -cells both in gestational female mice and in mice fed with a high-fat diet. Importantly, reactivated MafB expression was also observed in the early β -cell lesions and insulinomas that developed in β -cell specific *Men1* mutant mice, appearing in >80% of β -cells in hyperplastic or dysplastic islets from the mutant mice >4 months of age. Moreover, MafB expression could be induced by glucose stimulation in INS-1 rat insulinoma cells. The induction was further reinforced following *Men1* knockdown by siRNA. Furthermore, MafB overexpression in cultured β TC3 cells enhanced cell foci formation both in culture medium and on soft agar, accompanied with the increased expression of Cyclin B1 and D2. Conversely, MafB downregulation by siRNA transfection reduced BrdU incorporation in INS-1E cells. Taken together, our data reveal that *Men1* inactivation leads to MafB reexpression in mouse β -cells *in vivo*, and provides evidence that deregulated ectopic MafB expression may have a hitherto unknown role in adult β -cell proliferation and *Men1*-related tumorigenesis.

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Keywords: Maf; MafB; *Men1*; pancreatic β -cells; proliferation; insulinoma

Recent studies highlight that the adult pancreatic β -cells have, in general, limited capacity for cell replication (Teta *et al.*, 2007). However, in certain circumstances,

such as during gestation or under high-caloric diet, they may undergo adaptive cell proliferation. The identification of molecular mechanisms underlying the control of β -cell proliferation is a major issue in the field of islet biology, as their deregulation may lead to both metabolic and tumor disorders. Among different pathways and factors known to be involved in the control of β -cell proliferation, menin, the protein encoded by the *MEN1* gene, is of particular interest. It is not only known as a tumor suppressor in islet cells (Yang and Hua, 2007), but also for its role in adaptive β -cell proliferation (Karnik *et al.*, 2007). Menin is considered as a cofactor of transcriptional regulation, capable of interacting with many transcription factors and other protein partners. Although it has been demonstrated that menin inactivation led to acute β -cell proliferation (Schnepp *et al.*, 2006; Yang *et al.*, 2010), several studies of mouse *Men1* insulinoma models highlighted the fact that the menin-related tumorigenesis procedure may need the participation of other factors apart from *Men1* inactivation (Bertolino *et al.*, 2003b; Crabtree *et al.*, 2003; Loffler *et al.*, 2007; Yang and Hua, 2007).

Among different candidate genes that may participate in the control of β -cell proliferation by menin, the large Maf family in particular has drawn our attention, owing to their involvement both in oncogenesis and adult islet-cell function. Although their oncogenic role appears to depend on cell type and tissue (Pouponnot *et al.*, 2006; Rocques *et al.*, 2007), several reports including their translocation in human multiple myeloma (Chesi *et al.*, 1998) have firmly established the large Maf family members, MafA, MafB and c-Maf, as *bona fide* protooncogenes (Eychène *et al.*, 2008). It was shown that Maf proteins can transform primary cells (Nishizawa *et al.*, 2003; Pouponnot *et al.*, 2006). Their oncogenic activity was also demonstrated in multiple myeloma cell lines (Hurt *et al.*, 2004) and in mice (Morito *et al.*, 2006, 2011).

During mouse development and adulthood, the expression of large Mafs in the endocrine pancreas is tightly regulated. MafA is only expressed in insulin-producing cells during development and in adult life, and has been demonstrated as a critical factor in the regulation of genes crucial for adult β -cell function (Zhang *et al.*, 2005). MafB is expressed in both

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glucagon- and early insulin-secreting cells during embryonic development, whereas its expression is restricted to α -cells in the adult (Artner *et al.*, 2007). Constitutive MafB loss of function mutations in mice showed reduced number of cells expressing insulin and glucagon, and the blockade of β -cell maturation (Artner *et al.*, 2007; Nishimura *et al.*, 2008), indicating that MafB possesses an essential function in cell proliferation and differentiation of both α - and β -cells at the embryonic stage. Moreover, MafB has been recently shown to be induced in the islets of e15.5 pregnant female mice (Pechhold *et al.*, 2009), suggesting its relevant role in adult adaptive β -cell proliferation. However, the role of large Maf members in the control of adult islet-cell tumorigenesis remains unknown.

To gain insight into this issue, we decided to investigate the expression of Maf family members in mouse β -cells under different physiological and pathological conditions of proliferation. We first examined the *in vivo* expression of large Mafs in normal pancreas during the embryonic stage and adulthood. At E14.5, consistent with previous reports (Artner *et al.*, 2006; Nishimura *et al.*, 2006), MafA was detected only in insulin⁺ cells (Supplementary Figure S1a), whereas MafB was expressed in both glucagon⁺ and insulin⁺ cells (Supplementary Figure S1b). c-Maf was not detectable in the embryonic pancreas even at E17.5 (Supplementary Figure S1c). In 2-month-old islets, MafA was expressed in >95% of β -cells (Supplementary Figure S1d), whereas MafB expression was restricted to α -cells (Supplementary Figure S1e). c-Maf was weakly and strongly expressed in the endocrine and exocrine pancreas, respectively (Supplementary Figure S1f), and, therefore, not pursued for further investigation.

Next, we examined MafA and MafB expression in β -cells that underwent adaptive proliferation triggered by physiological or pathological needs, using islet sections from either pregnant or high-fat-fed mice. We observed that, albeit the expected MafA expression in β -cells (data not shown), MafB was aberrantly reexpressed in $31 \pm 4.2\%$ of β -cells in the islets of C57BL/6 female mice ($n=6$) on pregnant day 14.5 (Figure 1a) where adaptive β -cell proliferation reaches its highest level (Karnik *et al.*, 2007). Our data are consistent with recent findings by Pechhold *et al.* (2009). We noticed that the appearance of MafB reexpression was accompanied with an increase in Ki67⁺ cell number (Figures 1a and b). Interestingly, as shown in Figure 1c, most of Ki67⁺ β -cells co-expressed MafB. No MafB reexpression was found in the age-matched non-pregnant female littermates. Similarly, a substantial number of β -cells reexpressing MafB was found in all tested high-fat-fed adult mice as compared with the control mice (Figure 1d). Due to their diet, the former displayed increased body weight, normal fasting glucose and impaired glucose tolerance by IPGTT analysis (Figures 1e–g) and β -cell hyperplasia (Figure 1d). Taken together, the above results suggest a possible link between adaptive β -cell proliferation and reactivated MafB expression.

The above findings prompted us to question whether MafB was also 'switched-on' in mouse *Men1* insulinoma-

mas, another condition of pathological β -cell proliferation. We addressed this issue in insulinomas that developed in *Men1^{F/F}-RipCre⁺* mice, a β -cell-specific *Men1* knockout model previously generated in our laboratory (Bertolino *et al.*, 2003a, b). Unexpectedly, in insulinomas from 12-month-old mutant mice, MafB was found to be reexpressed in tumors with menin inactivation at both the mRNA (Figure 2a) and protein levels, latter being confirmed by western blot (Figure 2b) and immunostaining (Figure 2c). In contrast, Arx, another α -cell-specific transcription factor, and glucagon were virtually undetectable in the same insulinomas (Figures 2a and c), because of the disappearance of normal α -cells in tumor lesions. Therefore, our data excluded the responsive α -cell proliferation due to hypoglycemia as the cause of MafB reexpression.

To determine whether MafB reexpression is an early event in insulinoma development, we examined young *Men1^{F/F}-RipCre⁺* mice (Figure 2d). Interestingly, MafB expression was already detectable in a substantial number of β -cells in mice at 2 months of age ($23 \pm 3.8\%$, $n=4$), with the number of MafB-expressing β -cells increasing sharply at 4 months ($86 \pm 4.2\%$, $n=4$). Immunofluorescent staining clearly showed that the majority of MafB-positive cells seen in the lesions did not express glucagon (Figure 2d) but insulin (data not shown). Double immunofluorescent staining using anti-Pdx1 and MafB antibodies further confirmed the results, indicating that they are indeed β -cells as opposed to α -cells (Supplementary Figure S2).

As MafB reexpression was found to be induced in both adaptive β -cell proliferation and tumorigenesis related to menin inactivation, we sought to better define the interplay between menin expression, metabolic loading and MafB reexpression. To this end, INS-1E rat insulinoma cells (Merglen *et al.*, 2004) were firstly subjected to *Men1* knockdown by siRNA transfection under normal culture conditions (Figure 3a, Supplementary Figures S3a and b). Menin downregulation in these cells resulted in a 2.7-fold increase in MafB expression (Figure 3a). As menin was previously reported to be downregulated during adaptive β -cell proliferation (Karnik *et al.*, 2007), we also examined menin expression and its potential correlation with MafB reexpression in pregnant (Supplementary Figure S4) or high-fat-fed mice (data not shown). No decrease in menin expression was evident in both conditions, consistent with a recent report where menin expression levels in islets were found unchanged in mice fed with a high-fat diet (Yang *et al.*, 2010), thus no correlation could be found between ectopic MafB expression and menin expression levels.

To gain further insight into MafB regulation, INS-1E cells were subjected to glucose stimulation, after *Men1* knockdown by siRNA. After having assessed MafB expression in different glucose concentrations in INS-1E cells (Supplementary Figures S5a and b), we adopted a commonly used glucose-stimulation protocol with an overnight starvation in 0.1 mM glucose medium followed by a 6-h stimulation in 11 mM glucose medium (da Silva Xavier *et al.*, 2010), in order to examine the effect of *Men1* knockdown on MafB expression upon glucose

