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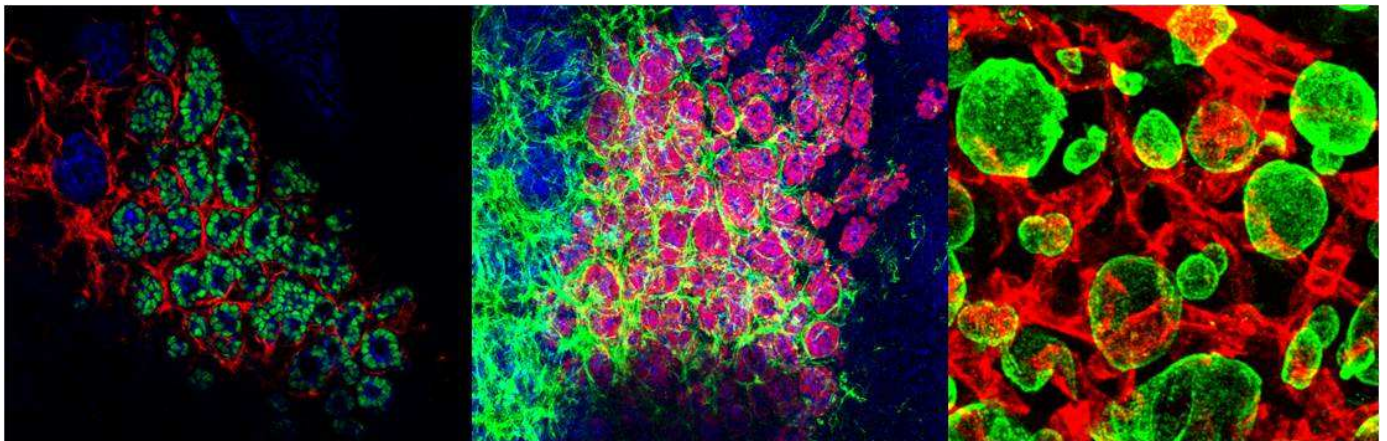
De Duve Institute (ICP)

Cell Biology Unit



UCL
Université
catholique
de Louvain

***Paracrine communications control
reorganization of epithelial masses into
polarized monolayers***



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*Thesis submitted in fulfillment of the requirement for the degree of
Ph.D. in Biomedical and Pharmaceutical sciences*

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III. ABBREVIATION LIST

A: aorta	FGFR: fibroblast growth factor receptor
ACh: acetyl-choline	FLK-1: fetal liver kinase 1
Adam: a disintegrin and metalloproteinase	Foxe1: forkhead box protein e1
ALK: activin receptor-like kinase	FRS2 α : fibroblast growth factor receptor substrate 2
AMIS: apical membrane initiation site	G: gut tube
Anx2: annexin2	GAG: glycosaminoglycans
AS: aortic sac	GDF: growth and differentiation factor
BAC: bacterial artificial chromosome	Gli: glioblastoma transcription factor
BMP: bone morphogenetic protein	HBEGF: heparin-binding epidermal growth factor
CA: carotid artery	Hes: hairy-related enhancer of split
CCh: carbachol	HGF: hepatocyte growth factor
cKO: conditional KO	Hhex: hematopoietically-expressed homeobox protein
Crb3: Crumbs3a	HIF: hypoxia-inducible factor
CPA: carboxypeptidase A	HNF-1 β : hepatocyte nuclear factor-1 β
CXCL: CXC chemokine ligand	HNF-6/OC-1: hepatocyte nuclear factor-6/onecut 1
CXCR: CXC chemokine receptor	HS: heparan sulfate
D: dorsal	HSPG: heparan-sulfate proteoglycan
DTR: diphtheria toxin receptor	HUVEC: human umbilical vein endothelial cells
e: embryonic day	IFN: interferon
ECM: extracellular matrix	IGF: insulin-like growth factor
eEPC: embryonic endothelial progenitor cell	ISH: <i>in situ</i> hybridization
EGF: epidermal growth factor	K5: cytokeratin-5
eGFP: enhanced green fluorescent protein	KI: knock-in
Eph: Eph receptor	KO: knockout
ERK: extracellular signal-regulated kinase	Lu: lung
ERM: ezrin-radixin-moesin	M1: muscarinic receptor M1
FGF: fibroblast growth factor	

MDCK: Madin-Darby canine kidney (cell)	PIP ₃ or PI(3,4,5)P ₃ : phosphatidylinositol (3,4,5)-trisphosphate
MG: mammary gland	
MMP: matrix metalloprotease	PPH3: phospho-histone H3
Muc1: mucin 1	RT-qPCR: reverse transcription - quantitative real time polymerase chain reaction
mRNA: messenger ribonucleic acid	Scl: stem cell leukemia
MS: methylsalicylate	SDF-1: stromal cell-derived factor-1
MV: membrane-derived microvesicle	Shh: sonic hedgehog
NC: neural crest	SMG: submandibular gland
Ngn3: neurogenin 3	Smo: smoothened
NIS: sodium iodide symporter	T: thyroid
NT: neural tube	T3: triiodothyronin
P0: postnatal day 0	T4: tetraiodothyronin, thyroxin
PAA: pharyngeal arch artery	TBX1: T-box 1
PAP: pre-apical patch	TGFβ: transforming growth factors beta
Pax: paired homeobox	Tg: thyroglobulin
PCX: podocalyxin	TPO: thyroperoxidase
PDGF: platelet-derived growth factor	TSH: thyroid-stimulating hormone
PDGFR: platelet-derived growth factor receptor	TSHr: TSH receptor
PECAM: platelet and endothelial cell adhesion molecule (CD31; blood capillary marker)	TTF-1/Nkx2.1: thyroid transcription factor 1
PLGF: placental growth factor	Ub: ultimobranhial body
Pdx1: pancreas and duodenal homeobox gene-1	V: vitelline vein
PSG: parasympathetic submandibular ganglia	Ve-cadherin: vascular endothelial cadherin
Ptc: patched	VEGF: vascular endothelial growth factor
Ptf1a: pancreas transcription factor 1 subunit alpha	VEGFR: vascular endothelial growth factor receptor (Kdr)
PIP: phosphatidylinositol phosphate	WM: whole-mount
PIP ₂ or PI(4,5)P ₂ : phosphatidylinositol 4,5 bis phosphate	ZO-1: zonula occludens-1

IV. SUMMARY

The pancreas, submandibular and thyroid glands originally derive from the endoderm and undergo two opposite epithelial transitions to reach their mature tissue organization. During the first transition, endodermal cells at specified sites lose polarity and proliferate so as to generate an undifferentiated three-dimensional cell mass. In the second transition, the bud reorganizes to form polarized epithelial monolayers. A final choice of epithelial architecture is lumen fusion into branched tubes (exocrine pancreas and submandibular glands) vs closed follicles (endocrine thyroid).

The aim of my thesis was to better understand the cellular and molecular mechanisms controlling the second epithelial transition. I first described precisely this transition in the three organs, and found that epithelial cells composing the mass are always in close contact with the mesenchyme and the endothelium during reorganization into polarized monolayers. This raised the possibility of paracrine communications between these different cell types.

In the pancreas and submandibular glands, we found that the chemokine Stromal cell-Derived Factor-1 (SDF-1) is produced by the mesenchyme while its main receptor is expressed by the epithelium. SDF-1 binding to CXCR4 is necessary for branching morphogenesis of both organs. Of further interest, SDF-1 production by the submandibular mesenchyme could also target the CXCR7-expressing blood vessels, which, in turn, produce an as yet unidentified signal that promotes submandibular gland branching.

Combining my results on thyroid development with those on pancreas development reveals that VEGF-A expression by the epithelium may be either regionalized (restricted to the trunk cells in the pancreas) or global (all epithelial cells of the thyroid). Accordingly, VEGFR2⁺ endothelial cells are either recruited only near trunk cells in the pancreas, or uniformly in the thyroid. Using transgenic mouse models and an original thyroid explant culture system, we showed that endothelial cells signal back to the epithelium. In the pancreas, they restrict acinar differentiation to tip cells and support endocrine progenitors. In the thyroid, endothelial cells promote folliculogenesis and expression of calcitonin by C-cells. We further demonstrated in the thyroid that these effects depend on a secreted factor released by endothelial cells, rather than on contacts.

In conclusion, we have shown that reciprocal paracrine interactions govern the reorganization of a mass of epithelial cells into polarized monolayers. In particular: (i) mesenchymal SDF-1 controls pancreas and submandibular glands branching; (ii) epithelial VEGFA recruits blood vessels; and (iii) blood vessels control pancreas differentiation and thyroid gland morphogenesis and differentiation.

V. INTRODUCTION

The aim of developmental biology is to understand the processes leading to the formation of a mature organism, starting from a single cell, the zygote. Vertebrate development may be subdivided into five steps: (i) fertilization of the large ovocyte, (ii) serial cleavage into smaller cells (i.e. morula formation), (iii) gastrulation i.e. establishment of the three germ layers (i.e. ectoderm, mesoderm and endoderm), (iv) neurulation or development of the early nervous system, and (v) organogenesis i.e. formation of internal organs. All cellular processes involved - proliferation, differentiation, adhesion, migration, communication, etc... - are finely regulated in time and space by extracellular signals coordinating differential gene expression.

During this work, I focused my attention on the last step of vertebrate development, organogenesis, in particular on that of three types of glands derived from the endodermal germ layer: the pancreas, the submandibular glands and the thyroid gland. These three organs caught our attention because of early similarities and late divergences in their development (also called morphogenesis), ensuring their mature three-dimensional structure.

The adult pancreas, the diverse salivary glands and the thyroid are all composed of monolayers of epithelial cells surrounding a lumen. Organization of cells around a central lumen is a widely used structure in the animal kingdom and is present in many organs within each organism. Respiratory, circulatory and secretory organs are all built of cells arranged around a central lumen. Most of the lumen-containing organs are organized in tubes, with a great variability in size and structure between organs to allow highly specialized functions. Tubes are convenient to allow transport and modification of gases, nutrients, hormones or cells; and serve as barriers between different tissues. As opposed to the tubes found in the pancreas and submandibular glands, the simplest organization around a lumen is a sphere, such as found in the thyroid follicles. Spheres are suitable for the storage of cellular products and their rapid recapture.