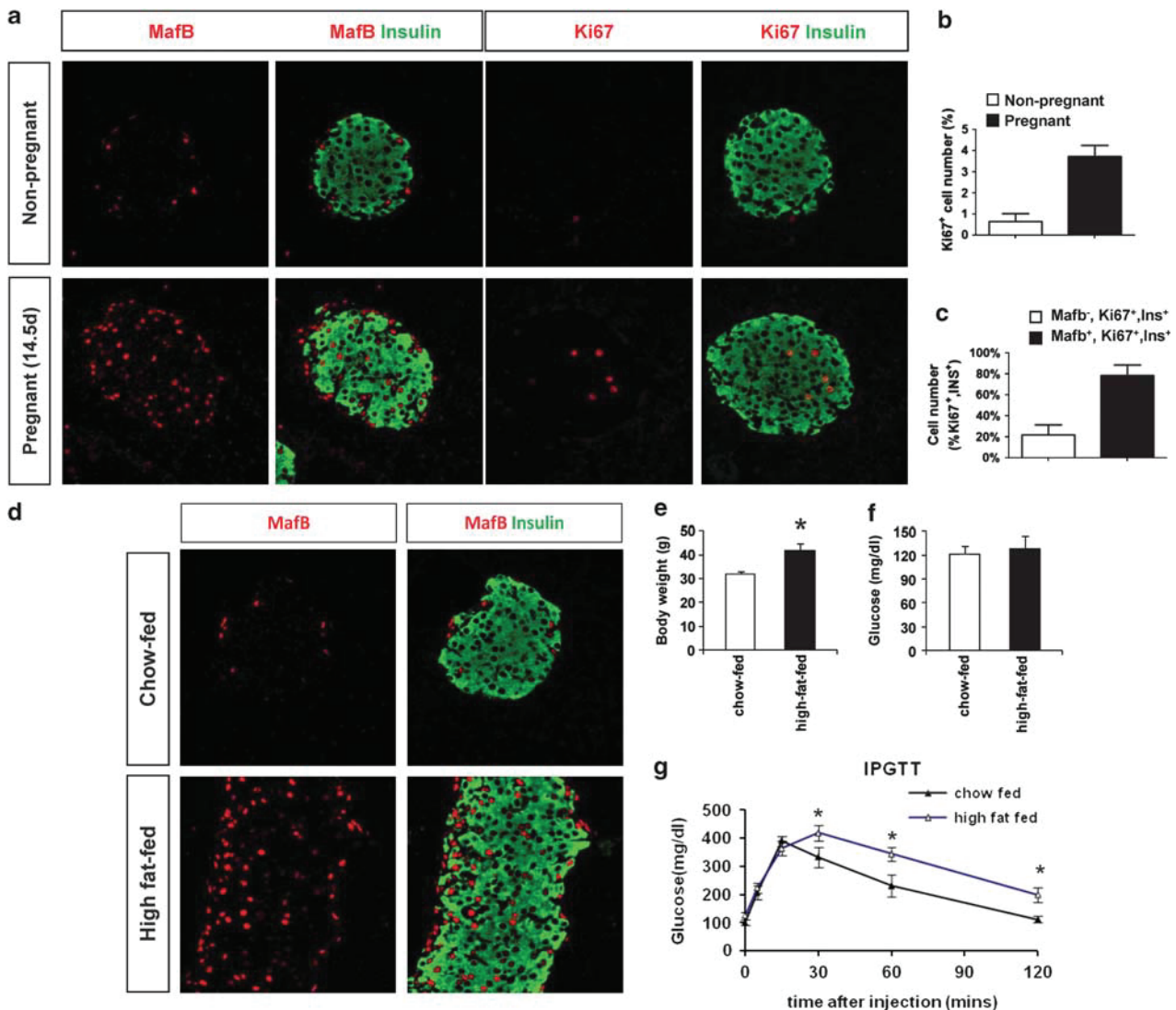


Figure 1 MafB is ectopically expressed in proliferative β -cells during pregnancy and in obesity. (a) Representative images of immunofluorescent staining from 14.5-dpc pregnant mice and non-pregnant female littermates using anti-MafB (1:4000, Bethyl, Montgomery, TX, USA), Ki67 (1:100, Santa Cruz Biotech, Santa Cruz, CA, USA) and insulin (1:500, DAKO, Carpinteria, CA, USA) antibodies. Immunofluorescent staining was performed as described previously (Fontaniere *et al.*, 2008; Lu *et al.*, 2010). Pregnant C57BL/6 wild-type mice were generated by timed-mating. (b, c) Graphs show the percentage of Ki67-positive β -cells per islet and the distribution of MafB-positive and MafB-negative β -cells over total Ki67-positive β -cells, respectively. (d) MafB expression in islets from high-fat-fed obese mice by immunofluorescent staining as in (a). 2-month-old male C57BL/6 mice purchased from Charles River Laboratories (St-Germain-sur-l'Arbresle, France) and fed with a high-fat diet (45 kcal% fat, 35 kcal% carbohydrate and 20 kcal% protein) (Research Diets, North Brunswick Township, NJ, USA) or a chow diet for 16 weeks were subject to the monitoring of body weight (e) and serum glucose levels after 5 h of fasting (f) and IPGTT (g, 2 mg per g body weight) ($n = 6$). * $P < 0.05$ (Student's t test). The data are presented as means \pm s.e.m. All animal experiments carried out in the current study were approved by the Regional Animal Experiments Committee of Centre National de la Recherche Scientifique and were conducted in accordance with the ethical guidelines.

stimulation. Six hours after acute glucose exposure, INS-1E cells treated with the non-targeting siRNA showed a marked 2.0-fold increase in MafB expression compared with the initial expression levels, whereas no significant change was detected in menin expression (Figure 3a). These data suggest that glucose stimulation alone can activate MafB expression without altering menin expression under the current experimental conditions. More interestingly, *Men1* knockdown by siRNA in these cells further increased glucose-induced MafB expression by a 2.7-fold increase (Figure 3a).

Similar results were obtained using INS-r β cells (Wang and Iynedjian, 1997), another subclone of INS-1 cells (data not shown). Using real-time RT-PCR analysis, an increase in *MafB* mRNA level was detected in all INS-1E cells with *Men1* knockdown cultured with different glucose concentrations (Figure 3b), suggesting that menin downregulation affects *MafB* transcription. This is reminiscent of what was observed in mouse *Men1* insulinomas (Figure 2a). Taken together, our data suggest that both glucose stimulation and menin downregulation can independently induce MafB reexpression

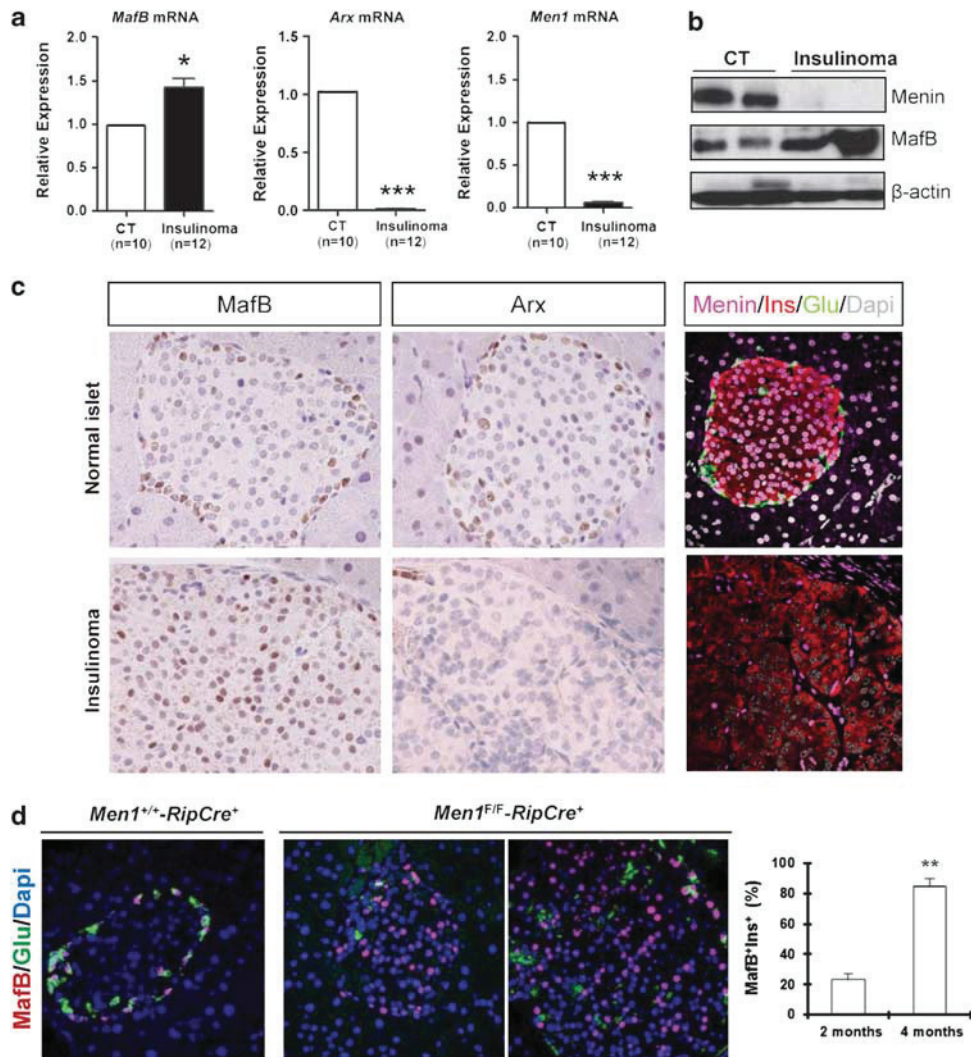


Figure 2 MafB is ectopically expressed in insulinomas and early β -cell lesions developed in $Men1^{F/F}-RipCre^+$ mice. **(a)** Analysis of *MafB*, *Arx* and *Men1* expression in islets from 12-month-old mutant and 10 littermate control mice (CT, seven $Men1^{+/+}-RipCre^+$ and three $Men1^{F/F}-RipCre^-$ mice, respectively) by real-time RT-PCR was carried out as previously described (Fontaniere *et al.*, 2006). Results are from representative experiments performed in triplicate. Bars represent the mean. Student's *t* tests were used to compare means between two groups. * $P < 0.05$ and *** $P < 0.001$. **(b)** Menin and MafB expressions in insulinomas derived from two 12-month-old $Men1^{F/F}-RipCre^+$ mice were analyzed and compared with two independent pools of islets from age-matched control mice by western blot analyses using anti-menin (1:8000, Bethyl), MafB (1:2000, Bethyl) and β -actin antibodies (1:50 000, Santa Cruz). **(c)** MafB, Arx and menin protein detection in islets from mutant and control mice by immunostaining. Representative images of immunohistochemical analysis using anti-MafB and Arx antibodies, respectively (1:1000, given by Pr Jacques Philippe) and triple-immunofluorescence analysis using anti-menin (1:4000, Bethyl), insulin and glucagon antibodies. **(d)** Detection of MafB expression in early β -cell lesions. Graphs show the percentage of MafB⁺Ins⁺ cells over total β -cell number. Representative images of double immunofluorescent staining of MafB and glucagon in the pancreas from 2- (left) and 4-month (right)-old $Men1^{F/F}-RipCre^+$ and control mice. The results represent the mean of three independent experiments \pm s.e.m. ** $P < 0.01$ (Student's *t* test).

in INS-1 cells, whereas they exert a synergistic effect when applied simultaneously.

To understand the biological significance of MafB reexpression in the control of β -cell proliferation, we overexpressed human MAFB protein in the β TC3-cell line that has no detectable endogenous MafB expression. Colony formation assay showed that exogenous MAFB overexpression significantly increased cell proliferation by $\sim 30\%$ as compared with the control (Figures 3c and 4a). We further investigated the effect of MAFB overexpression on anchorage-independent growth and found that MAFB-overexpressing β TC3-

cells formed more than eightfold more colonies in soft agar compared with control cells (Figure 3d). Furthermore, as shown in Figure 4a, we observed that MAFB-overexpressing β TC3-cells displayed the upregulated expression of both *Cyclin D2*, a previously identified MafB target gene in myeloma cells (van Stralen *et al.*, 2009), and *Cyclin B1* (Figure 4a). Factors in both Cyclin D and Cyclin B families were previously reported to be upregulated in mouse *Men1* insulinomas (Fontaniere *et al.*, 2006) and in mouse β -cells, with acute *Men1*-ablation (Yang *et al.*, 2010). To confirm the above results, MafB was knocked-down by siRNA transfect-