How do tubes form and grow are interesting questions in biology and medicine because many diseases arise from a defect in tube architecture maintenance. Polycystic kidney disease is characterized by dilated tubules and perturbed renal function; hypertension is associated with narrowing of arteriolar lumen; and many carcinomas (such as in the pancreas and thyroid) are associated with lumen filling by malignant cells and loss of the tubular architecture. The understanding of the molecular and cellular mechanisms controlling tube formation is a prerequisite to improve prevention, diagnosis and treatment of epithelial dysgenesis (Datta *et al.*, 2011).

For organogenesis studies, the mouse is our favorite model organism because processes and molecular actors governing mammals' development are highly similar. Moreover, mice have a relatively short gestation period, deliver multiple embryos at the same time, and a variety of transgenesis methods allows the analysis of gene function *in vivo*, if necessary at defined times and sites.

This introduction will provide the tools to understand our work on pancreas, submandibular and thyroid gland development. Accordingly, I shall first elaborate on general principles of polarized organs and their formation, then review pancreas, submandibular and thyroid gland histology and development. The last part of the introduction will focus on paracrine interactions governing epithelial morphogenesis. I shall expand on some known stromal factors involved in pancreas, submandibular and thyroid gland development, then concentrate on blood vessels, and finally summarize current knowledge on the role of endothelial cells during organs development.

1 General principles of polarity and tubulogenesis

1.1 Key features of epithelial polarity

Depending on their function, epithelia can be composed of one (i.e. simple epithelia) or several layers of cells (i.e. stratified epithelia) surrounding a lumen. The mature pancreas, submandibular and thyroid glands are simple epithelia.

Epithelial cells that compose simple epithelia adhere to adjacent cells of the monolayer and are highly polarized (Fig.1). Contacts with neighboring cells occur *via* tight junctions, adherens junctions and desmosomes. Tight junctions (TJs) act as fences separating the apical membrane, specialized in absorption and secretion, from the baso-lateral membrane. This site is thus crucial for apical expansion during epithelial thickening. TJs further serve as diffusion barriers between the external and the internal milieu. This barrier can be regulated, allowing controlled paracellular transport. Finally, TJs also play a role in signaling during development (polarity complexes Par and crumbs, see below) and differentiation (ZO1-ZONAB; Balda and Matter, 2009). Of interest, TJ transmembrane proteins such as claudin-1 are subverted for epithelial crossing by viruses such as hepatitis C. It is believed that establishment of TJs follows development of circumferential adherent junctions.

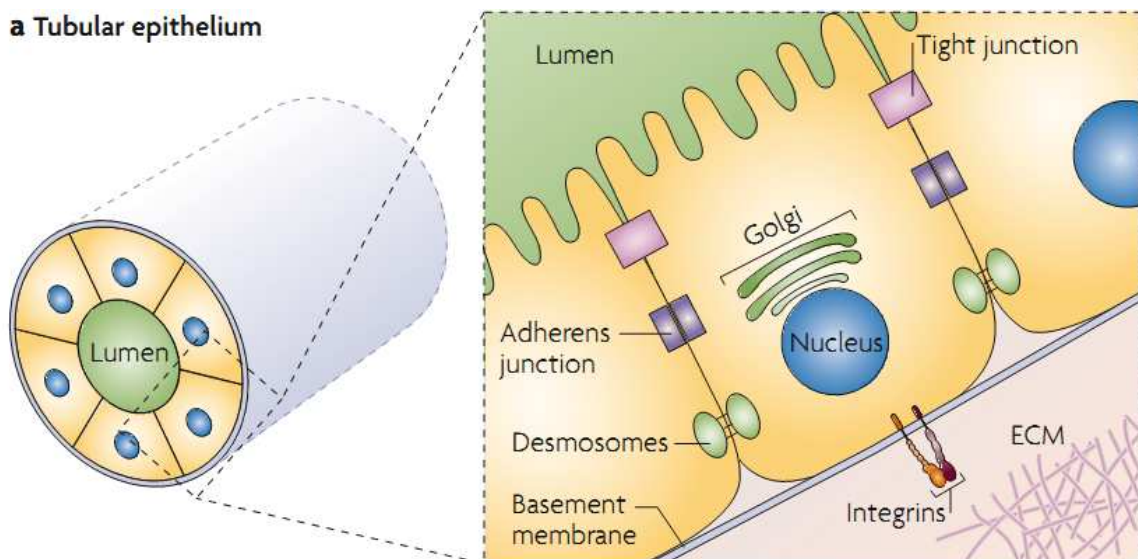


Figure 1 Epithelial cell polarization. Cells organized in monolayers surrounding a central lumen are highly polarized. The apical pole is delimited by tight junctions. Two other junctions localized at the lateral membrane, the adherens junctions and desmosomes, establish contacts with adjacent cells of the monolayer. The basal pole interacts with the basement membrane and ECM (reproduced from Bryant and Mostov, 2008).

The basal membrane of the cell lies on the basal lamina and interacts with the extracellular matrix (ECM) *via* integrins. Segregation of apical vs basolateral membranes allows specialization thus vectorial transport of ions and polarized secretion (Davies, 2002; Lubarsky and Krasnow, 2003; Bryant and Mostov, 2008; Andrew and Ewald, 2009; Datta *et al.*, 2011).

Thus, polarized epithelial cells display asymmetrical distribution of protein complexes that are involved in the establishment and maintenance of polarity. The three main polarity complexes, PAR (CDC42-PAR3-PAR6-aPKC), Crumbs (Crb-PALS-PATJ) and Scribble (Scrib-Dlg-Lgl), promote the development of the cell pole they associate with (Fig. 2). PAR and Crumbs complexes promote apical domain while Scribble complex supports the basolateral domain. Based on fine localization, the PAR complex can itself be subdivided into two groups: CDC42-PAR6-aPKC is found at the apical membrane, whereas PAR3-aPKC and the lipid phosphatase PTEN are confined to the tight junctions. Reciprocal exclusions between these protein complexes allow the maintenance of discrete membrane domains. For example, the Par-3-mediated phosphorylation of Lgl restricts Lgl localization to the basolateral pole while, in turn, the Scribble complex inhibits the Par-3 complex in the basolateral domain. How these complexes promote asymmetry is still unclear, but their establishment is a prerequisite for microtubule cytoskeleton and membrane traffic asymmetrical organization and phosphatidylinositol-phosphate (PtdInsPs) regulation (Bryant and Mostov, 2008).

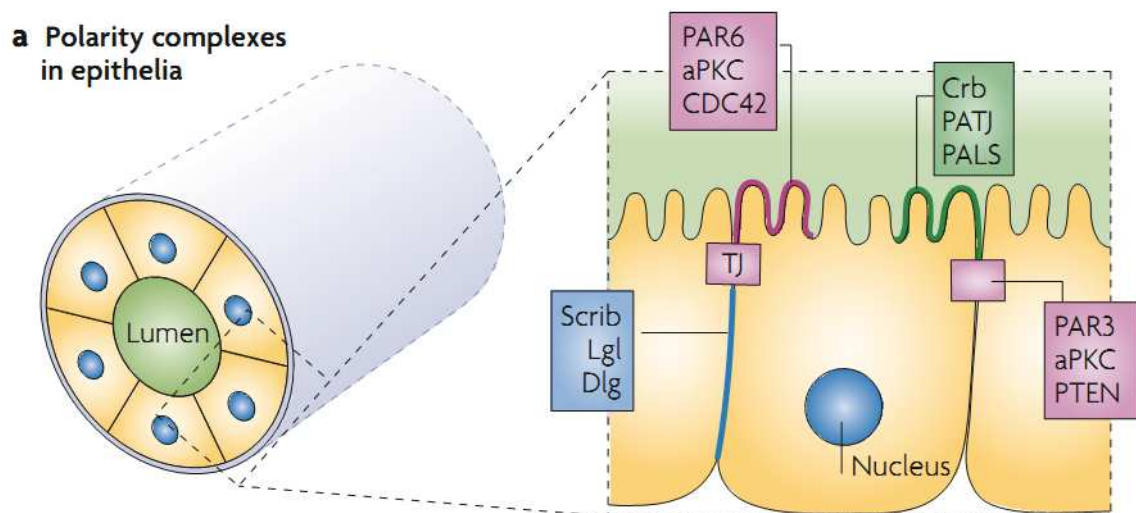


Figure 2 Polarity complexes in mature epithelia. Polarity complexes display asymmetrical distribution and promote the development of the cell pole they associate with. PAR6-aPKC-CDC42 and Crumbs complexes promote apical polarity and are localized at the apical pole, PAR3-aPKC-PTEN complex is localized at tight junctions, and Scribble complex is localized to and promotes the basolateral domain (from Bryant and Mostov, 2008).

1.2 Structural mechanisms of tubulogenesis

Before describing the various mechanisms that can give rise to a tube, it is important to note the great variability in tube size and structure. Tubes can be extremely narrow (e.g. capillaries) or quite large (e.g. the large intestine). Tubes can be strictly linear (e.g. the intestine) or highly branched (e.g. the pancreas, the submandibular glands, and particularly the lungs). Branched organs are tree-like structures, composed of a network of tubes, whose diameter decreases towards blind ends. Branches (also termed ducts) end in open spherical structures called acini (e.g. in the pancreas and submandibular glands) or alveoli (e.g. in the lungs).

Five mechanisms to form simple epithelial tubes have been described (Fig. 3-5, Lubarsky and Krasnow, 2003). In two of these mechanisms (wrapping and budding), the newly formed tube originates from polarized epithelia, and the cells maintain their polarity throughout organogenesis. In the three other cases, lumen formation occurs from non-polarized epithelial cells and requires *de novo* establishment of polarity, i.e. the definition of apical and basal domains.

1.2.1 Extension of pre-existing lumen/tube formation from polarized epithelia

The wrapping process starts when polarized cells from a simple epithelial sheet evaginate and curl. When the two edges arrive in close proximity, they fuse, thereby creating a tube disconnected from, and parallel to the plane of the parental epithelial sheet. This process is typical of neural tube formation in many mammals. Budding occurs when polarized cells from an existing tube divide and grow perpendicular to the plane of the epithelia. The nascent tube remains connected to the parental tube and their lumen is always continuous. Iterative budding gives rise to highly branched epithelial organs, like the mammalian lung (estimated at 14 divisions in man) and mammary glands (Fig. 3). It is important to remember that these two mechanisms occur without cell polarity loss.

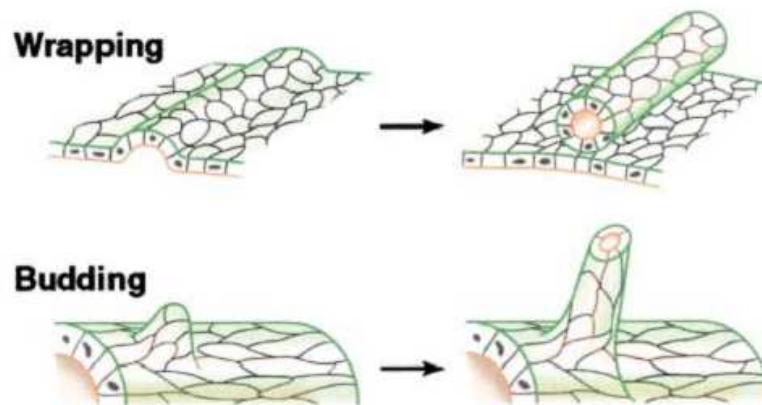


Figure 3 Histological mechanisms of tube formation: wrapping and budding (from Lubarsky and Krasnow, 2003)

1.2.2 *De novo* generation of epithelial lumen

The cell hollowing mechanism allows the formation of the smallest capillaries, in which a single cell surrounds the lumen. Non-polarized cells, organized in chains, first produce small cytoplasmic vesicles. Coalescence of the small vesicles forms an elongated structure that spans the length of the cell and eventually fuses with the plasma membrane at both ends, thereby establishing a connection between intracellular lumina of neighboring cells (Fig. 4).

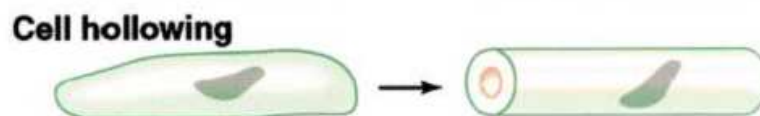


Figure 4 Cell hollowing (from Lubarsky and Krasnow, 2003)

In cord hollowing, a lumen is created between two cells. Similarly to cell hollowing, small cytoplasmic vesicles form in the cells that face each other in the cord. Concerted fusion of the intracellular vesicles of each cell at the membrane facing the opposite cell will create a lumen. Extension of this process to the length of the cord generates the tube. (Lubarsky and Krasnow, 2003; Bryant and Mostov, 2008; Andrew and Ewald, 2009).

Cavitation occurs between more than two non-polarized cells. This process implies the death of the cells at the center of the mass, and their clearance, to generate a hole (i.e. the lumen) surrounded by a monolayer of polarized cells. Although submandibular glands morphogenesis is reported to occur by cavitation we did not observe cell death during submandibular glands development, as will be described in our first paper (Hick *et al.*, 2009).

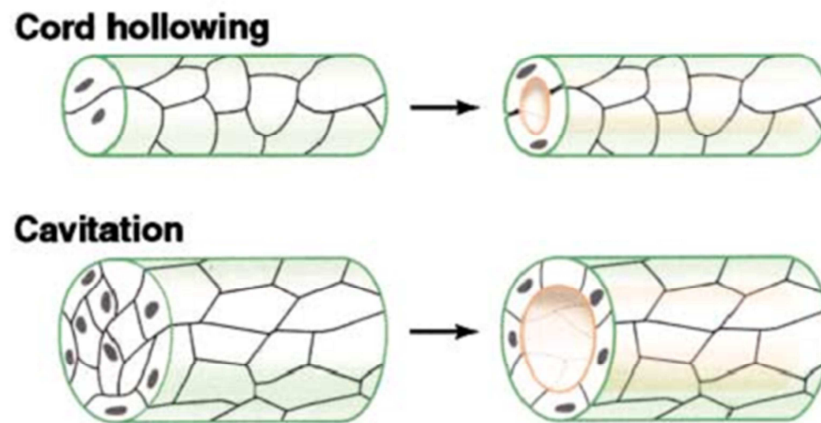


Figure 5 Cord hollowing and cavitation (from Lubarsky and Krasnow, 2003)

1.3 Molecular mechanisms of tubulogenesis

The molecular mechanisms of *de novo* lumen formation have been extensively studied using the Madin-Darby canine kidney (MDCK) cell line. Indeed, when MDCK cells are cultured in three-dimensions within extracellular matrix (ECM), they form masses of cells that eventually polarize and form simple epithelial cysts surrounding a lumen (Bryant and Mostov, 2008).

In this experimental system, *de novo* lumen formation involves three steps: orientation, polarization and expansion.

Orientation: Cells in the cord or at the periphery of the mass must orient themselves to form a tube. Contacts with the ECM (e.g. laminins, collagen; green line in Fig. 5) and the neighboring cells provide two axes to orient cells and lumen positioning. Signals from the ECM are sensed by integrin-based complexes, which can transduce signals and regulate cytoskeletal tension (see section 3.2), while signals from the adjacent cells are sensed by the junctional complexes (Bryant and Mostov, 2008; Datta *et al.*, 2011).

Polarization: As we have seen, apico-basal polarization requires the polarity complexes. These allow the coordinated delivery of apical membrane components to a discrete common site, the apical membrane initiation site (AMIS) where lumen initiation takes place. At the molecular level, this implies the accumulation of polarity proteins [Crumb3a (Crb3), podocalyxin (PCX) and mucin 1 (Muc1)] in Rab8/11-positive intracellular vesicles (Bryant *et al.*, 2010). Oriented exocytosis of those vesicles to the AMIS is in part controlled by the CDC42-Par3-aPKC complex (Bryant *et al.*, 2010) and by the PIP₂-binding protein annexin2 (anx2) (Fig. 6) (Belmonte *et al.*, 2007). The AMIS then matures in a pre-apical patch (PAP), an early apical domain that forms in adjacent cells before lumen opening. When the PAP is formed, Par3-aPKC and the exocyst, a complex that regulates vesicle docking and delivery to the cell surface, are relocalized to the tight junctions (Bryant *et al.*, 2010).

Lipid distribution in membranes is also important for the maintenance of epithelial polarity. Indeed, phosphoinositides are asymmetrically distributed in the membrane domains: PI(4,5)P₂ (PIP₂) is localized to, and specifies the apical membrane domain, while PI(3,4,5)P₃ (PIP₃) is enriched in the basolateral domain. Exclusion of PIP₃ from the apical membrane is achieved by the apical phosphatase PTEN that converts back PIP₃ into PIP₂ (Belmonte *et al.*, 2007).

Lumen expansion: Once these apical vesicles have fused with the cell surface, extracellular space must be expanded between neighboring cells. A dilatation mechanism, analogous to that exploiting hydrophilicity of hyaluronan, relies on strong hydration of negatively-charged proteins, like mucin and podocalyxin, upon glycosylation or sialylation. This implies a supply of water and salts through the activation of channels proteins and pumps, like aquaporins. For example, the uncontrolled activation of CFTR channel in the zebrafish gut leads to the accumulation of fluid and expansion of the gut tube lumen (Bagnat *et al.*, 2010). A second way for lumen expansion is cell removal via apoptosis. Indeed, it has also been shown in the *in vitro* MDCK system that cells within the mass which lose contact with the ECM undergo apoptosis, the so-called anoikis. Finally, recruitment of ERM (ezrin-radixin-moesin) proteins at the lumen initiation site triggers the F-actin/ERM/RhoA/myosin-II network that generates microfilament-based force for lumen rounding (Bryant and Mostov, 2008; Datta *et al.*, 2011).

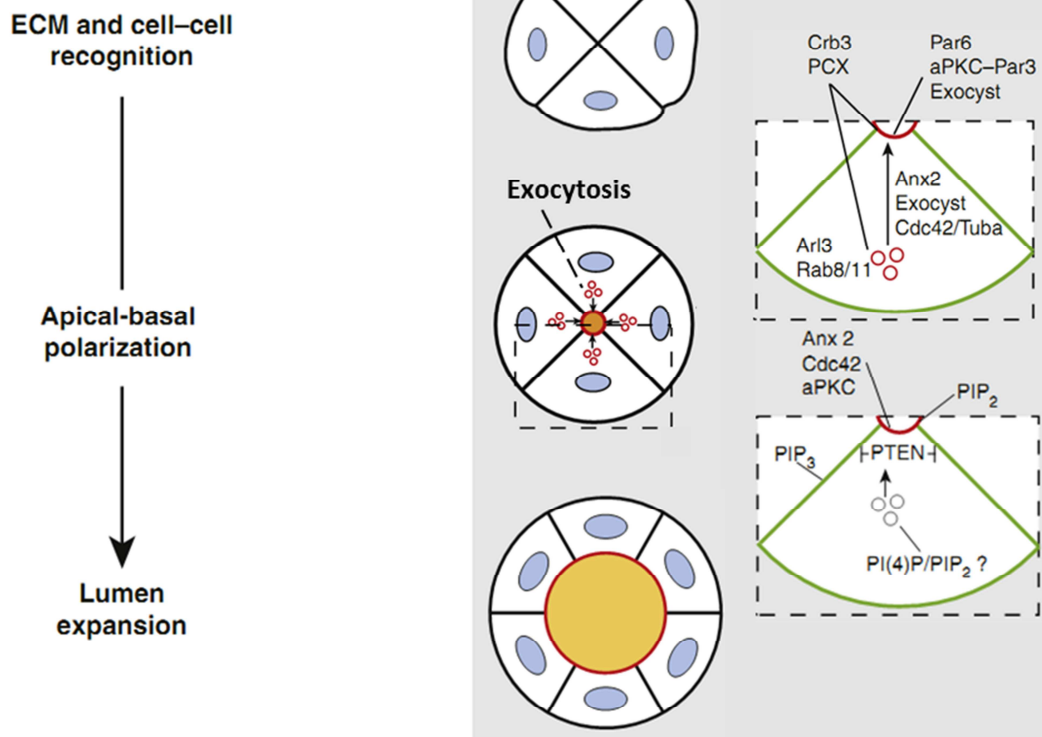


Figure 6 *De novo* lumen formation. Lumen formation in various contexts involves three fundamental design principles: ECM and cell-cell recognition, apico-basal polarization and lumen expansion. In the illustrated four-cell cluster, cells in contact with the ECM produce vesicles that contain luminal components (such as Crumbs 3a (Crb3), podocalyxin (PCX) and muc1). Vesicles are exocytosed via Rab8/1-positive vesicles to generate a central luminal region and allow subsequent apico-basal polarization. Exocytosis to the AMIS (apical membrane initiation site) requires the action of the exocyst, Par3-aPKC and Cdc42-Par6 complexes. As polarization takes place, phosphoinositides distribute asymmetrically, with PIP₂ enriched at the apical pole and PIP₃ at the basolateral pole. Then, lumen expansion takes place (adapted from Datta *et al.*, 2011).