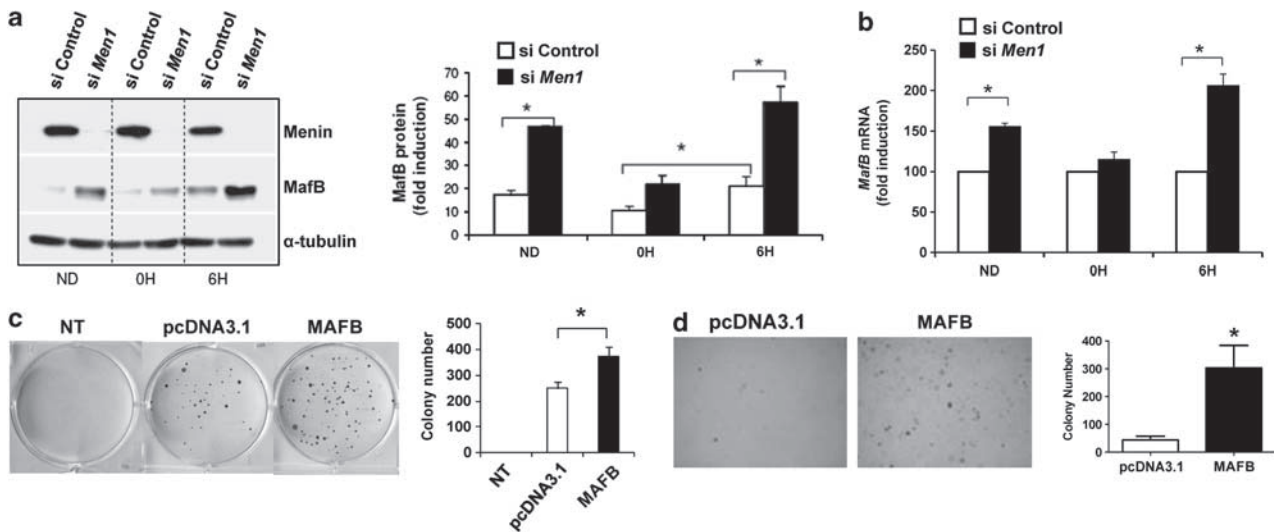


Figure 3 MafB expression in cultured β -cells. (a) MafB expression can be induced by acute glucose exposure and is further activated by *Men1* knockdown. INS-1E cells were seeded in 6-well plates and transiently transfected by either non-targeting siRNA or anti-*Men1* siRNA (Thermo Scientific, Waltham, MA, USA) in RPMI1640 medium containing 5 mM glucose (ND). After 48 h, cells were starved overnight in a deprivation medium containing 0.1 mM glucose. This was replaced by a stimulation medium containing 11 mM glucose and the cells were harvested at 0 and 6 h. A total of 10 μ g cellular extracts in RIPA buffer (Santa Cruz) were used in western blot analysis. Quantification of densitometrically analyzed protein bands in different conditions and indicated time points, from four independent experiments. The data represent mean \pm s.e.m. analyzed by Mann-Whitney *U*-tests. (b) Analysis of *MafB* mRNA levels in INS-1E cells by real-time RT-PCR was carried out as mentioned above using primers *MafB*-FW: 5'-CACCTGCGGGGCTTACC-3' and *MafB*-Rev: 5'-GCTGCTCCACCTGCTGAATG-3'. Results were from a representative experiment performed in triplicate. Bars represent the mean. Mann-Whitney *U*-tests were used to compare means between two groups with $*P < 0.05$ considered as significant. (c, d) MafB ectopic expression in β TC3 line promotes cell proliferation. Colony formation test (c) was carried out as previously described (Hussein *et al.*, 2007). Cells were either untransfected (NT) or transfected with 2 μ g empty vector pcDNA3.1 or with 2 μ g pcDNA-MafB. After 14 days of G418 selection, giemsa-stained colonies were photographed. The results represent the mean of two independent experiments \pm s.e.m. $*P < 0.05$. (d) Soft agar test was performed as previously described (Poupponnot *et al.*, 2006). Graphs show the quantification of colony number of three independent experiments performed in triplicate \pm s.e.m. $*P < 0.05$ (Student's *t* test).

tion in INS-1E cells. We found significantly less BrdU incorporation and less *Cyclin D1* and *D2* expression levels in the cells with reduced MafB expression (Figure 4b). Interestingly, co-expression analysis of *Cyclin D2* and MafB in islets from gestational mice (Figures 4c and d) or from the high-fat-fed mice (data not shown) revealed that $> 60.5\%$ of MafB-reexpressing β -cells co-expressed *Cyclin D2*, whereas only 11.8% of MafB-negative β -cells expressed *Cyclin D2*. Taken together, these data suggest that MafB overexpression promotes β -cell proliferation by controlling the expression of important cell cycle regulators.

In the present study, we showed that MafB, which is normally 'switched-off' in normal adult β -cells, was reexpressed in both pregnant and high-fat-fed mice. We further demonstrated that MafB reexpression also occurred in the early neoplastic lesions of menin-deficient β -cells and in mouse *Men1* insulinomas. Our work strongly suggests that MafB reexpression could be a key factor for β -cell proliferation, both in the context of physiopathological metabolic needs and that of tumorigenesis. Although previous reports have identified several genes with altered expression in mouse *Men1* insulinoma (Fontaniere *et al.*, 2006; Mould *et al.*, 2007), the current study describes for the first time the altered expression of a transcription factor known to have a critical role during normal islet development in early

Men1 β -cell neoplastic lesions. Considering the known oncogenic function of MafB in other tissues (Eychène *et al.*, 2008) and the effects of its overexpression on growth behavior of β TC3-cells observed in this study, we believe that MafB reexpression may fulfill a tissue-specific molecular link between menin inactivation and β -cell proliferation by increasing the expression of the cell cycle regulators. It should be noted that the lack of specific anti-human-MafB antibodies prevented us from extending our current investigation into human MEN1 insulinomas and proliferative β -cells.

In particular, our data demonstrated for the first time that silenced MafB expression in β -cells can be reactivated not only by glucose stimulation but also by menin inactivation. MafB reactivation has been reported in adaptive β -cells (Pechhold *et al.*, 2009), although the underlying mechanism remains elusive. We may speculate that both glucose and insulin pathways could be involved. It appears that menin inactivation is the major factor leading to MafB reactivation in *Men1* mouse insulinomas, as the glucose levels are decreased in the mutant mice because of high insulin levels (Bertolino *et al.*, 2003b), precluding a causal role for hyperglycemia. In contrast, the *in vivo* MafB reexpression in adaptive β -cells appears to be mainly dependent on the metabolic needs rather than that of menin expression, as no correlation was detected

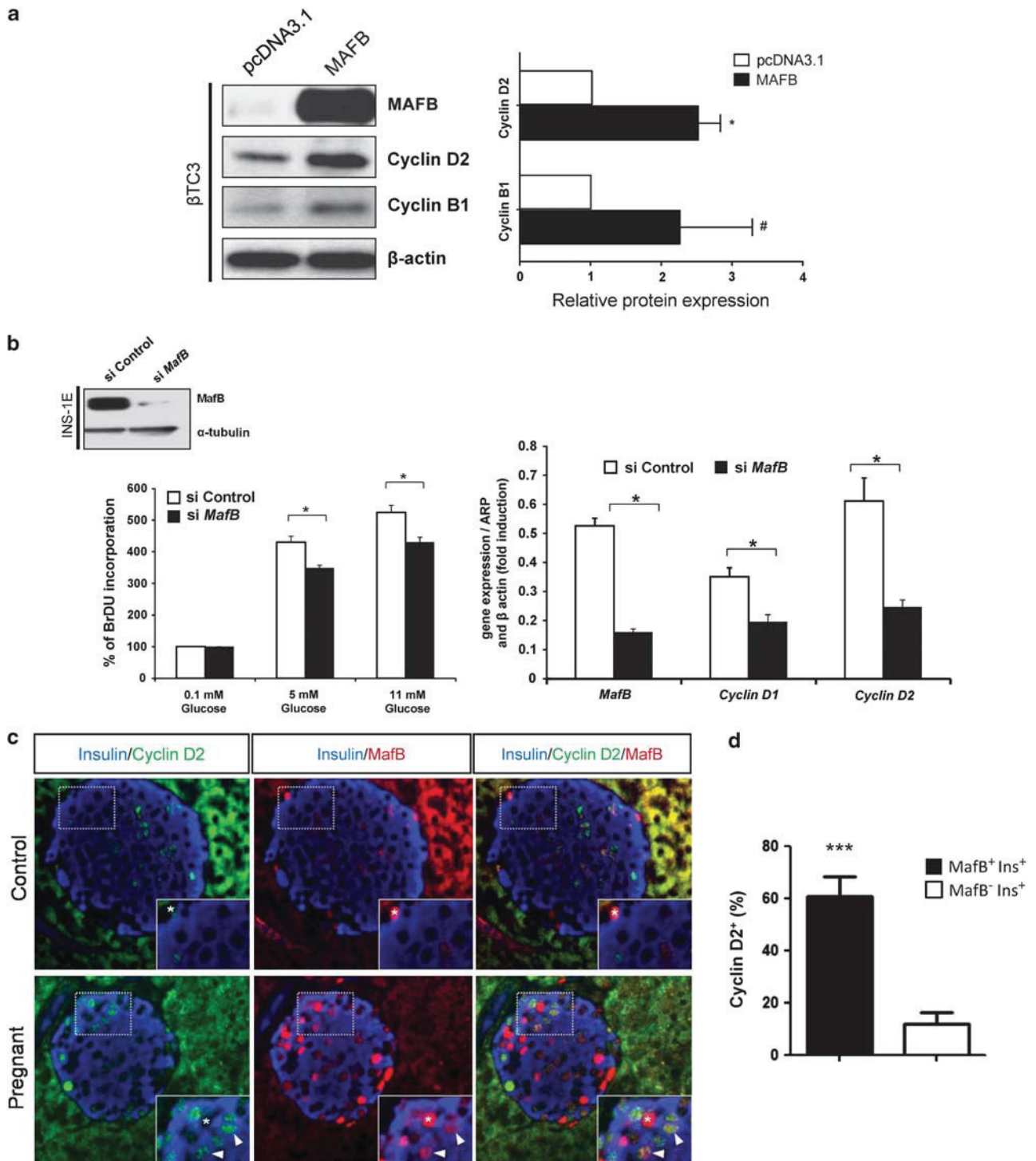


Figure 4 Altered MafB expression in β -cell lines deregulates the expression of Cyclins. **(a)** MafB-overexpressing β TC3-cells displayed increased expression of Cyclins. Total cell lysates of the above β TC3-cells were analyzed for MAFB and Cyclin expression by western blot with anti-MAFB, Cyclin D2 (1:2000, Santa Cruz) and Cyclin B1 (1:1000, Santa Cruz). Quantification of densitometrically analyzed bands from four independent experiments were analyzed by Mann-Whitney *U*-test, * $P < 0.05$, # $P < 0.001$. **(b)** *MafB* knockdown in INS-1E cells resulted in decreased cell proliferation and Cyclin expression. INS-1E cells transiently transfected by either non-targeting siRNA or anti-*MafB* siRNA (Thermo Scientific) were subjected to BrdU incorporation assay and detection of Cyclin expression by real-time RT-PCR (*Cyclin D1*-FW: 5'-GGATTCAGGACGACTCTT-3', *Cyclin D1*-Rev: 5'-AACCTTCCCAATAAA-TACTCTTC-3'; *Cyclin D2*-FW: 5'-TTTACACCGACAATTCTG-3', *Cyclin D2*-Rev: 5'-TAGGATGTGCTCAATGAA-3'). Note that, in order to better illustrate MafB knockdown, MafB detection by western blot was performed with a protein loading threefold more than that used in Figure 3a. The results represent the mean of three independent experiments \pm s.e.m. * $P < 0.05$ (Student's *t* test). **(c)** Detection of MafB and Cyclin D2 expression in proliferative β -cells from 14.5-dpc pregnant mice and non-pregnant female littermates using anti-MafB, Cyclin D2 (1:5000, Santa Cruz) and insulin antibodies. **(d)** Graph shows the percentage of Cyclin D2-expressing cells in MafB⁺Ins⁺ and MafB⁻Ins⁺ cell populations, respectively. *** $P < 0.001$ (Student's *t* test).

between ectopic MafB expression and menin expression levels. To better understand the regulation of MafB expression by menin, we attempted to investigate the potential influence of menin overexpression on the known *MafB* promoter (Huang *et al.*, 2000) by luciferase reporter assay in INS-1E cells. However, we were unable to detect any effect (data not shown). This could be because of the MafB promoter tested in the current study or the complex nature of MafB regulation, including likely indirect regulation of MafB by menin. Therefore, it will be relevant to elucidate how menin participates in the regulation of MafB expression in β -cells in the future and to determine whether MafB reexpression is essential for the *Men1* insulinoma development *in vivo*.