Reorganization of non-polarized epithelial cell masses into polarized monolayers occurs during pancreas, submandibular and thyroid development. Understanding the mechanisms involved in these reorganization events is not only important for developmental biology, but also for human health since the opposite transition, i.e. reversion of polarized monolayers to a mass of non polarized cells, is a frequent histological hallmark of some carcinomas (Larsen *et al.*, 2006).

2 Exocrine pancreas, submandibular and thyroid glands: exocrine vs endocrine monolayers

2.1 The exocrine pancreas

2.1.1 Histology

The adult pancreas is a heterogeneous gland, composed of exocrine and endocrine tissues, respectively specialized in digestion and glucose homeostasis. The exocrine compartment is constituted of two cell types: acinar and ductal cells. Acinar cells, organized as open spherical structures called the acini, synthesize digestive (pro-)enzymes (amylase, trypsinogene ...) and secrete zymogen granules into the acinar lumen upon appropriate stimuli. Ductal cells not only drain secretions from the acini into large ducts such as Wirsung's duct, then to the duodenum but also secrete bicarbonate, which neutralizes the acidity produced by the stomach so as to reach the optimal pH for the pancreatic hydrolases. The endocrine compartment, representing around 2% of the pancreatic mass, is organized in the islets of Langerhans. Five endocrine cells types (α , β , δ , ϵ and PP) synthesize hormones (glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide, respectively) and deliver them to the blood circulation (Kim *et al.*, 2002 ; Murtaugh *et al.*, 2003; Pan & Wright, 2011).

2.1.2 Morphogenesis and transcription factors

In the mouse, pancreas specification occurs at embryonic day (e) 8.5 in two regions of the endoderm, ventral and dorsal (Wilson *et al.*, 2003). Proliferation of the cells committed to a pancreatic fate leads to the formation of two visible pancreatic buds around e9.5. Epithelial cells of the buds grow and invade the surrounding mesenchyme before initiating branching morphogenesis around e12.5. At this stage, the dorsal and ventral buds fuse to generate a single organ. Exocrine and endocrine differentiation starts from the e13.5 multipotent progenitors and continue until birth, to generate mature acini, ducts and islets of Langerhans (Kim *et al.*, 2002; Pan & Wright, 2011).

In 2007, the group of Melton identified and characterized a proliferating cell population in the pancreas. Based on their histological localization, they called this population “tip” cells. They also demonstrated that tip cells behave as multipotent progenitors, able to give rise to acinar, endocrine and ductal cells (Fig. 7, purple). Tip cells co-express a set of transcription factors, among which the pancreas and duodenal homeobox gene-1 (Pdx1) and p48/Ptf1a. The progenitors also express carboxypeptidase A (CPA1+ in Fig. 7), but not amylase. In their model, asymmetric division of multipotent/tip cells ensures lengthening of the branches, while pushing the tip cells away from the center of the pancreas. Daughter cells forming the branches will later differentiate into endocrine (green) or ductal (blue) cells, while tip cells retain multipotency, and continue to express Pdx1, p48/Ptf1a and CPA (purple). Around e14, tip cells lose multipotency and become restricted to an acinar fate (orange), marked by the expression of amylase and the loss of Pdx1 (Zhou *et al.*, 2007). Ductal cells express the transcription factors HNF-1 β and HNF-6, while endocrine cells transiently express the key transcription factor Ngn3, whose expression culminates at e15 (Puri and Hebrock, 2010).

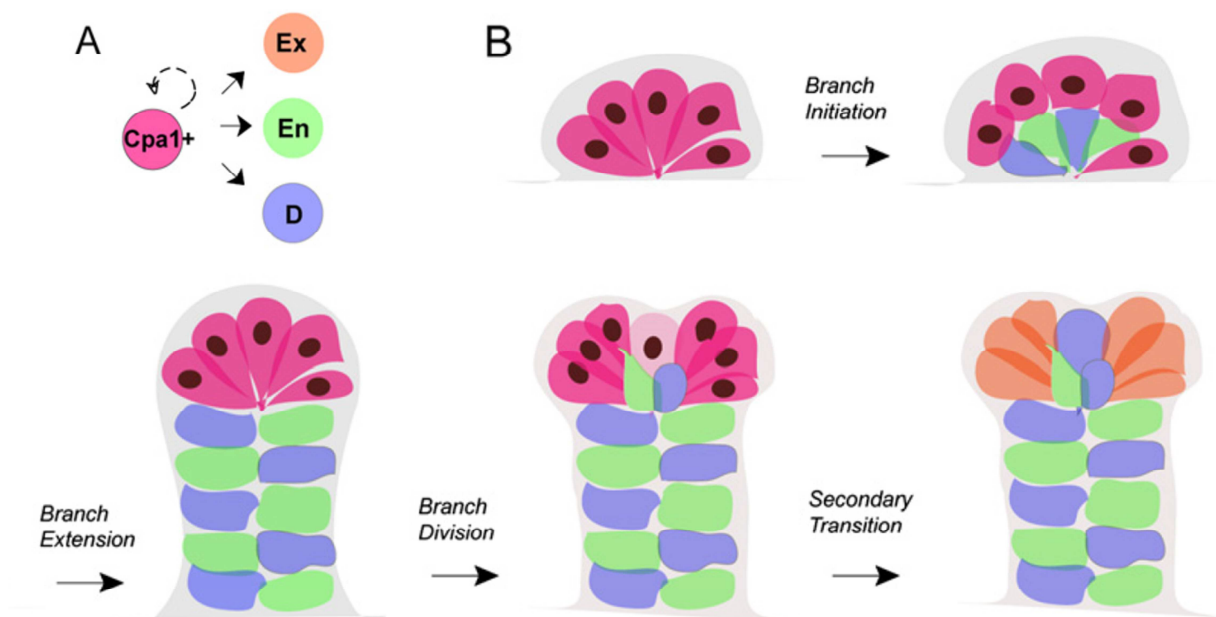


Figure 7 Multipotent progenitors guide pancreatic organogenesis. (A) Progenitor cells expressing CPA (carboxypeptidase A) are multipotent (purple). They give rise to acinar (orange), endocrine (green) and ductal (blue) cells and may self-renew. (B) During development, the multipotent pancreatic tip cells divide asymmetrically from e12 onwards. Each division generates a new tip cell and another cell that participates to the extension of the branch. After e14, tip cells become acinar cells and the central cells of the branches differentiate into endocrine (green) or ductal (blue) cells (from Zhou *et al.*, 2007).

The ramification of the exocrine pancreas (ductal and acinar cells) is classically described as resulting from iterative branching of a polarized monolayer, like in the lungs (budding in Fig. 3). In this view, epithelial cells would be organized in polarized monolayers, from the emergence of the first bud until the end of development (Kim *et al.*, 2002 ; Cano *et al.*, 2007). Data obtained during my PhD demonstrate that this model is incorrect. We, and others, have recently shown that early pancreas morphogenesis involves a step of depolarization of the endodermal cells after their pancreatic specification. Pancreatic budding thus generates a mass of unpolarized epithelial cells. Reorganization of the cells composing the mass and their repolarization give rise to the primitive network of ducts (Hick *et al.*, 2009; Kesavan *et al.*, 2009). From these primitive polarized monolayers, branching morphogenesis continues, as described for the lung.

2.1 Submandibular glands

2.1.1 Histology

Salivary glands are exocrine glands composed of a network of ducts ending in acini. They lubricate the food, aide tasting, initiate digestion and serve as a pH buffer. In the mouse, there are three pairs of major salivary glands that differ in their architecture and function: the submandibular and sublingual glands, that have grapes-like structures, are located beneath the tongue, and the parotid glands, with a tree-like architecture, is located at the back of the tongue, near the jaws. Submandibular glands are composed of a mix of serous cells [i.e. proteins (like amylase)-producing cells] and mucous cells (producing mucous), while the sublingual and parotid glands are respectively composed of mucous and serous cells (Tucker, 2007).

2.1.2 Morphogenesis

Submandibular development begins by a thickening of the oral endoderm on each side of the tongue, which leads to the formation of two “prebuds” at e11.5. Similarly to the pancreas, the prebud epithelial masses grow caudally and invade the surrounding mesenchyme to form the first epithelial bud. At e12.5, the bud is connected to the oral surface by a stalk that will become the main duct of the gland. The first signs of branching morphogenesis appear around e13.5, with the formation of clefts at the periphery of the mass. The high proliferation rate coupled to cleft formation form the first 4-5 secondary buds. The increase in the number of buds is called pseudoglandular stage. At the canalicular stage (e15.5-e16.5), many small lumina form at the future apical pole (i.e. on the opposite pole of the basal membrane; black line in Fig. 8) of the cells within the non-polarized epithelial mass. Coalescence of the small lumina into an almost continuous

extracellular lumen generates the network of ducts linking the acini to the oral cavity. The accepted model of submandibular gland tubulogenesis is cavitation, with cell death (Jaskoll and Melnick, 1999; Melnick *et al.*, 2001). The total continuity of the network is achieved after birth and differentiation pursuits until puberty (Tucker, 2007).

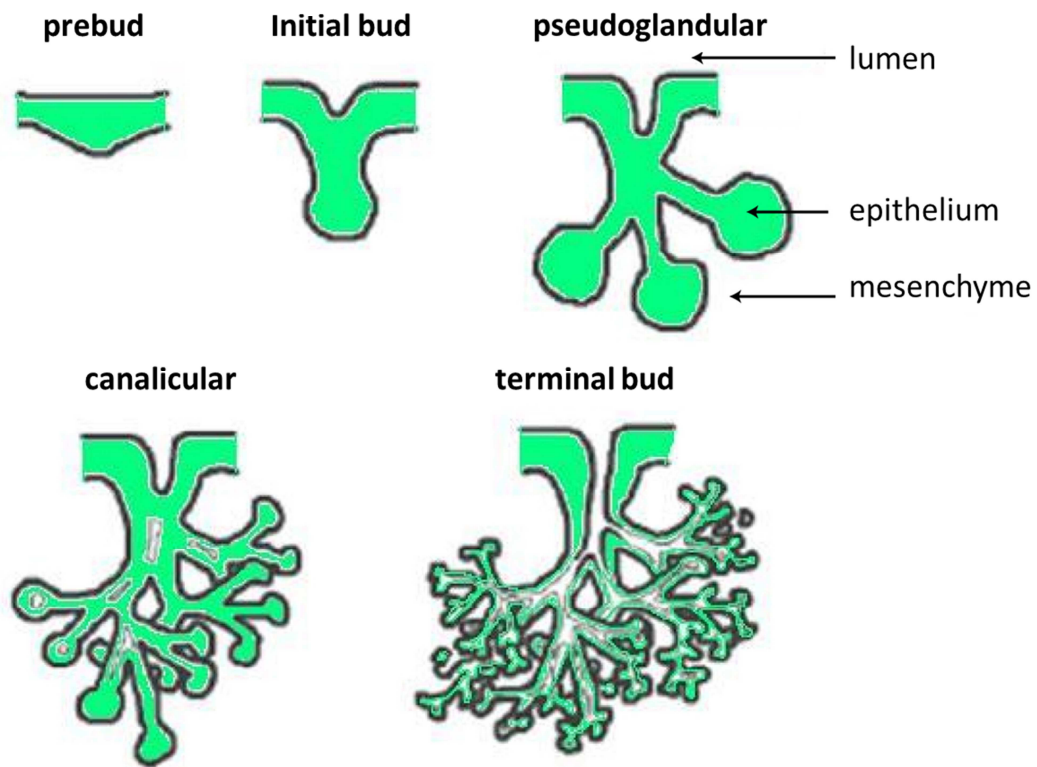


Figure 8 Stages of submandibular gland branching morphogenesis (adapted from Tucker, 2007).

Very few transcription factors involved in submandibular glands development have been identified. Given that their precise roles have not been fully investigated, we will not detail these preliminary informations (which can be found in Patel *et al.*, 2006 and Knosp *et al.*, 2012).

2.2 The thyroid gland

2.2.1 Histology

The thyroid gland in mammals is a composite endocrine gland located in the neck that produces thyroid hormones and calcitonin by two distinct cell types: the follicular cells and the C cells. C cells, a minor population dispersed within the gland, produce the calcium-lowering hormone calcitonin. Follicular cells, also called thyrocytes, are organized in closed polarized monolayers surrounding a lumen (Fig. 9). Thyrocytes are able to actively capture iodine at their basal pole *via* the sodium iodide symporter (NIS), which transports two sodium ions together with one iodide ion inside the cell. The sodium gradient required for the symporter activity is generated by Na^+/K^+ -ATPase. Iodide is then transported to the apical pole of the cell and excreted most probably by the chloride/iodide transporter pendrin. Thyroglobulin (Tg) iodination takes place by incorporation of atomic iodine on tyrosyl residues, after a step of iodide oxidation catalyzed by thyroperoxidase (TPO) and mediated by hydrogen peroxide on the outer apical membrane. Iodinated thyroglobulin is stored in the colloid. Upon stimulation by the pituitary-derived TSH (thyroid-stimulating hormone) that binds to the TSH receptor (TSH-R) at their basal pole, thus activates cyclic AMP response, thyrocytes reabsorb iodinated-thyroglobulin from the colloid. Upon arrival in lysosomes, the huge iodinated-thyroglobulin (330 kDa/monomer) is digested and on the average three molecules of thyroxine, i.e. tetraiodothyronin (T4) and triiodothyronin (T3; about 1/100 of the Tg mass) are released from lysosomes (Dunn and Dunn, 2001) by an unknown mechanism and are selectively transported across the basal pole of the thyrocytes into the blood circulation *via* monocarboxylate transporter 8 (Mct8) (Di Cosmo *et al.*, 2010). All thyrocytes of a follicle operate as a unit since they are all well equipped with two types of gap junctions. However, their behavior varies between adjacent follicles (Munari-Silem *et al.*, 1991). The overall thyroid hormones production is tightly regulated by a negative feedback regulation by serum T4 and T3 at both hypothalamo-and hypophyseal levels, thus decreasing TSH release, and depends of adequate iodine concentration in the thyrocytes.

Moreover, since thyrocytes function requires capture of iodide from, and delivery of thyroid hormones into, the blood vessels, a close association between thyroid follicles and capillaries is crucial. This organization is sometimes described as “angio-follicular units” (Gerard *et al.*, 2002).

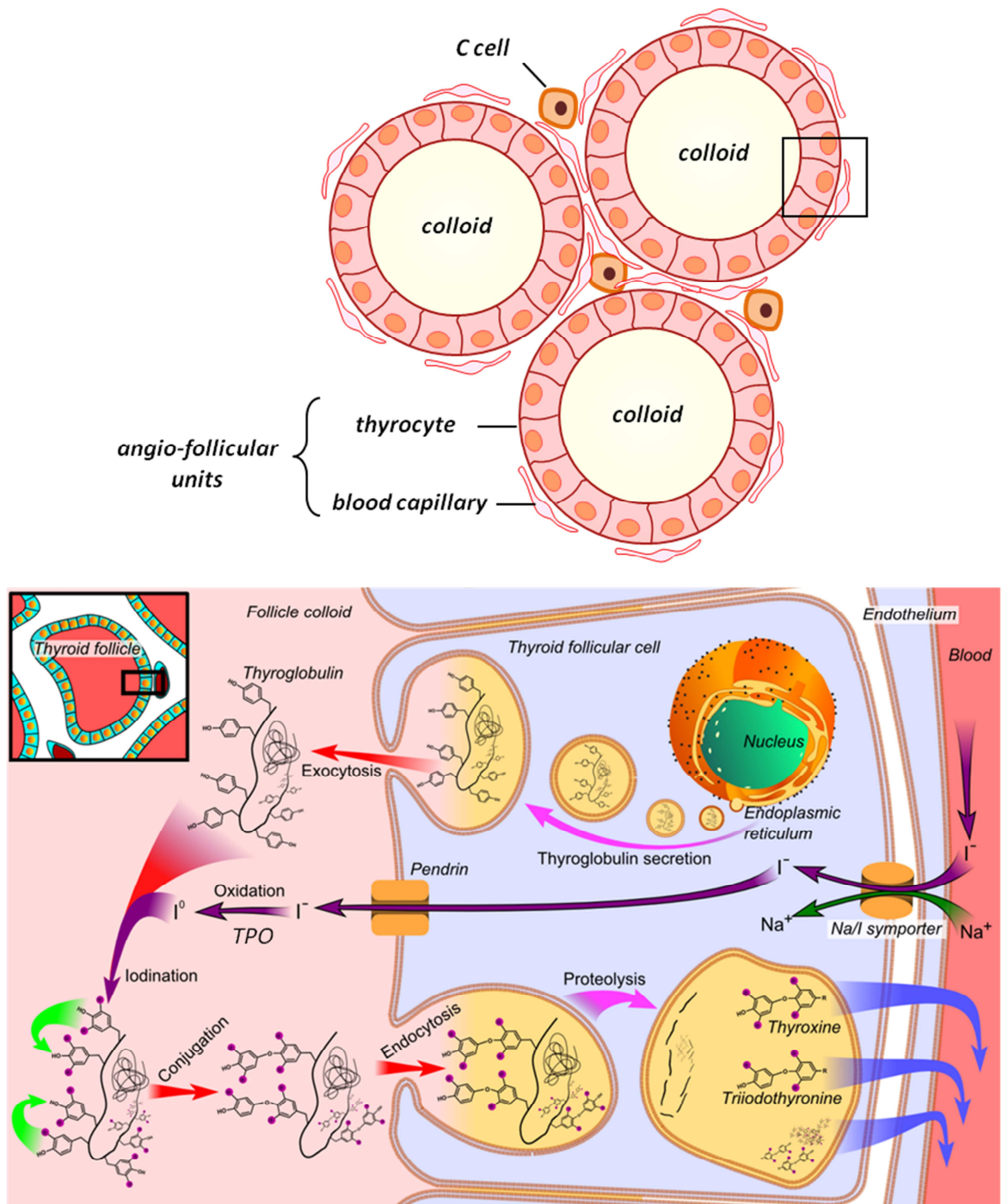


Figure 9 Schematic representations of thyroid follicular organization and of thyroid hormone synthesis by follicular cells. Follicles are composed of monolayers of thyrocytes surrounded by a dense network of endothelial cells. This close association is sometimes called angio-follicular units. C cells (in orange) are scattered between follicles. Thyroid hormone production involves the capture of iodide from blood vessels at the basal pole of the thyrocyte via Na^+/I^- symporter (NIS) and its excretion to the apical pole possibly by pendrin. Iodide is oxidized by thyroperoxidase (TPO) and incorporated on selected thyrosyl residues of thyroglobulin. Upon stimulation by TSH, iodinated thyroglobulin is endocytosed and cleaved by lysosomal proteases to release thyroxine (T4) and triiodothyronine (T3) into the blood.

2.2.2 Morphogenesis and transcription factors

The two endocrine cell types originate from distinct embryonic structures. Follicular cells differentiate from the main thyroid anlage that originates from the most anterior portion of the ventral foregut endoderm (De Felice and Di Lauro, 2004; Fagman and Nilsson, 2011). C cells originate from the ultimobranchial bodies, which derive from the fourth pharyngeal pouch (Kameda *et al.*, 2007).

2.2.2.1 Specification and budding

The main thyroid anlage appears as a thickening of the anterior endoderm by e8-8.5 (Fig. 10). Endodermal cells specified for a thyroid fate are characterized by the co-expression of four transcription factors *Nkx2.1 (Ttf1)*, *Pax8*, *Hhex* and *Foxe1* and by the transition from the monolayered endodermal germ layer to a pseudostratified state. Factors controlling thyroid specification are presently unknown but one can postulate that inductive signals coming from the adjacent mesenchyme and aortic sac might be critical (De Felice and Di Lauro, 2004; De Felice and Di Lauro, 2007; Fagman and Nilsson, 2011).

Similarly, and concomitantly, to pancreas and submandibular glands budding, the thickening evaginates from the pharyngeal floor and deepens caudally in the surrounding mesenchyme to form the thyroid bud by e9.5. At this stage, the thyroid bud is still connected to the endoderm by a thin structure, the thyroglossal tract that is meant to regress and disappear by e11.5 (De Felice and Di Lauro, 2007); while the most caudal part of the thyroid bud remains closely associated with the aortic sac (Fig. 10) (Fagman *et al.*, 2006; Alt *et al.*, 2006). By e10.75, cells within the thyroid primordium begin to proliferate. Also, few mesenchymal cells are now interposed between the thyroid bud and the aortic sac. At e11.5, the thyroid moves ventrally away from the pharyngeal floor and lies on the cranial surface of the aortic sac. The first endothelial cells project into the thyroid epithelium at that stage (Fagman *et al.*, 2006).