In conclusion, our results demonstrate that the silenced MafB expression is reactivated in *Men1* insulinomas in mice, and establish a correlation between MafB expression level and cell proliferation capacity of β -cell lines. Considering the specific function of MafB in islets, the current finding could be useful for better understanding the tissue-specificity of MEN1 pathology. Further studies elucidating the underlying mechanisms involved in this process may provide insights into the regulation of β -cell proliferation, which is vital for

treating both metabolic diseases and tumors affecting β -cells.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)

α Cell-Specific *Men1* Ablation Triggers the Transdifferentiation of Glucagon-Expressing Cells and Insulinoma Development

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BACKGROUND & AIMS: The tumor suppressor menin is recognized as a key regulator of pancreatic islet development, proliferation, and β -cell function, whereas its role in α cells remains poorly understood. The purpose of the current study was to address this issue in relation to islet tumor histogenesis. **METHODS:** We generated α cell-specific *Men1* mutant mice with *Cre/loxP* technology and carried out analyses of pancreatic lesions developed in the mutant mice during aging. **RESULTS:** We showed that, despite the α -cell specificity of the *GluCre* transgene, both glucagonomas and a large amount of insulinomas developed in mutant mice older than 6 months, accompanied by mixed islet tumors. Interestingly, the cells sharing characteristics of both α and β cells were identified shortly after the appearance of menin-deficient α cells but well before the tumor onset. Using a genetic cell lineage tracing analysis, we demonstrated that insulinoma cells were directly derived from transdifferentiating glucagon-expressing cells. Furthermore, our data indicated that the expression of Pdx1, MafA, Pax4, and Ngn3 did not seem to be required for the initiation of this transdifferentiation. **CONCLUSIONS: Our work shows cell transdifferentiation as a novel mechanism involved in islet tumor development and provides evidence showing that menin regulates the plasticity of differentiated pancreatic α cells in vivo, shedding new light on the mechanisms of islet tumorigenesis.**

Keywords: *Men1* Ablation; Pancreatic α Cells; Transdifferentiation; Insulinoma.

Previous clinical studies have highlighted the complexity of pancreatic islet tumors, at least one-third of all functioning islet tumors secreting multiple hormones, and have suggested controversial hypotheses about their histogenesis.^{1–3} Despite their rarity, islet tumors have long been attractive for clinicians and investigators because of the insights they may provide into islet-cell replication, survival, regeneration, and the actions of islet hormones in glucose homeostasis.

The most important insights on the molecular pathogenesis of islet tumors have come from the studies of inherited pancreatic endocrine tumor syndromes.⁴ Among the involved genetic factors, menin, encoded by the *MEN1*

gene, has drawn particular attention. Menin is a tumor suppressor, and its mutation causes multiple endocrine neoplasia type 1 (MEN1), characterized by multiple occurrences of endocrine tumors affecting the parathyroid, endocrine pancreas, and anterior pituitary.^{5,6} Malignant islet tumors account for the main cause of death in patients with MEN1.⁷ β cell-specific *Men1* disruption in mice, which results in the development of insulinomas, has confirmed the function of menin as a tumor suppressor in these cells.^{8–10} Studies of MEN1 syndrome have defined menin's role solely in the context of tumor pathogenesis. However, recent progress enlarged this view. Interestingly, Karnik et al¹¹ reported that, during pregnancy, menin was down-regulated by prolactin in maternal islets, which led to islet mass expansion to meet the increased metabolic demand. Recently, we showed that, during embryogenesis, *Men1* ablation in mice led to the abnormal development of both pancreatic α and β cells, which was accompanied by a decrease in the size of the neuroginin3 (Ngn3)⁺ cell population.¹²

Although menin has been recognized as a key regulator in β -cell proliferation and endocrine function by extensive studies,^{13,14} its potential role in α cells remains unknown. Recent advances highlighted the pathophysiology of the α cell in hyperglucagonemia, representing an important component of the metabolic abnormalities associated with diabetes mellitus.^{15,16} In addition to its effect on insulin secretion, glucagon has also been suggested to play a role in the development of β cells¹⁷ and islet microcirculation.¹⁸ Thus, studying the mechanisms that regulate α -cell growth and endocrine function is useful to better understand the biology of these cells and the related diseases.

Among islet tumors that develop in patients with MEN1, glucagonomas only rarely occur. Intriguingly, Perren et al¹⁹ and Anlauf et al²⁰ have shown that in patients with MEN1, most of the early islet lesions with

Abbreviations used in this paper: β -gal, β -galactosidase; KO, knock-out; MEN1, multiple endocrine neoplasia type 1; Ngn3, neuroginin 3; PCR, polymerase chain reaction.

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a loss of heterozygosity at chromosome 11q13 were glucagon positive, whereas the advanced hormone-secreting islet tumors detected in these same patients were mainly insulinomas, suggesting that the glucagon-positive early lesions may be replaced or overtaken by insulinomas and mixed islet tumors during tumorigenesis.

To investigate the role of menin in the pancreatic α cells and further to study MEN1 pathology, we generated an α cell-specific *Men1* knockout (KO) mouse model. Surprisingly, in addition to glucagonomas, insulinomas and mixed glucagon- and insulin-expressing islet tumors developed in these *Men1* mutant mice. A comparative analysis showed that these insulinomas displayed a different expression profile of endocrine pancreatic markers than that seen in the insulinomas that developed in a β cell-specific *Men1* mutant mouse model.⁸ Further analyses have shown that *Men1* ablation in α cells resulted in their proliferation, thereby triggering the transdifferentiation of menin-deficient α cells into insulin-expressing cells and the insulinoma formation. Therefore, our study highlights the role played by menin in the control of α -cell plasticity and establishes transdifferentiation as a novel mechanism involved in islet tumor development.

Materials and Methods

Animals

Men1^{+/-}, *Men1*^{F/F}, *Men1*^{F/F}-*RipCre*⁺, *GluCre*⁺, and *R26R* mice have been previously described.^{8,21-24} Mice were housed in pathogen-free facilities. All animal experiments were approved by the Regional Animal Experiments Committee of Centre National de la Recherche Scientifique and were conducted in accordance with the ethical guidelines.

Histologic and Immunostaining Analyses

Pancreata were harvested at different ages and fixed in 4% buffered formaldehyde. Immunohistologic analyses were performed on paraffin serial section as described previously.¹² For more information, see Supplementary Materials and Methods.

Quantitative PCR Analysis

Total RNA extraction was done on 2- to 10-month-old hand-picked islets with the use of an RNeasy Mini kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. Quantitative real-time polymerase chain reactions (PCRs) were performed as previously described.²⁵ Primer sequences are provided in Supplementary Materials and Methods.

Metabolic Measurements

Serum glucagon and insulin measurements were analyzed from collected blood with the use of a mouse endocrine kit (LINCOPlex, Multiplexed Biomarker Immunoassays; Linco Research, St. Charles, MO). Animals were deprived of food 5 hours before blood collection,

and duplicate measurements were repeated twice for each sample.

Double Immunoelectron Microscopy

Pancreata were fixed overnight in 4% paraformaldehyde and 0.1% glutaraldehyde and then processed as described in Supplementary Materials and Methods.

Statistical Analysis

All results were reported as means \pm standard errors of the mean. Each variable was analyzed with the unpaired Student's *t* test. For all analyses, *P* values of $<.05$ were considered significant. All analyses were performed with the use of GraphPad Prism software (GraphPad Software Inc, San Diego, CA).

Results

Generation of an α Cell-Specific *Men1* Knockout Mouse Model

To disrupt the *Men1* gene in the pancreatic α cells, *Men1*^{F/F} mice⁸ were crossed with *GluCre* mice expressing Cre recombinase driven by the *Proglucagon* promoter.²³ We first checked the α -cell specificity of *GluCre* in the endocrine pancreas with the use of *GluCre-R26R* mice²¹ (Figure 1A). Consistent with previous reports,^{26,27} β -galactosidase (β -gal) expression was exclusively detected in the glucagon-producing cells in the pancreata of *GluCre-R26R* mice either at embryonic day 18.5 or at 5 months of age (Figure 1B). No labeling was found in insulin-producing cells (Figure 1B) or in δ , pancreatic polypeptide, or acinar cells (data not shown).

Cell-specific *Men1* ablation was then examined in *Men1*^{F/F}-*GluCre*⁺ mice. As expected, the excision of the floxed *Men1* allele was detected by PCR analysis in the pancreatic islets and intestinal tissues isolated from 6-week-old *Men1*^{F/F}-*GluCre*⁺ mice but not in other tissues (Figure 1C). *Men1* inactivation was further confirmed by quantitative reverse transcription-PCR (Figure 1D) and immunofluorescence (Figure 1E). Menin expression was lost in 35% of glucagon-expressing cells in the pancreas of *Men1*^{F/F}-*GluCre*⁺ mice at 6 weeks of age. Conversely, the insulin-expressing cells remained menin positive at this stage. Altogether, the results confirmed α cell-specific *Men1* disruption in the mutant mice.

α Cell-Specific *Men1* Ablation Leads to Glucagonoma, Insulinoma, and Mixed Islet Tumor Development

Men1^{F/F}-*GluCre*⁺ mice were monitored over a 12-month period and necropsied at different ages. At 2 to 3 months of age, α -cell proliferation was clearly observed in *Men1*^{F/F}-*GluCre*⁺ mice, with a 40% increase in the proportion of glucagon-expressing cells over total islet cells. Menin detection showed that the increased number of α cells was due to the expansion of menin-deficient α cells (Supplementary Figure 1A and B). Glucagonomas were