2.2.2.2 Migration of follicular precursors and fusion with ultimobranchial bodies

In contrast to the pancreas and submandibular glands, the thyroid bud loses its connection with the endoderm around e11.5. It then starts expanding bilaterally while ultimobranchial bodies appear as spherical protrusions of the endoderm, located dorso-laterally and caudally to the thyroid anlage. Cells of the ultimobranchial bodies express the transcription factor *Nkx2.1 (Ttf1)* but are devoid of *Pax8*. One day later, the thyroid midline anlage has a bar-like shape. It keeps on migrating laterally along the track of pharyngeal arch arteries to fuse with the ultimobranchial bodies. At e13.5, the thyroid gland has reached its definitive characteristic shape: two lobes, on each side of the

trachea, connected by a narrow isthmus. Despite inclusion of the ultimobranchial bodies within the midline thyroid anlage around e13, mixing of the two epithelial cell types is only observed a few days later (Fagman *et al.*, 2006).

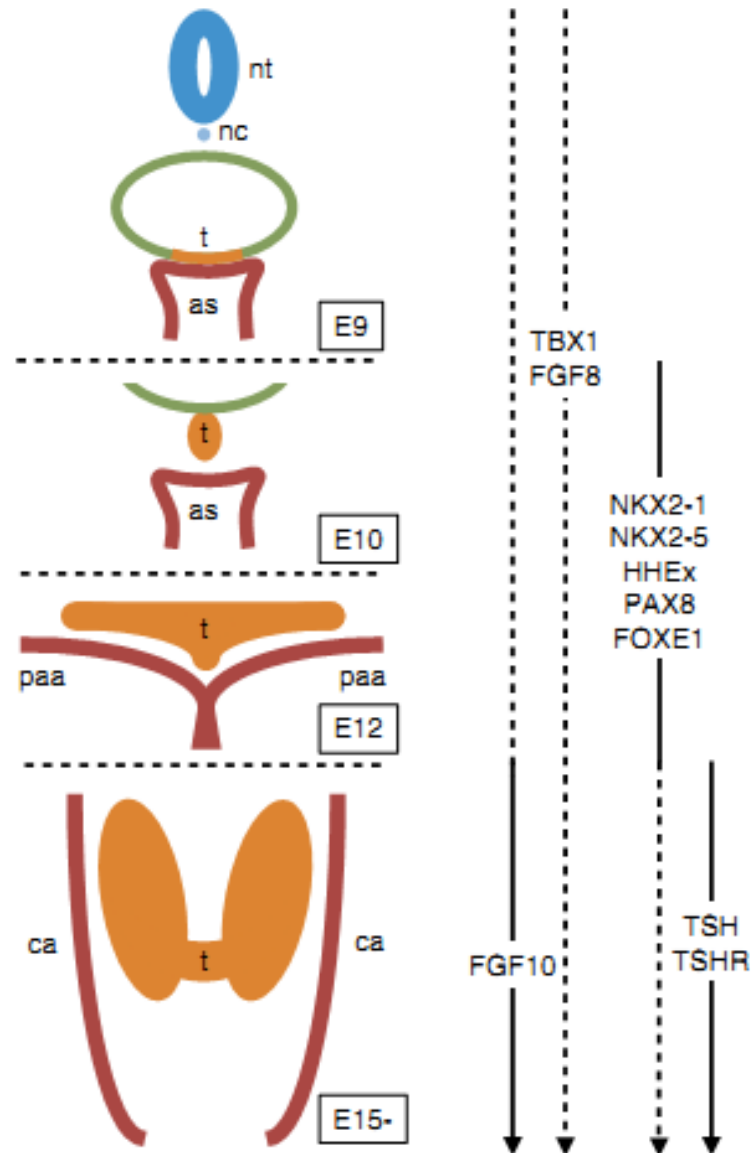


Figure 10 Schematic representation of mouse thyroid development. At e9, cells co-expressing Nkx2.1, Pax8, Foxe1 and Hhex form a placode (orange) in the ventral region of the foregut (green) adjacent to the aortic sac (“as”, red). Thyroid precursor cells invade the surrounding mesenchyme and form the first bud. Around e12, the thyroid midline anlage migrates laterally along the course of the pharyngeal arch arteries (paa). The midline anlage thyroid fuses with the ultimobranchial bodies around e13 (not depicted), and the thyroid gland reaches its final shape by e15: two lobes connected by an isthmus (ca, carotid arteries). On the right, arrows represent the expression pattern of factors that control thyroid development, with dashed lines indicating time periods when the effect on thyroid morphogenesis is not well defined (from Fagman and Nilsson, 2011).

2.2.2.3 Lobe expansion and folliculogenesis

Lobe expansion, around e15.5, is due to extensive proliferation of peripheral epithelial cells. At this stage, epithelial cells have become organized in cords, emerging from the center of the lobe; which is mainly composed of cells originating from the ultimobranchial bodies. Concomitantly appear the first signs of polarization (organization of cells in pre-follicular structures and appearance of small lumina) and early differentiation (thyrocyte precursors start expressing thyroglobulin, while cells at the center of the lobes that will give rise to C-cells, start expressing calcitonin). At e17.5, small individualized follicles are clearly visible and genes involved in thyroid hormones synthesis (Tg, TPO, TSHr, NIS, etc.) are strongly expressed. C cells are now scattered throughout the lobes (De Felice *et al.*, 2004; Fagman *et al.*, 2006). Expansion of the lobes continues, with a generalized proliferation of epithelial cells (Fagman *et al.*, 2006). Arch arteries give rise to the carotid arteries that are positioned in the immediate vicinity of the two mature thyroid lobes (Fig. 9) (Fagman *et al.*, 2006; Alt *et al.*, 2006).

2.2.2.4 Evidence for a transcriptional network

As mentioned above, four transcription factors -*Nkx2.1 (Ttf1)*, *Pax8*, *Hhex* and *Foxe1*- are expressed from the time of specification of the thyroid anlage to the adult follicular cells. They control the different stages of embryonic thyroid development (De Felice *et al.*, 2004), but as their genetic loss perturbs the earliest stages of thyroid development, their roles in the subsequent steps have been poorly studied.

Nkx2.1 (Ttf1) is necessary for the maintenance of the thyroid primordium, but not for its specification. Indeed, in *Nkx2.1* knockout (KO) embryos, the primordium is specified but disappears by e11. Since *Nkx2.1* is also expressed in other tissues, including lung, trachea and pituitary, knockout mice show severe alterations such as pulmonary dysplasia, absence of septation between trachea and oesophagus, thyroid agenesis ("athyreosis"), absence of pituitary, etc... leading to death at birth. Later on, *Nkx2.1* controls the expression of thyroid follicular-specific genes, such as TPO and thyroglobulin. *Nkx2.1* inactivation in adults shows that this factor is essential for the maintenance of thyroid architecture and function (Kusakabe *et al.*, 2005). Moreover, folliculogenesis is impaired in thyroids expressing a non-phosphorylatable *Nkx2.1* protein (Silberschmidt *et al.*, 2011).

Pax8 is expressed in the thyroid bud from e8.5 but is absent from the ultimobranchial bodies. Other tissues expressing *Pax8* include the neural tube and kidneys. Similarly to the *Nkx2.1* KO, the thyroid primordium of *Pax8* KO embryos is specified but disappears by e12. Due to its particular expression pattern, the remaining gland is mainly composed of C cells. *Pax8* KO mice show

athyreosis leading to death by 2-3 weeks of age, unless pups are supplemented with thyroxin. Importantly, these mice do not show defects in other organs, such as kidneys, and heterozygous mice are normal. *Pax8* is also involved in the transcriptional regulation network that controls thyroid development and differentiation.

Foxe1 (Ttf2) enjoys a wider expression pattern than *Nkx2.1* and *Pax8*: it is found in the pharyngeal arches and pharyngeal wall, and all their derivatives, including thyroid, tongue, palate and esophagus. The thyroid primordium is specified in *Foxe1* KO embryos, but by e9.5 the thyroid precursor cells are still on the floor of the pharynx, instead of budding into the surrounding mesenchyme. As development proceeds, these precursor cells either remain attached to the pharyngeal floor and differentiate, or completely disappear. Therefore, *Foxe1* seems crucial for thyroid precursor cells migration. *Foxe1* KO mice die within 48h after birth, probably due to severe cleft palate.

Hhex is expressed in the primordium of many endoderm-derived organs, such as that of the thyroid, pancreas, liver and lungs. At e9.5, *Hhex* null embryos are either devoid of thyroid primordium or the primordium remains located on the floor of the pharynx. In this case, *Nkx2.1*, *Pax8*, and *Foxe1* are down-regulated, which further indicates that the four transcription factors are integrated into a regulatory network (Parlato *et al.*, 2004). Later, *Hhex* could repress the expression of differentiation genes, such as thyroglobulin (De Felice *et al.*, 2004; Fagman and Nilsson, 2009).

The role of mesenchymal signals, in particular FGF8, FGF10 and TBX1 will be discussed in the next section.

3 Paracrine interactions control epithelial morphogenesis

3.1 The key role of the “stroma”/“mesenchyme” has been established on tissue recombination experiments

Each epithelial organ depends on a typical architecture, acquired during organogenesis, that defines its function. Acquisition of the appropriate structure and proper functioning of this epithelium depends on interactions between epithelial cells and their local “stromal” environment. The latter comprises other cell types, such as fibroblasts, endothelial cells and nerves, secreted paracrine factors and ECM which can reversibly trap or release such factors, the so-called matricrine regulation.

The key morphogenetic importance of the tissue environment for final epithelial organization was first hypothesized almost two centuries ago by Parder but only confirmed more than a century later. Its clear demonstration was provided by tissue recombination experiments (Fig. 11). For example, the mammary gland mesenchyme induces the typical branching of mammary epithelium and lactation when recombined to another epithelium, such as the epidermis. Conversely, if mammary epithelium is recombined with salivary glands mesenchyme, it undergoes salivary gland morphogenesis; yet keeping its ability for lactation. These recombination experiments, which have been performed on many other organs, including lung, kidney and limb, suggest that epithelial cells are flexible and respond to their instructing environment (for a review, see Nelson and Bissel, 2006). Of note, the term “mesenchyme” used in these pioneering experiments refers to the whole supporting tissue of the organ, thus has exactly the same meaning as “stromal environment” defined above. In the following sections, we will focus on the ECM, the secreted factors and the major cell types that constitute the pancreatic, submandibular and thyroid environment.

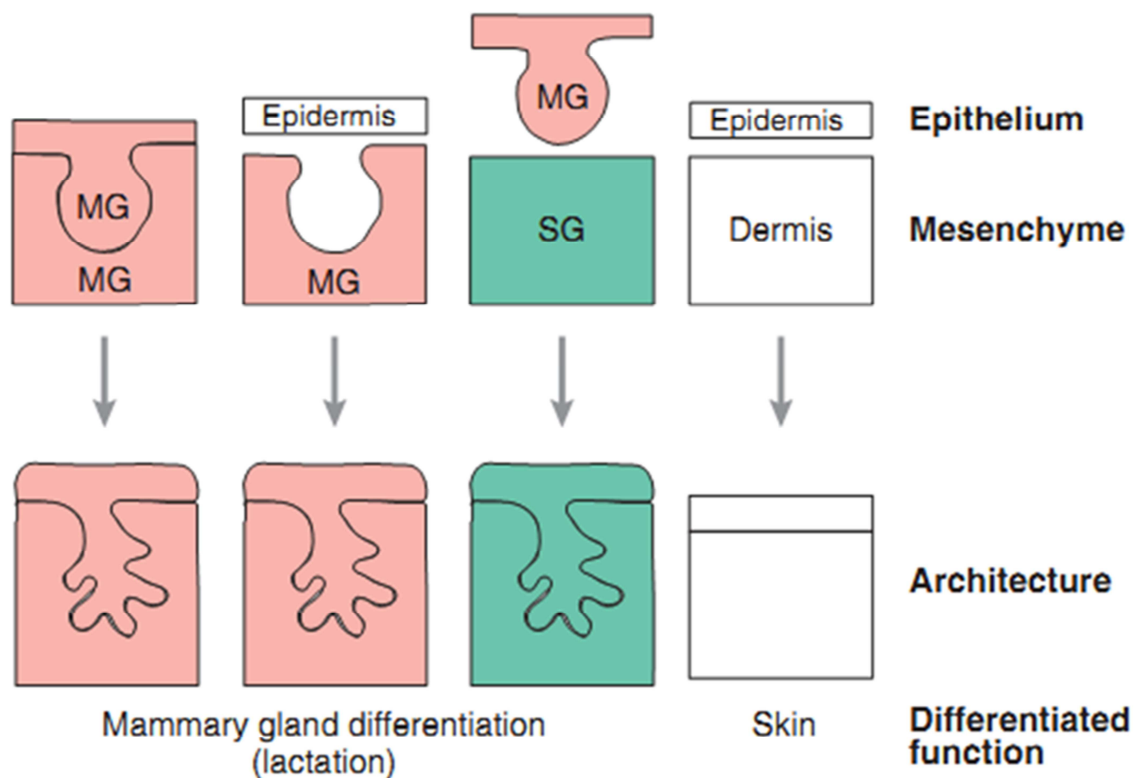


Figure 11 Demonstration of the instructing role of the mesenchyme onto the epithelial structure by tissue recombination experiments. Mammary gland (MG) mesenchyme can induce non-mammary epithelial cells (e.g. epidermis) to produce a lactation-competent gland. Conversely, mammary gland epithelium recombined with salivary gland mesenchyme adopts the salivary gland architecture, yet retaining its ability to produce milk (from Nelson and Bissel, 2006).

3.2 The extracellular matrix (ECM)

As its name implies, the extracellular matrix (ECM) is the structured extracellular part of tissue. ECM is composed of fibrous proteins (collagens, elastin, fibronectin, laminin...) and associated proteoglycans (heparan, chondroitin and keratan sulfates). These components are produced by resident cells and secreted by exocytosis. The ECM not only provides epithelial support, in particular at “basement membranes” or basement laminae (marker: laminin), but is also important for tissue compartmentation and for matricrine regulation. During embryonic development, and in particular during organogenesis, the ECM acts on several cellular processes: migration, proliferation and survival, polarization (as mentioned in section 1.3) and differentiation. In particular, ECM generates space by hydration, regulates growth factor bioavailability, modulates cell adhesion and force *via* linkage to the actin cytoskeleton as well as signaling *via* integrin-based focal adhesions, all properties that can be modulated by matrix metalloproteases (MMPs) (Kim *et al.*, 2011).

The role of ECM proteins in submandibular glands development has been extensively studied. For example, collagen accumulation at sites of cleft formation serves both as a physical constraint and as a factor promoting morphogenesis. Although its *in vivo* role needs further investigation, *in vitro* degradation of collagen types I and III inhibits branching. Glycosaminoglycans (GAGs) also accumulate at the cleft sites. In addition, cleavage of the GAG heparan sulfate (HS) by heparanase allows the release of HS-bound growth factors accumulated in the ECM, such as FGFs and VEGFs (Patel *et al.*, 2006). Accordingly, inhibition of heparanase activity reduces FGF10-mediated submandibular glands branching, while addition of exogenous heparanase increases branching (Fig. 12 A, B) (Patel *et al.*, 2007). Fibronectin is another ECM component that accumulates at sites of cleft formation and stimulates submandibular glands clefting (Larsen *et al.*, 2006). The role of laminins in submandibular glands morphogenesis, in particular laminin $\alpha 5$, is also interesting (Fig. 12 C, D). Laminin $\alpha 5$ is thought to regulate FGFR (FGFR1b and FGFR2b) expression through binding and activation of the $\beta 1$ integrin receptor. In turn, FGFRs also regulate laminin $\alpha 5$ expression. These observations suggest that a positive feedback loop between FGFs and integrin signaling controls submandibular glands morphogenesis (Rebustini *et al.*, 2007). Finally, MMPs, the ECM remodeling enzymes, have numerous functions in submandibular glands morphogenesis because they cleave ECM proteins (collagen, laminins, etc.), as well as cell surface receptors [FGFRs, Heparan-sulfate proteoglycans (HSPG)], and release or activate growth factors (HBEGF) and enzymes (heparanase). Not surprisingly, analysis of various MMP knockout embryos (MT1-MMP, Adam17) disclosed perturbed submandibular glands development (Patel *et al.*, 2006).

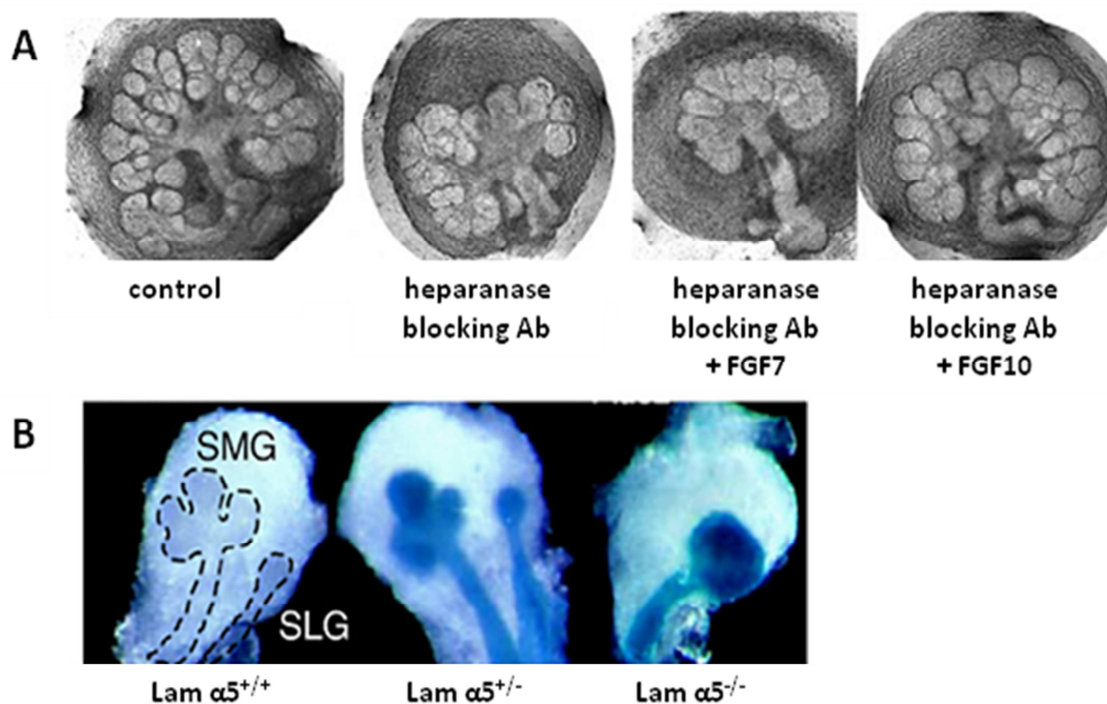


Figure 12 Extracellular matrix components control submandibular glands morphogenesis. A) Inhibition of heparanase function reduces branching morphogenesis. This effect is specifically rescued by the addition of FGF10 but not by FGF7. B) Development of submandibular glands is blocked in laminin $\alpha 5^{-/-}$ as compared to WT mice (adapted from Rebutini *et al.*, 2007).

Several reports likewise indicate a role for the ECM in early pancreas development. Laminin-1, produced by the mesenchyme and localized at the interface between epithelial and mesenchymal cells, was shown to promote pancreatic duct morphogenesis, but not differentiation (Crisera *et al.*, 2000). Slightly later, $\alpha v\beta 5$ and $\alpha v\beta 3$ integrins interact with several ECM components (fibronectin, collagen-IV, vitronectin) to allow migration of putative endocrine progenitors out of the ductal epithelium (Cirulli *et al.*, 2000). The role of integrin:ECM interactions in pancreas development was also shown to be mediated by netrins. Netrins are secreted molecules resembling laminin that were initially described as factors involved in axon guidance. However, additional roles for netrins in adhesion, migration and differentiation in other tissues were discovered. This is the case for Netrin-1, produced by pancreatic ductal cells and deposited in basal membranes. They bind to integrin receptors ($\alpha 3\beta 1$ and $\alpha 6\beta 4$) at the surface of epithelial cells to mediate pancreatic cell adhesion and migration (Yebra *et al.*, 2003). Finally, a few roles of endothelial-derived ECM were recently discovered. They will be tackled in section 3.4.4.

To our knowledge, there has been no report on the possible role of ECM components during thyroid morphogenesis.