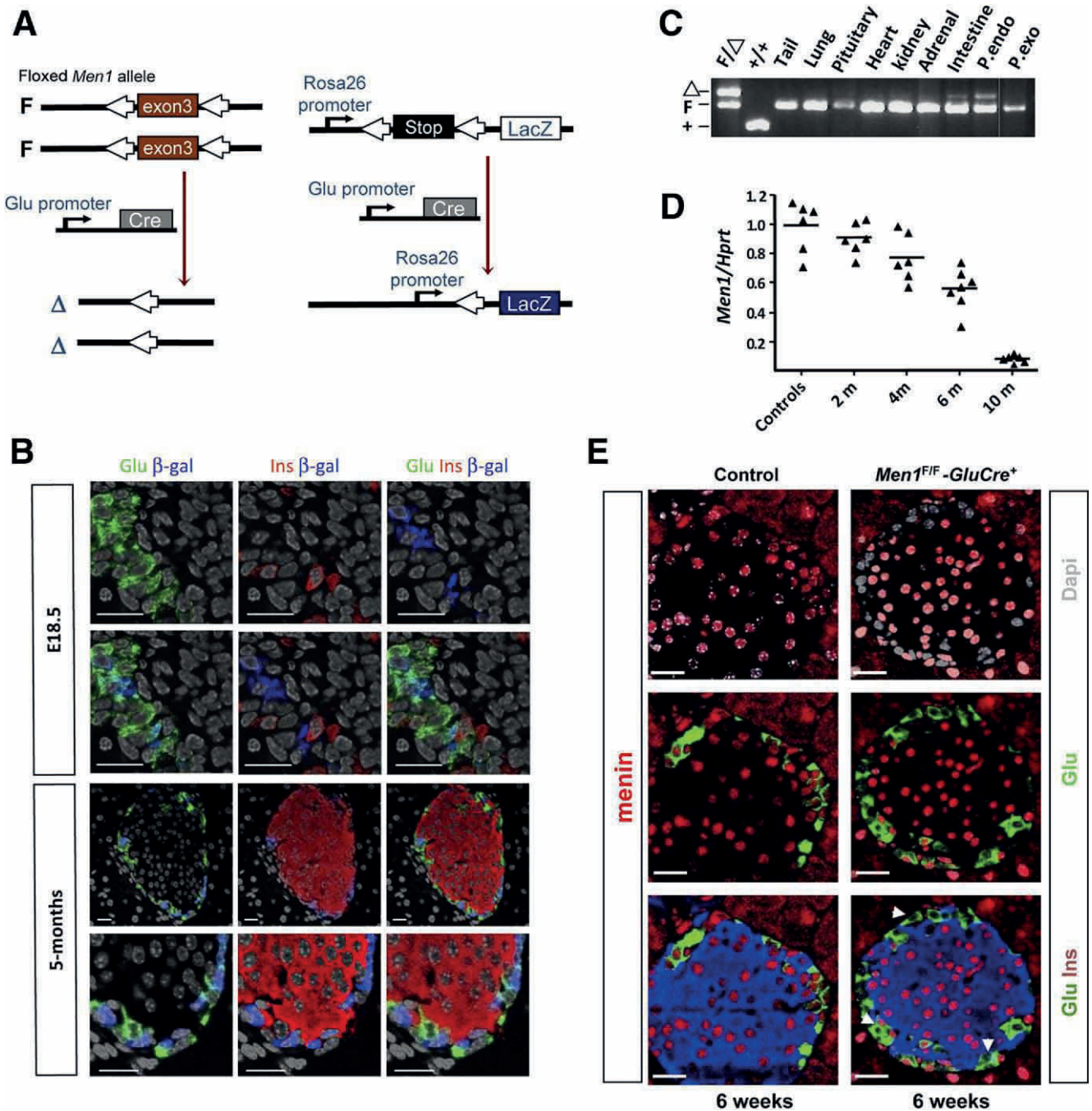


Figure 1. α cell-specific disruption of the *Men1* gene in *Men1*^{F/F}-*GluCre*⁺ mice. (A) Schematic view of the experimental strategy. *R26R* mice were crossed with both *GluCre*⁺ and *Men1*^{F/F}-*GluCre*⁺ mice to trace recombined α cells. (B) Representative images of the lineage-tracing analysis with the use of immunofluorescence in E18.5 embryonic and 5-month-old islets of *GluCre*-*R26R* mice. Note β -gal is expressed only in glucagon (Glu)-but not in insulin (Ins)-expressing cells. Scale bars, 20 μ m. (C) PCR analysis of tissue-extracted DNA from 6-week-old *Men1*^{F/F}-*GluCre*⁺ mice. Δ , deleted *Men1* allele; F, floxed *Men1* allele; +, wild-type *Men1* allele (n = 4). (D) *Men1* mRNA level assessed by quantitative reverse transcription-PCR analysis in pancreatic islets of 2- to 10-month-old *Men1*^{F/F}-*GluCre*⁺ mice (n \geq 6 for each group). (E) Immunofluorescence analysis of menin expression in 6-week-old *Men1*^{F/F}-*GluCre*⁺ and control pancreatic islets (n = 5). Arrows indicate menin-deficient glucagon-expressing cells. Scale bars, 20 μ m.

detected from 7 months of age (Supplementary Table 1; Figure 2A; Supplementary Figure 1C).

Surprisingly, islet adenomas nearly exclusively expressing insulin were also detected in *Men1*^{F/F}-*GluCre*⁺ mice

starting at 7 months of age (Supplementary Table 1; Figure 2A), whereas no pancreatic polypeptide- or somatostatin-secreting islet tumors were found (data not shown). There were also a substantial number of islet

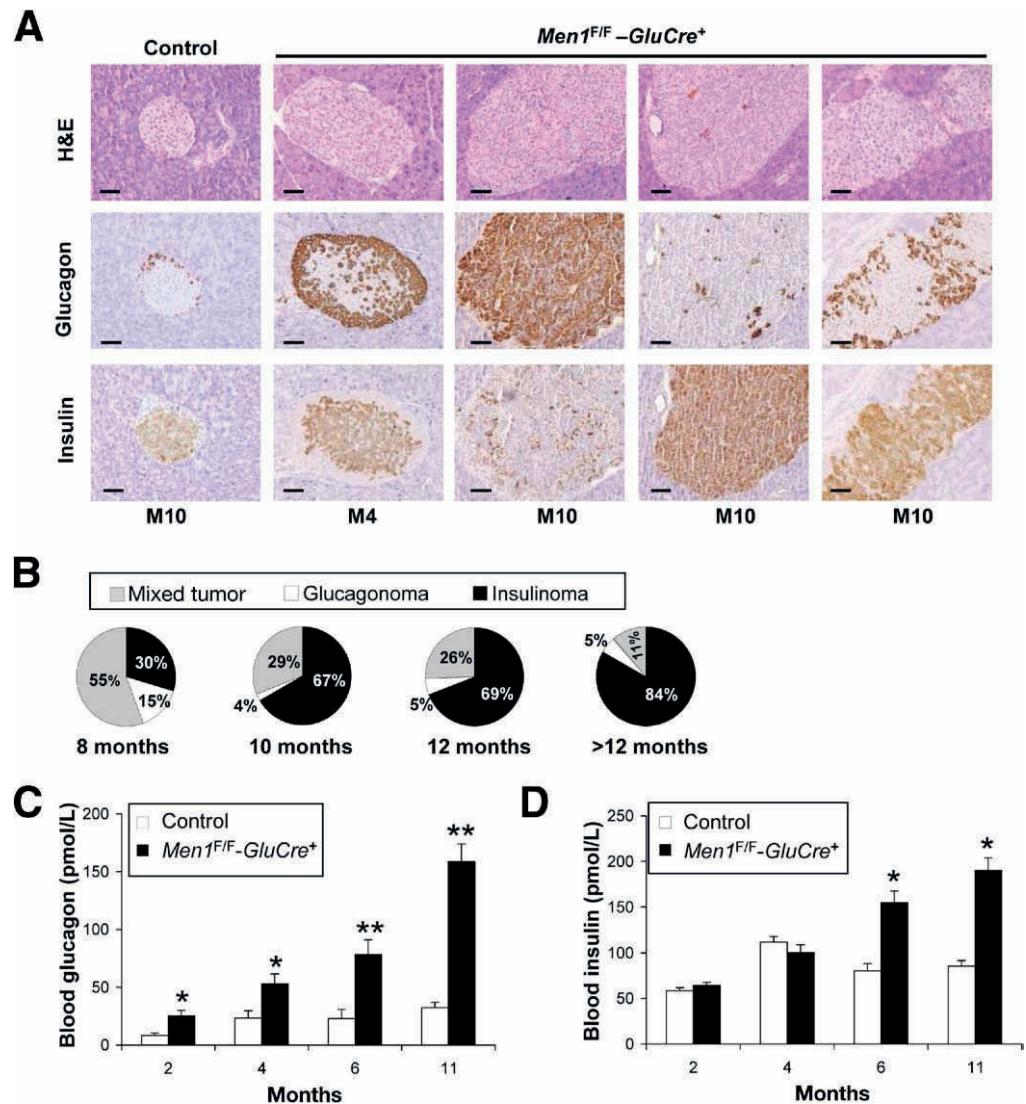


Figure 2. Disruption of the *Men1* gene in α cell leads to glucagonoma, insulinoma, and mixed islet tumor development. (A) Representative micrographs of H&E, glucagon, and insulin stainings, showing, from left to right, a normal islet from a control mouse, an α -cell hyperplasia (4 months old), a glucagonoma, an insulinoma, and a mixed islet tumor from 10-month-old mutant mice. Scale bars, 50 μ m. (B) Proportions of the different islet tumors observed at 8, 10, and 12 months of age and older in *Men1*^{F/F}-*GluCre*⁺ mice (n = 5–10 for each age group). (C–D) Increase in glucagon (C) and insulin (D) serum concentration in *Men1*^{F/F}-*GluCre*⁺ mice (n = 8), compared with the control *Men1*^{+/+}-*GluCre*⁺ mice (n = 6). Data are presented as means \pm standard errors of the mean; **P* < .05, ***P* < .01.

tumors expressing both insulin and glucagon (Figure 2A). At 8 months of age, the proportion of islet tumors exclusively expressing glucagon or both insulin and glucagon was 15% and 55%, respectively. However, these proportions decreased to 4%–5% and 29%–26%, respectively, at 10 to 12 months of age. In contrast, the proportion of insulinomas markedly increased over the same period (Figure 2B). No islet tumors were observed in *Men1*^{+/+}-*GluCre*⁺ (Supplementary Table 1) or *Men1*^{F/F}-*GluCre*⁻ control mice (data not shown). The insulinomas expressed both proprotein convertase 1/3 and glucose transporter 2, 2 mature β -cell markers (Supplementary Figure 2A). To examine islet cell proliferation, we assessed bromodeoxyuridine incorporation and Ki67 expression in 10-month-old *Men1*^{F/F}-*GluCre*⁺ mice. Compared with the control mice, the number of cells positively labeled was increased, respectively, by 11- and 10-fold in the islet tumors (Supplementary Figure 2B). No difference in the level of bromodeoxyuridine and

Ki67 labeling was observed between the insulinomas and glucagonomas (data not shown).

Hyperglucagonemia was detected in mutant mice at the age of 2 months and increased with age (Figure 2C), whereas hyperinsulinemia was found starting from 6 months of age (Figure 2D). The blood glucose concentration was increased in young *Men1*^{F/F}-*GluCre*⁺ mice but decreased, starting at 6 months of age, and reached the same concentration as that in the control by 10 months of age (Supplementary Figure 2C). Intraperitoneal glucose tolerance tests conducted in 2-month-old mice indicated a mildly impaired glucose tolerance in mutant mice (Supplementary Figure 2D), whereas the impaired glucose tolerance was no longer present in mutant mice older than 10 months (data not shown). Taken together, the hormonal and metabolic alterations observed in the mutant mice were consistent with the early α -cell proliferation and late insulinoma development in these mice.

Occurrence of Insulinomas Is Not Due to Adaptive β -Cell Proliferation or Cell Fusion

Because β -cell growth can be triggered by physiologic needs,^{11,28} we asked whether the development of insulinoma in *Men1^{F/F}-GluCre⁺* mice could be due to adaptive β -cell proliferation that was triggered by the

hyperglycemia that occurred in young mutant mice. To this end, we analyzed menin expression and the status of the *Men1* gene in the insulinomas, because the insulinoma cells with eventual adaptive proliferation should not be subjected to *Men1* ablation. As shown in Figure 3A, menin was not expressed in insulinomas. Genotyping

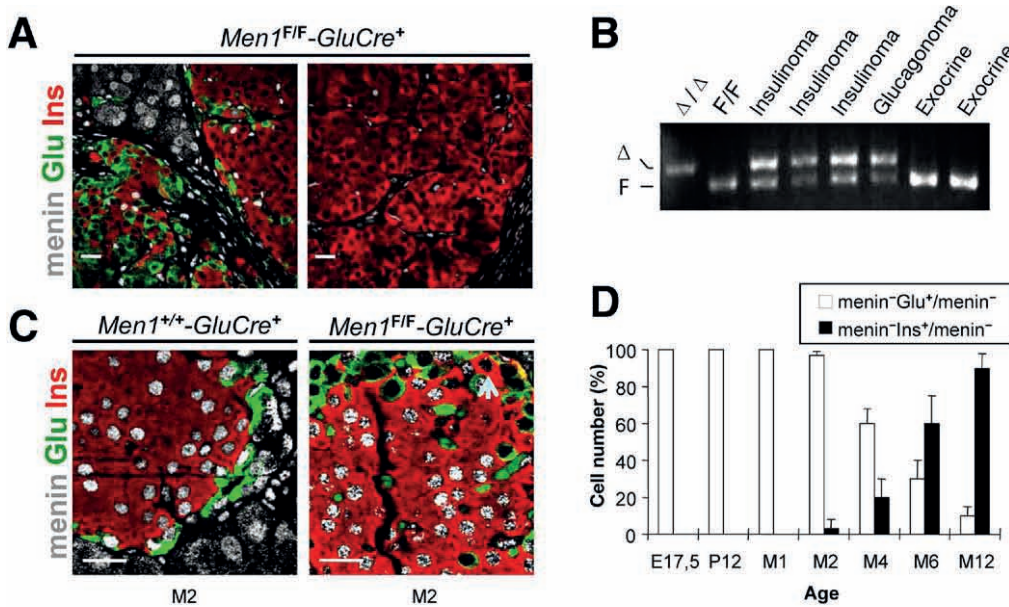
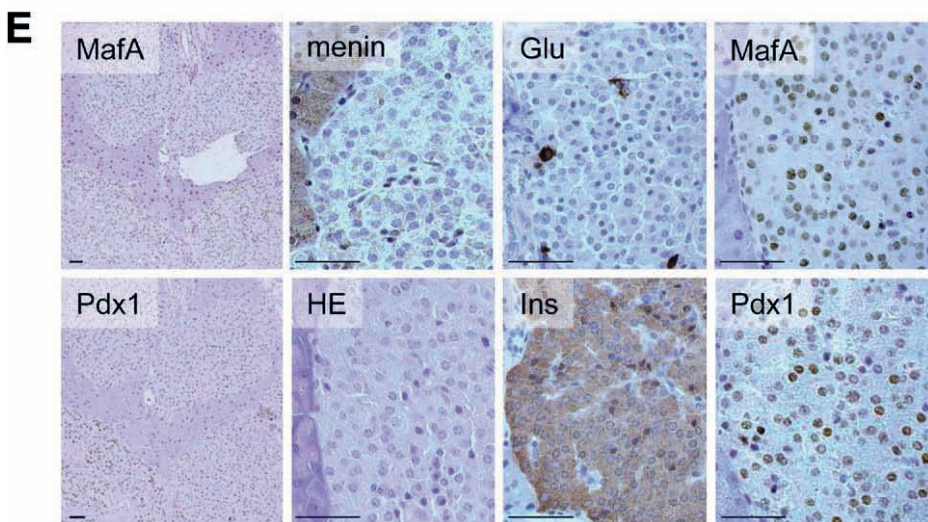


Figure 3. Insulinoma development is not due to adaptive β -cell proliferation or to *GluCre* transgene misexpression. (A) *Men1* inactivation is found in insulinomas that developed in *Men1^{F/F}-GluCre⁺* mutant mouse. Shown are 2 representative triple-immunofluorescence micrographs of insulin (Ins), glucagon (Glu), and menin (menin) immunoreactivity in pancreatic sections of a 12-month-old mutant mouse. Scale bars, 50 μ m. (B) Deletion of the *Men1* allele (Δ) detected by PCR in both microdissected α - and β -cell tumors. F, floxed *Men1* allele. (C) Representative confocal micrograph of insulin (Ins), glucagon (Glu), and menin expression in control and *Men1^{F/F}-GluCre⁺* 2-month-old mice (M2). The arrow indicates menin-deficient insulin-expressing cells. Scale bars, 20 μ m. (D) The number of menin-deficient insulin (Ins⁺)- or glucagon (Glu⁺)-expressing cells are presented as the percentage of the total number of menin-deficient islet cells. For each animal, 100 (E17.5 embryos) or 400 (P12, 12 days old; M2, M4, M6, and M12 are, respectively 2-, 4-, 6- and 12-month-old mice) islet cells were analyzed (n = 3 mice per age group). (E) Homogenous expression of Pdx1 and MafA in insulinomas that developed in 12-month-old *Men1^{F/F}-RipCre⁺* mutant mice. Representative micrographs of pancreatic sections stained for H&E, menin, MafA, Pdx1, Glu, and Ins. Scale bars, 50 μ m. (F) Heterogeneous expression of pancreatic endocrine cell markers in the insulinomas developed in *Men1^{F/F}-GluCre⁺*. Three different subtypes (I to III) were categorized for Pdx1, MafA, and MafB expression.



	Normal islet		Glucagonoma (<i>Men1^{F/F}-GluCre⁺</i>)	Insulinoma (<i>Men1^{F/F}-GluCre⁺</i>)		
	α cells	β cells		Type I	Type II	Type III
menin	+	+	-	-	-	-
Glucagon	+	-	+	-	-	-
Insulin	-	+	-	+	+	+
Pdx1	-	+	-	+	+	+
MafA	-	+	-	-	+	+
MafB	+	-	+	+	-	+

of microdissected insulinomas further showed that the insulinoma cells, similar to the glucagonoma cells, contained the deleted *Men1* allele, whereas the exocrine pancreatic cells uniquely displayed the intact floxed *Men1* allele (Figure 3B). The data indicate that the insulinoma cells underwent the same genetic modification of the *Men1* gene as the glucagonoma cells, which excludes the possibility that the insulinoma development in *Men1^{F/F}-GluCre⁺* mice resulted from adaptive β -cell proliferation.

To further detail insulinoma development in mutant mice, we investigated the appearance of menin-deficient glucagon- (menin⁻Glu⁺) and insulin-expressing (menin⁻Ins⁺) cells with the use of immunofluorescence (Supplementary Table 2). Menin⁻Ins⁺ cells were not detectable in *Men1^{F/F}-GluCre⁺* mice until 2 months of age and accounted for <2% of the total menin-deficient cells and only 1% of the total Ins⁺ cells in 2-month-old *Men1^{F/F}-GluCre⁺* mice (Figure 3D; Supplementary Table 2). At the same stage, nearly half of the α cells lacked menin expression. In 4-month-old mutant mice, menin⁻Ins⁺ cells accounted for approximately 20% of the total menin-deficient cells, and the percentage increased sharply thereafter. Overall, menin⁻Ins⁺ cells appeared after the proliferation of menin-deficient glucagon-expressing (menin⁻Glu⁺) cells but before the development of islet tumors. The number of menin⁻Ins⁺ cells increased with age and gradually exceeded the number of menin⁻Glu⁺ cells.

Furthermore, no multinucleated menin⁻Ins⁺ cells were observed in *Men1^{F/F}-GluCre⁺* mice at different ages (data not shown). In addition, there was no irregular nuclear menin expression in insulinomas (Figure 3A). These results suggest that the development of insulinomas in mutant mice was not caused by cell fusion.

Heterogeneous Expression of β - and α -Cell Markers in Insulinomas

We next asked whether the altered cellular context resulting from *Men1* disruption in α cells could lead to the mis-expression of the *GluCre* transgene in the β cells of *Men1^{F/F}-GluCre⁺* mice. We reasoned that, if this was indeed the case, the insulinomas that developed should be similar to the insulinomas that developed in *Men1^{F/F}-RipCre⁺* mice, which is a β cell-specific *Men1* KO model, because the initial genetic event (*Men1* ablation) would be the same. Therefore, we compared the expression profile of pancreatic endocrine markers between the insulinomas from these 2 models. Both Pdx1 and MafA, 2 β -cell markers, were homogeneously expressed in all of the tested insulinomas from *Men1^{F/F}-RipCre⁺* mice (Figure 3E). In contrast, the insulinomas that developed in *Men1^{F/F}-GluCre⁺* mice displayed a heterogeneous expression of these markers (Figure 3F; Supplementary Figure 3), with at least 3 different expression profiles detected. One subtype (type I) expressed MafB and Pdx1, but not MafA, a typical β -cell marker. The second subtype (type II) expressed Pdx1 and MafA, but not

MafB, whereas the third subtype (type III) expressed all 3 markers. Such a heterogeneous expression profile of pancreatic endocrine markers, particularly the type I insulinoma (Pdx1⁺MafA⁻MafB⁺), makes these insulinomas largely different from those that developed in the β cell-specific *Men1* mutant mice. Therefore, it is unlikely that these insulinomas were caused by the leaky expression of the transgene in the β cells of *Men1^{F/F}-GluCre⁺* mice.

Appearance of Menin-Deficient Cells Having the Characteristics of Both α and β Cells in Young Mutant Mice

Another potential explanation for the development of insulinomas in *Men1^{F/F}-GluCre⁺* mice is that *Men1* disruption results in not only α -cell proliferation but also the transdifferentiation of menin-deficient α cells into insulin-expressing cells. If this is the case, we should be able to detect intermediate menin-deficient cells sharing characteristics of both α and β cells during the transdifferentiation procedure. With the use of triple immunofluorescence staining, 2 types of intermediate cells were observed in pancreata of young mutant mice: (1) menin-deficient cells coexpressing glucagon and insulin (menin⁻Glu⁺Ins⁺, designated as A1 cells), that started to appear in 3- to 4-month-old mutant mice (Figure 4A middle), and (2) menin-deficient cells expressing only insulin (menin⁻Glu⁻Ins⁺, namely A2 cells), localized in the same islet area with menin⁻Glu⁺Ins⁻ cells together with some residual A1 cells and found in 4- to 6-month-old mice (Figure 4A right). Noticeably, the menin⁻Glu⁻Ins⁺ (A2) cells displayed a sharp and narrow insulin-staining pattern, resembling that seen in neighboring menin⁻Glu⁺Ins⁻ cells, but completely distinct from the diffuse insulin staining seen in normal β cells. This pattern was never detected in normal control islets (Figure 4A left). Therefore, the sequential appearance of menin-deficient cells coexpressing glucagon/insulin and cells expressing insulin in young *Men1^{F/F}-GluCre⁺* mice was observed before the development of islet tumors.

To further confirm the existence of menin⁻Glu⁺Ins⁺ cells, ultrastructural double immunogold staining for glucagon and insulin was analyzed by electronic microscopy. In control mice, α cells contained only the typical high-density α granules that were positively stained with antiglucagon antibody, whereas β cells contained only the β granules stained with anti-insulin antibody (Figure 4B left). In 3- to 5-month-old mutant mice, the density and structure of these secretory granules were altered compared with the controls (Figure 4B middle and right). Furthermore, we observed granules stained with both glucagon and insulin antibodies (Figure 4B right), as well as granules either glucagon or insulin positive within the same cells (Figure 4B middle). These data provided the further evidence for the existence of intermediate cells during transdifferentiation.