3.3 Molecular signals

Many signals derived from the epithelial micro-environment are common to most of the branched organs in vertebrates, suggesting a high conservation. Among the secreted factors, fibroblast growth factors (FGF), hepatocyte growth factors (HGF) and epidermal growth factors (EGF) tend to promote branching; conversely, signaling by transforming growth factors β (TGF β) and activins *via* smads inhibits branching in various systems (Davies 2002). We will now summarize our knowledge on major secreted signals that control organ development, focusing on pancreas, submandibular and thyroid glands.

3.3.1 FGF signaling

The fibroblast growth factors family regulates a large diversity of biological activities like proliferation, differentiation and branching morphogenesis. The 23 members of this family bind to four tyrosine kinase receptors (FGFR1-4) and have a high affinity for ECM heparan sulfate (HS), as mentioned in the previous section. Upon ligand binding, FGFRs and heparan sulfate form a 2:2:2 FGF:FGFR:heparan dimer that induces the phosphorylation of the receptors. This activates cytoplasmic signal transduction pathways, such as the Ras/ERK, Akt, or PKC pathways. FGFs and their receptors are usually expressed in a complementary fashion in the mesenchyme and epithelium, respectively (Dorey and Amaya, 2010).

In the pancreas, FGF1, 7 and 10 are expressed in the mesenchyme while their common receptor FGFR1B is expressed in the epithelium. Binding of FGFs to FGFR1B induces epithelial proliferation of progenitor cells at the expense of pancreatic differentiation (Miralles *et al.*, 1999; Elghazi *et al.*, 2002). FGFs could favor the multipotent tip cells progenitors (Ptf1a+, see section 2.1.2) of the pancreas, since FGF10 from the mesenchyme maintains *Ptf1a* expression in the pancreatic epithelium (Jacquemin *et al.*, 2006). FGFs also favor exocrine differentiation at the expense of endocrine differentiation (Miralles *et al.*, 1999; Elghazi *et al.*, 2002).

The role of FGFs in submandibular glands development has been widely studied *in vitro* using whole submandibular glands explants culture on filters (see Fig. 12) but also using mesenchyme-depleted epithelium cultured in matrigel. In the latter system, where “pure” matrigel replaces the ECM, the epithelium does not branch unless FGFs are added to the culture medium. This leads to

different phenotypes: FGF2 and 7 induced branching morphogenesis, while FGF1, 4 and 10 promoted the elongation of ducts (Fig. 13) (Steinberg *et al.*, 2005). Also, total genetic deletion of some FGFs and FGFRs impaired submandibular glands development, demonstrating their importance during *in vivo* development: *Fgf10* and *Fgfr2b* KO display submandibular glands agenesis, while the submandibular glands of *Fgf8* KO do not branch (Patel *et al.*, 2006).

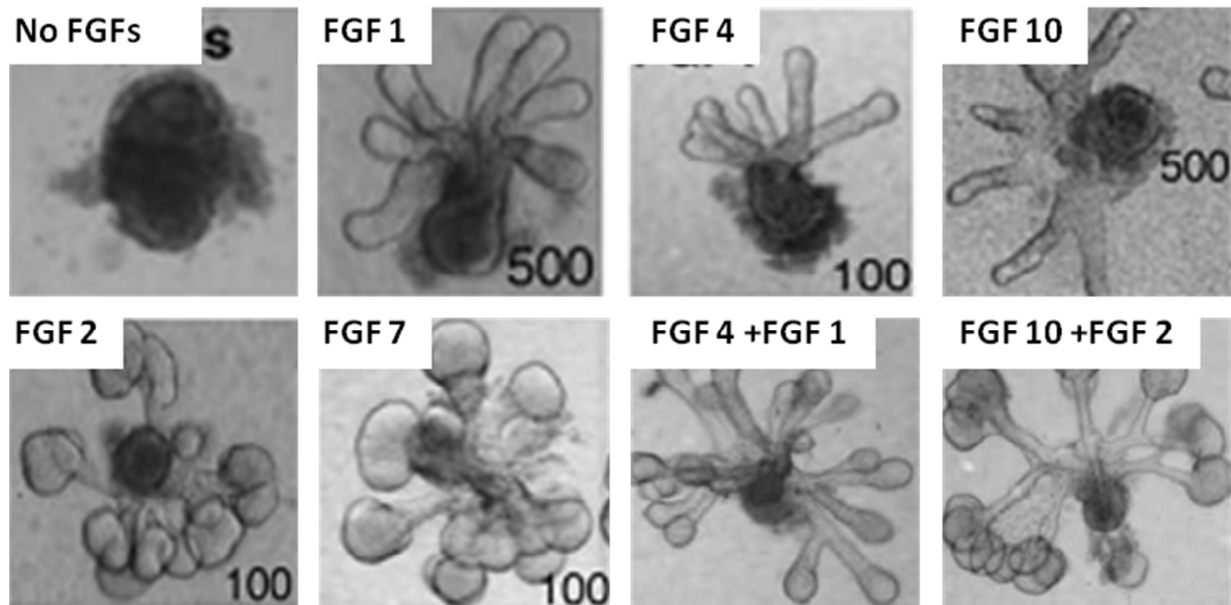


Figure 13 FGFs have distinct morphological effects on isolated epithelium cultured in growth factor-reduced matrigel. Epithelium treated with FGF1, FGF4 or FGF10 form duct-like structures while FGF2 and FGF7 promote budding. Combinations of FGFs induce more complex branching. For example, FGF10+FGF2-treated epithelium form elongated branches with enlarged acini (adapted from Steinberg *et al.*, 2005).

Fgf10 and *Fgfr2b* KO mice also display thyroid agenesis (Ohuchi *et al.*, 2000; Revest *et al.*, 2001), suggesting either a role of this signaling pathway, or a role of the FGF10-producing tissues (pharyngeal mesoderm) in thyroid development. Genetic deletion of *Tbx1*, a regulator of *Fgf8* expression that is expressed in the mesoderm surrounding the thyroid, induces thyroid hypoplasia mostly composed of one lobe and devoid of ultimobranchial bodies (Liao *et al.*, 2004; Lania *et al.*, 2009). Perturbation of FGF-induced intracellular signal transduction by the inactivation of the docking protein FRS2 α also causes thyroid aplasia or hypoplasia with few or absent C cells, further supporting the role of the FGF family in controlling thyroid development (Kameda *et al.*, 2009).

3.3.2 TGF β signaling

Transforming growth factor β (TGF- β) superfamily contains around 30 members (3 TGF- β s, 10 bone morphogenetic proteins (BMPs), 11 growth and differentiation factors (GDFs), 4 Activins and Nodal) that bind to two different types of serine/threonine receptors (7 type I receptors termed ALKs 1-7 and 5 type II receptors), thereby creating a huge diversity of cellular responses. In the canonical pathway, ligand dimers bind to a type II receptor with high affinity, leading to the recruitment and phosphorylation of a type I receptor. In turn, type I receptor phosphorylates intracellular mediators, the receptor-regulated Smads (R-Smads) Smad-1, -2, -3, -5, and -8. R-Smads form complexes with the co-Smad, Smad 4, and regulate the transcription of target genes. Part of the specificity in signaling depends on the ligands : BMPs/GDFs signal through ALK-2, -3, -6 and R-Smad-1, -5 and -8 while TGF- β s, Nodal, Activins signal through ALK-4, -5, -7 and R-Smads-2 and -3. However, this is a simplified view since interconnections between the two branches were shown and TGF- β s and BMPs can also activate non-Smad signaling pathways like the ERK or PI3K-AKT signaling cascades (Wu and Hill, 2009).

The various roles of TGF- β s during development depend on where and how the TGF- β members are expressed. In the pancreas, expression of a dominant negative form of TGF- β receptor II increases proliferation and accumulation of periductal endocrine cells, suggesting that TGF- β s restrict ductal or periductal cells recruitment into an endocrine fate (Tulachan *et al.*, 2007). By contrast, addition of activin A on pancreas explants inhibits branching of the exocrine compartment (Ritvos *et al.*, 1995) and induces a moderate increase in insulin producing cells (Demeterco *et al.*, 2000) suggesting that activin promotes endocrine differentiation at the expense of exocrine differentiation and branching. Smads 2/3 seem to be important for the balance between alpha and beta endocrine cells (Gittes, 2009).

In the submandibular glands, deletion of *Bmp7* impairs branching and lumen formation (Jaskoll *et al.*, 2002; Patel *et al.*, 2006). Consistent with this observation, addition of BMP7 in submandibular glands cultures stimulates branching, while an opposite role of BMP4 has been unraveled (Tucker, 2007; Davies *et al.*, 2002). Similarly to the pancreas, addition of activins to submandibular glands explants inhibits branching (Ritvos *et al.*, 1995).

Given the importance of the TGF- β superfamily in multiple organs development, it is tempting to postulate that they regulate thyroid development. During her undergraduate work in our lab, Mahé Bouquet has performed preliminary experiments on this superfamily (see discussion).

3.3.3 Sonic hedgehog (Shh) signaling

The hedgehog family members (Sonic, Indian and Desert hedgehog) are highly conserved between species, from fruitfly to humans, and are crucial for numerous cell differentiation and morphogenesis events during development. These secreted proteins bind to their membrane receptor Patched (Ptc), thereby relieving the inhibitory effect of Patched on Smoothened (Smo), another transmembrane protein. Subsequent intracellular signaling is mediated by glioblastoma (Gli) transcription factors: Gli1, Gli2 and Gli3 (Van Den Brink, 2007).

The Shh pathway is worth mentioning here for at least two reasons. First, the ligand is produced by the epithelium and signals to Ptc, expressed in the surrounding mesenchyme. Second, specific exclusion of *Shh* from the pancreas and thyroid regions is important for their development.

From the time of specification onwards, the ventral and dorsal pancreatic regions do not express *Shh*, while nearly all epithelial cells of the foregut endoderm express *Shh* by e10 (Fig. 14 A). A supportive observation is the exclusion of the receptor Ptc from the pancreatic mesenchyme (Fig. 14A). Ectopic expression of *Shh* in the pancreatic region using the *Pdx1* promoter perturbs both epithelial and mesenchymal pancreatic development. The mesenchyme then adopts intestinal mesenchymal characteristics, and the epithelium forms neither islets nor acinar structures but adopts duodenal-like structures producing duodenal-specific mucins (Apelqvist *et al.*, 1997). *Shh* KO embryos display a relative increase in pancreatic mass and endocrine differentiation markers (Hebrok *et al.*, 2000).