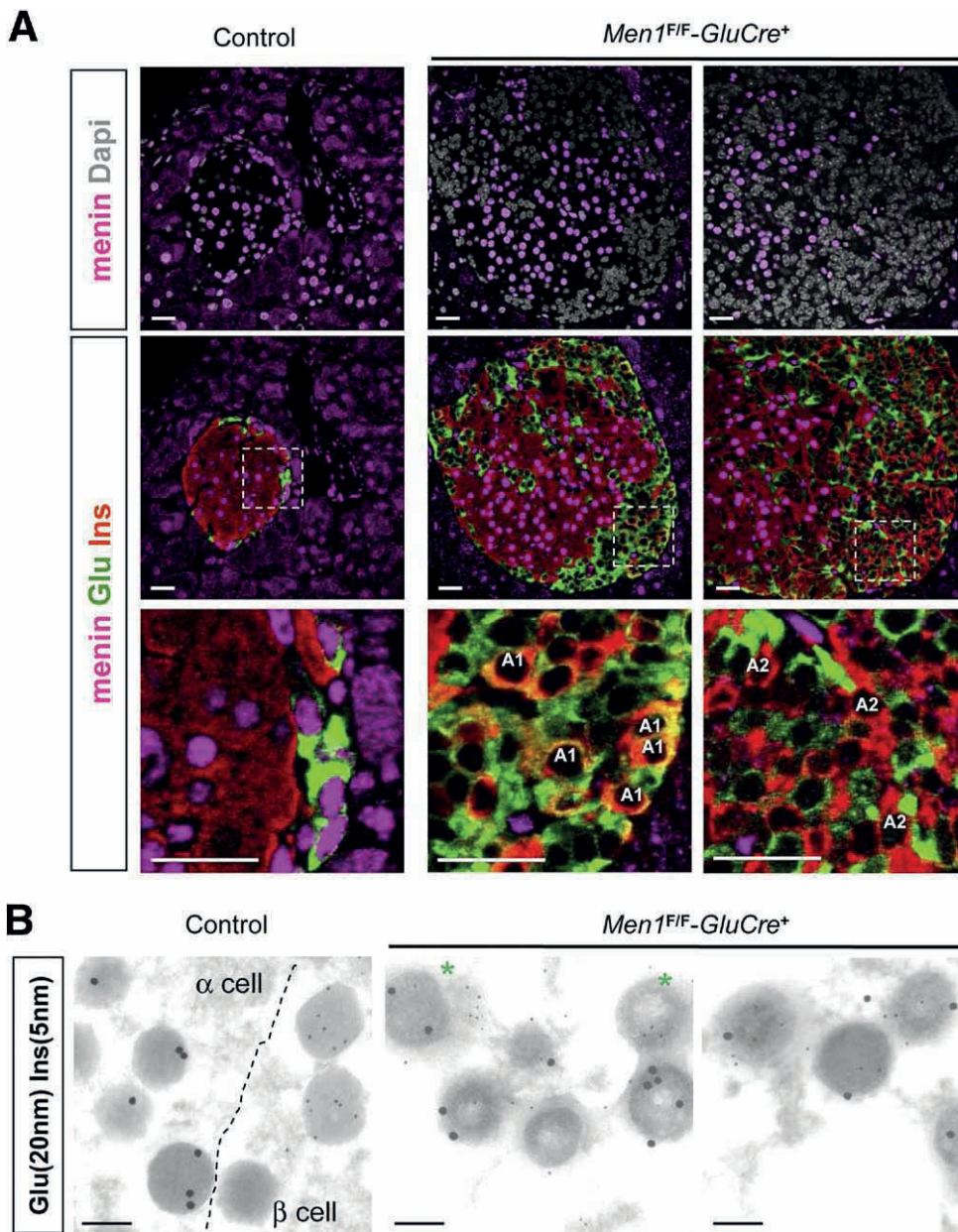


Figure 4. α -cell *Men1* disruption leads to the sequential appearance of menin⁻Glu⁺Ins⁺ and menin⁻Glu⁻Ins⁺ cells. (A) Menin-deficient islet lesions display unusual hormone expression patterns. Representative immunofluorescence micrographs of glucagon (Glu), insulin (Ins), and menin expression in control and mutant mice. Note the menin-deficient cells coexpressing glucagon and insulin (A1) in early lesions (3- to 4-month-old mutant mice) (middle). Shown in the right panel are sections from 6-month-old mutant mice displaying dysplasia lesions with mixed insulin (A2) or glucagon-expressing cell populations as well as residual cells coexpressing insulin and glucagon. Magnified views of the dashed area are shown in the lower panel. Scale bars, 20 μ m. (B) Representative pictures of ultrastructural double-immunogold staining for insulin (5 nm; Ins) and glucagon (20 nm; Glu) performed on pancreas sections. Note the presence in mutant mice of islet cells containing secretory granules with immunoreactivity both to glucagon and insulin (right) or positively stained by either glucagon or insulin (middle; granules with green asterisk). Scale bars, 200 nm.

Menin-Deficient Ins⁺ Cells Are Derived From Glucagon-Expressing Cells

The transdifferentiation of menin-deficient α cells into insulin-expressing cells was further confirmed with a genetic cell lineage-tracing analysis. The *Men1^{F/F}-GluCre⁺-R26R* mice allowed us to trace all cells derived from *Men1*-deficient glucagon-expressing cells. β -Gal expression was never detected in insulin-expressing cells from *GluCre⁺-R26R* control mice (Figure 5A), nor in *Men1^{F/F}-GluCre⁺-R26R* mutant mice younger than 2 months (Figure 5B). However, it could be detected in a few insulin-expressing cells in *Men1^{F/F}-GluCre⁺-R26R* mutant mice, starting at 2 months of age, the proportion of β -gal⁺Ins⁺ cells increasing with age in the mutant mice. In fact, β -gal staining was seen in all the islet lesions found in these

mice, including the initial menin-deficient α -cells (Figure 5B), the early islet lesions containing menin⁻Glu⁺Ins⁺ or menin⁻Glu⁻Ins⁺ cells (Figure 5C), the insulinomas (Figure 5D; Supplementary Figure 4), and the glucagonomas (data not shown). These data indicate that the identified menin-deficient insulin-expressing cells are derived from glucagon-expressing pancreatic endocrine cells, confirming therefore the origin of insulinoma cells in the mutant mice.

This interesting finding in α cell-specific *Men1* KO mice prompted us to explore whether the transdifferentiation could also occur in heterozygous *Men1* mutant mice (*Men1^{+/-}*), particularly in menin-deficient lesions with α -cell proliferation. Note that these *Men1* mutant mice were generated without the use of any Cre transgene. We analyzed insulin and glucagon expression in the

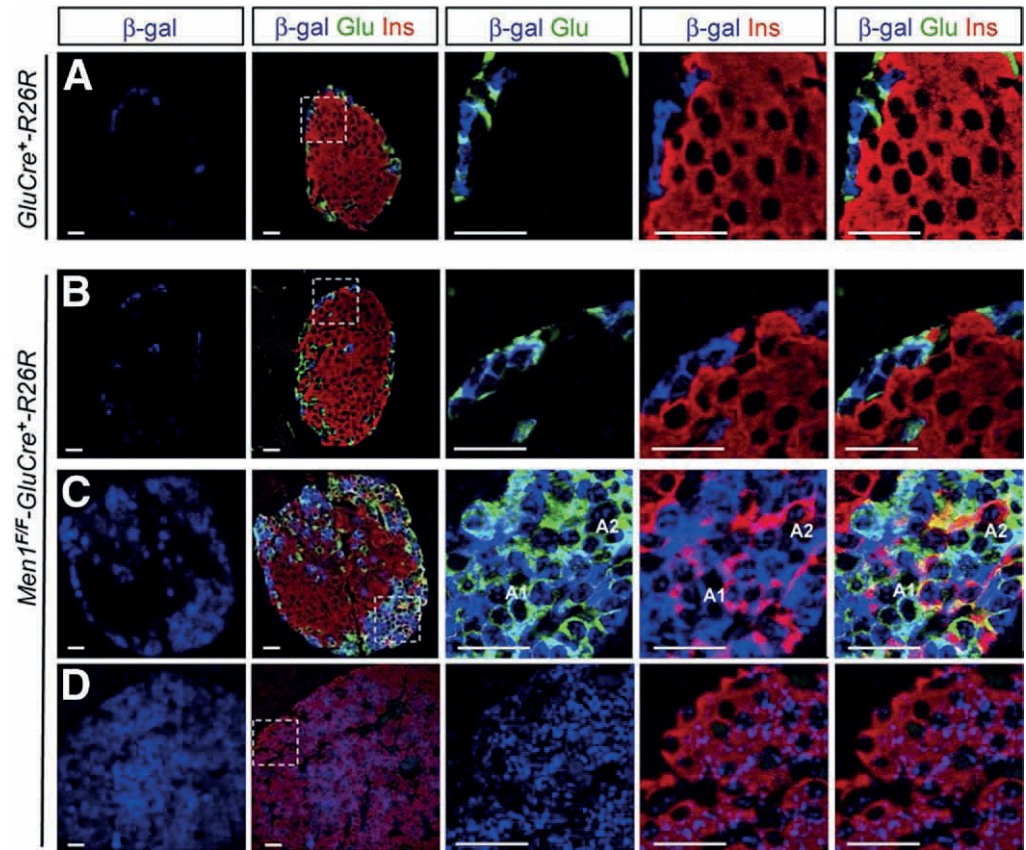


Figure 5. Insulinoma cells originate from glucagon-expressing cells in *Men1^{F/F}-GluCre⁺* mice. Lineage-tracing study with *Men1^{F/F}-GluCre⁺-R26R* mice showed that both menin-deficient α and β cells were positively stained with a β -gal-specific antibody. Shown are β -gal, insulin (Ins), and glucagon (Glu) triple immunofluorescence micrographs in control mouse (A), a very early lesion (B; 6-week-old), a typical early lesion (3-month-old) with A1 and A2 cells (C), and an insulinoma (D; 10-month-old) from *Men1^{F/F}-GluCre⁺* mice. Magnified views of the dashed area are shown in the 3 right panels. Scale bars, 20 μ m.

glucagon-positive islet lesions from 9- to 24-month-old *Men1^{+/-}* mice.²² Remarkably, coexpression of insulin and glucagon was indeed detected in 5 of 6 *Men1^{+/-}* mice. The lesions ranged from early lesions almost uniquely consisting of menin-deficient α cells (Supplementary Figure 5A) to glucagonomas and mixed islet tumors (Supplementary Figure 5B and C, respectively). These observations suggest that the transdifferentiation from α cells to insulin-expressing cells is a common phenomenon in *Men1*-related α -cell tumorigenesis.

Early Transdifferentiating Cells Express *MafB*, but Not *Pdx1*, *MafA*, *Pax4*, or *Ngn3*

To gain insights into the molecular mechanisms of the transdifferentiation, the expression of several pancreatic endocrine cell-specific transcriptional factors was further examined throughout the tumor development procedure. Noticeably, the early transdifferentiating cells, including both menin⁻Glu⁺Ins⁺ (A1 cells; Figure 6A) and menin⁻Glu⁻Ins⁺ cells (A2 cells; Supplementary Figure 6A), expressed *MafB*, but neither *Pdx1* nor *MafA*. Importantly, this expression profile was different from that of normal β cells (Figure 6A) but was identical to that of normal and menin-deficient α cells (Supplementary Figure 6B). Moreover, menin⁻Glu⁻Ins⁺*MafB*⁺*MafA*⁻*Pdx1*⁻ cells have never been detected in the insulinomas that developed in β cell-specific *Men1* KO mice (see above). Therefore, our data provide further evidence that both menin⁻Glu⁺Ins⁺ and

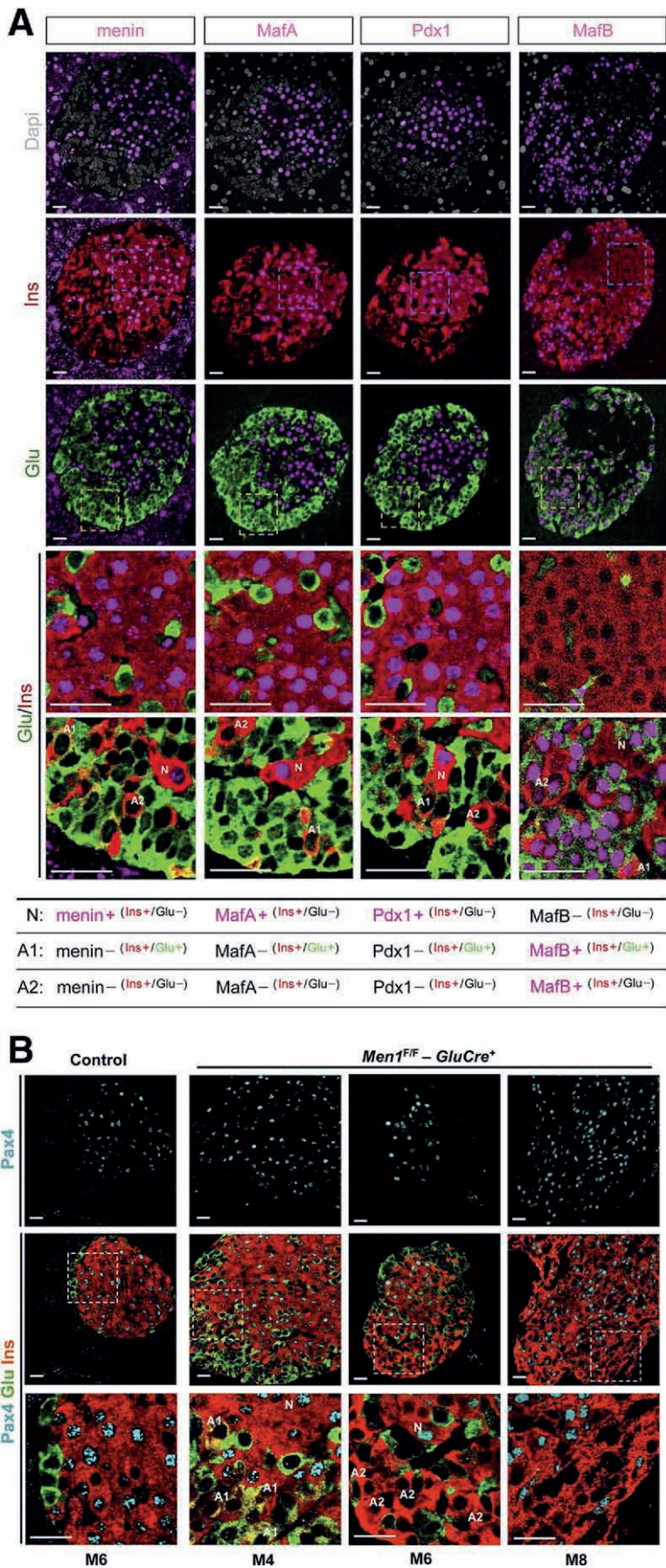
menin⁻Glu⁻Ins⁺ cells are closely related to menin⁻Glu⁺Ins⁻ cells and that the former cells may be derived from the latter through transdifferentiation.

Given the important role played by *Ngn3* in the differentiation of pancreatic endocrine cells during development,^{29,30} we examined its expression in *Men1^{F/F}-GluCre⁺* mice. No *Ngn3* expression was detected in the pancreata of 3- or 8-month-old mutant mice (data not shown). *Pax4* is another important factor essential for β -cell differentiation in the mammalian pancreas.³¹ Moreover, a recent study reported the effect of ectopic *Pax4* expression leading to the conversion of α cells into insulin-secreting cells.^{32,33} We examined therefore *Pax4* expression in the mutant mice at 2–12 months of age. The data showed that *Pax4* expression was found expressed in most normal β cells, but neither in initial menin-deficient α cell nor in the early transdifferentiating cells in mutant mice at 3–6 months of age, whereas its expression could be detected in insulinomas in the mutant mice older than 7 months (Figure 6B; Supplementary Figure 7). Altogether, our data indicated that the initiation of the transdifferentiation from menin-deficient α cells into insulin-expressing cells may not imply *Pdx1*, *MafA*, *Pax4*, and *Ngn3* expression.

Discussion

Here we demonstrate that α cell-specific *Men1* ablation in mice leads to the development of glucagono-

BASIC-LIVER, PANCREAS, AND BILIARY TRACT



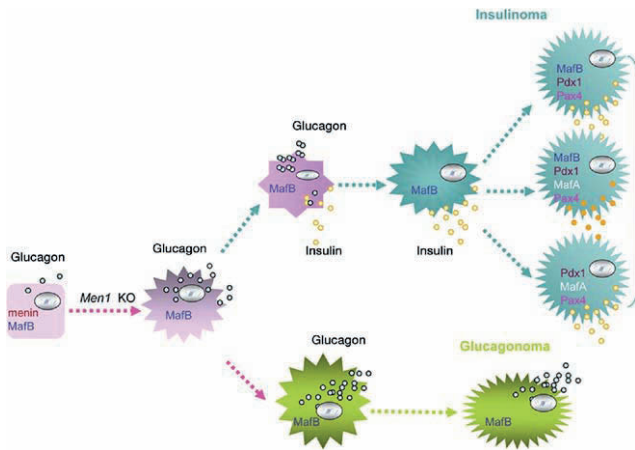


Figure 7. Schematic model depicting the development of insulinomas by transdifferentiation on α cell-specific *Men1* disruption. In adult mice, α cells produce glucagon and express MafB, but not Pdx1, MafA, or Pax4. α -cell *Men1* ablation leads to the proliferation of α cells but also to α -cell transdifferentiation. Initially, some of the *Men1*-deficient α cells coexpress insulin and glucagon and then become insulin-expressing cells. *Menin*-deficient insulin-expressing cells that have a profile similar to α cells subsequently develop into insulinomas that have a profile closer to β cells with the variable expression of pancreatic endocrine cell markers.

mas, insulinomas, and mixed islet tumors, and all the cells of islet lesions derive from cells having previously expressed the *Proglucagon* gene. The *GluCre*-mediated lineage tracing analyses, together with the sequential appearance of *menin*⁻*Glu*⁺*Ins*⁺*MafB*⁺*Pdx1*⁻*MafA*⁻ and *menin*⁻*Glu*⁻*Ins*⁺*MafB*⁺*Pdx1*⁻*MafA*⁻ cells before tumor development, provided strong evidence showing that *menin*-deficient α cells transdifferentiate into insulin-expressing cells with time (Figure 7), which further develop into insulinomas. Our work establishes for the first time *menin* as an *in vivo* regulator of pancreatic α -cell plasticity, and the transdifferentiation, induced by the deregulation of α -cell plasticity because of *menin* inactivation, as a novel mechanism involved in islet tumor development. Interestingly, the reverse phenomenon, ie, a β - to α -cell transdifferentiation, was not observed in β cell-specific *Men1* KO mice (data not shown).

The complexity of MEN1 and non-MEN1 pancreatic endocrine tumors is well known, and their histogenesis remains elusive.^{19,34,35} The tumor phenotype found in *Men1*^{F/F}-*GluCre*⁺ mice, characterized by glucagon-positive early lesions, islet tumors secreting multiple hormones, and the occurrence of insulinomas in aged mice, is reminiscent of the recent clinical observations.^{19,20} Therefore, the finding of involvement of transdifferentiation in islet tumor histogenesis in our study paves new ground for further investigation of the complex nature of islet tumors. Transdifferentiation is seldom mentioned as an underlying mechanism of tumorigenesis. Nevertheless, epithelial-to-mesenchymal transition, a form of transdifferentiation, plays an important role in cancer formation and progression.³⁶ Squamous metaplasia, seen in breast

and prostate cancers, and other forms of metaplasia observed in esophageal and stomach cancers are well-known precancerous lesions.³⁷ However, the transdifferentiation between different hormone-secreting cell types has never been experimentally established in the framework of endocrine tumors.

The plasticity of fully mature cells in the pancreas has been an attractive research area and of controversy. Recent studies implied that several types of mature pancreatic cells may possess more potential in cell plasticity than previously thought.³⁸⁻⁴⁰ Interestingly, both massive loss of β cells^{41,42} and the blockade in α -cell function by either gene targeting⁴³ or glucagon receptor antagonists⁴⁴ resulted in cells coexpressing glucagon and insulin. Although hormonal coexpression per se is not a proof of cell lineage conversion, these studies may have provided some hints on α -cell plasticity. By different analyses, we have shown that glucagon-expressing cells can indeed transdifferentiate into insulin-expressing cells on *menin* inactivation. Note that the transdifferentiation that we report here relates to islet tumorigenesis. Nevertheless, common features may exist among transdifferentiation events occurring in different contexts.

The finding of *menin*⁻*Glu*⁻*Ins*⁺*MafB*⁺*Pdx1*⁻*MafA*⁻ cells in the early lesions that developed in *Men1*^{F/F}-*GluCre*⁺ mice is intriguing, because they have been seen neither in normal adult islet cells nor in β cell-specific *Men1* mutant mice. The lack of Ngn3, Pdx1, and MafA expression in these cells makes them more similar to the immature *MafB*⁺*Ins*⁺ cells that have been documented during development.⁴⁵ However, further work is needed to clarify the similarities and the differences between the transdifferentiated cells observed in the current study and the *MafB*⁺*Ins*⁺ cells that occur during development. Interestingly, Collombat et al³² recently reported that ectopic Pax4 expression could convert pancreatic progenitors to α cells and subsequently into β cells. The occurrence of such a conversion is associated with α -cell deficiency, Ngn3 re-expression, and ectopic Pax4 expression.^{32,33} We noticed that this is manifestly different from the transdifferentiation reported in the current work which is featured by α -cell proliferation and lack of Ngn3 and Pax4 expression in the initial transdifferentiating cells. Markedly, their work highlighted the importance of the balance among islet-specific transcriptional factors in specifying cell fate. Considering the known functions of *menin*, we speculate that the deregulation of these factors could be involved in the mechanisms leading to the transdifferentiation observed in the current work. It will be important in the future to identify the genetic and epigenetic factors involved in the procedure.

In conclusion, the current study shows that *menin* regulates α -cell growth and, more importantly, α -cell plasticity. Our results provide, therefore, novel insights into the genetic events that regulate plasticity in differentiated α cells and shed new light on the histogenesis

and biology of multihormone-secreting islet tumors. In addition, given the need for cell-replacement therapy for diabetes, we believe that the modulation of α -cell plasticity could be explored in the future to develop alternative strategies for β -cell regeneration.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.01.046.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Glucose Tolerance Test

Glucose tolerance tests were performed after depriving animals of food. Animals were injected (intraperitoneally) with dextrose 2 g/kg of body weight (Sigma-Aldrich, St. Louis, MO). Glucose concentrations were measured from tail blood collected immediately before and 5, 15, 30, 60, and 120 minutes after the injection.

Quantitative PCR Analysis

Primer sequences were used as follows: *Men1*-fw 5'-GAAGATCATGCTTGGGTGGT-3', *Men1*-rev 5'-CAGTCTGCCAAGTTCCTA-3', *Hprt*-fw 5'-TGTTGTTGGA-TATGCCCTTG-3', and *Hprt*-rev 5'-AACTTGCGCTCATC-TTAGGC-3'.

Antibodies and Reagents for Histologic Analyses

Antibodies used for immunocytochemistry are as follows: menin (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), insulin (1:4000; Dako, Carpinteria, CA), glucagon (1:5000; NovoCastra, Newcastle, United Kingdom), bromodeoxyuridine (BrdU; 1:1000; Sigma-Aldrich), Ki67 (1:500; Santa Cruz Biotechnology). For immunofluorescence analysis, primary antibodies against insulin (1:1000, guinea-pig, Dako; 1:2000, mouse, Sigma-Aldrich); glucagon (1:2000, rabbit, Linco Research, St Charles, MO; 1:1000, mouse, Abcam, Cambridge, MA); MafA, MafB, and menin (1/4000, rabbit, Bethyl Laboratories, Montgomery, TX); glucose transporter 2 (Glut2; 1:2000, rabbit, Chemicon International, Temecula, CA); Pdx1 and anti-protein convertase 1/3 (PC1/3; 1:1000, rabbit, kind gifts from Dr Raphaël Sharfmann); Pax4 (1:500, rabbit,

kind gift from Dr Beatriz Sosa-Pineda); β -galactosidase (β -gal; 1:1000, rabbit, Cortex Biochem, San Leandro, CA) were used in combination with cyanine 2 (Cy-2)-, Cy-3-, and Cy-5-conjugated secondary antibodies (1/200; Jackson ImmunoResearch Laboratories Inc, West Grove, PA) as appropriate. Tyramide amplification (PerkinElmer Waltham, MA) was used according to the manufacturer's instructions.

Double Immunoelectron Microscopy

Immunoelectron microscopy was performed on ultrathin pancreas sections (70-nm thick) cut on a Reichert ultracut E ultramicrotome (Leica, Wetzlar, Germany) and mounted on polylysine-coated 200-mesh nickel grids. Gold immunolabeling was carried out at room temperature in 1% bovine serum albumin (BSA). Grids carrying samples were blocked (10 minutes in 1% BSA) and incubated 30 minutes with anti-glucagon primary antibody (1/1000, mouse, Abcam). The grids were subsequently washed in phosphate-buffered saline (PBS; 4 times, 15 minutes) and incubated with 20 nm gold-conjugated goat anti-mouse immunoglobulin G (IgG; 1/80; Tebu-bio, Le Perray en Yvelines, France) for 20 minutes. Grids were post fixed 5 minutes in 0.2% glutaraldehyde and washed in PBS/0.15 M glycine prior to the incubation with the anti-Insulin antibody (1/1000, guinea-pig, Dako). 5 nm gold-conjugated anti-guinea-pig IgG (1/80, Tebu-bio) were subsequently applied. Finally, the labeled grids were contrasted on ice in 0.3% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) within 2% methyl cellulose (Sigma-Aldrich) for 10 minutes and observed on a JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan).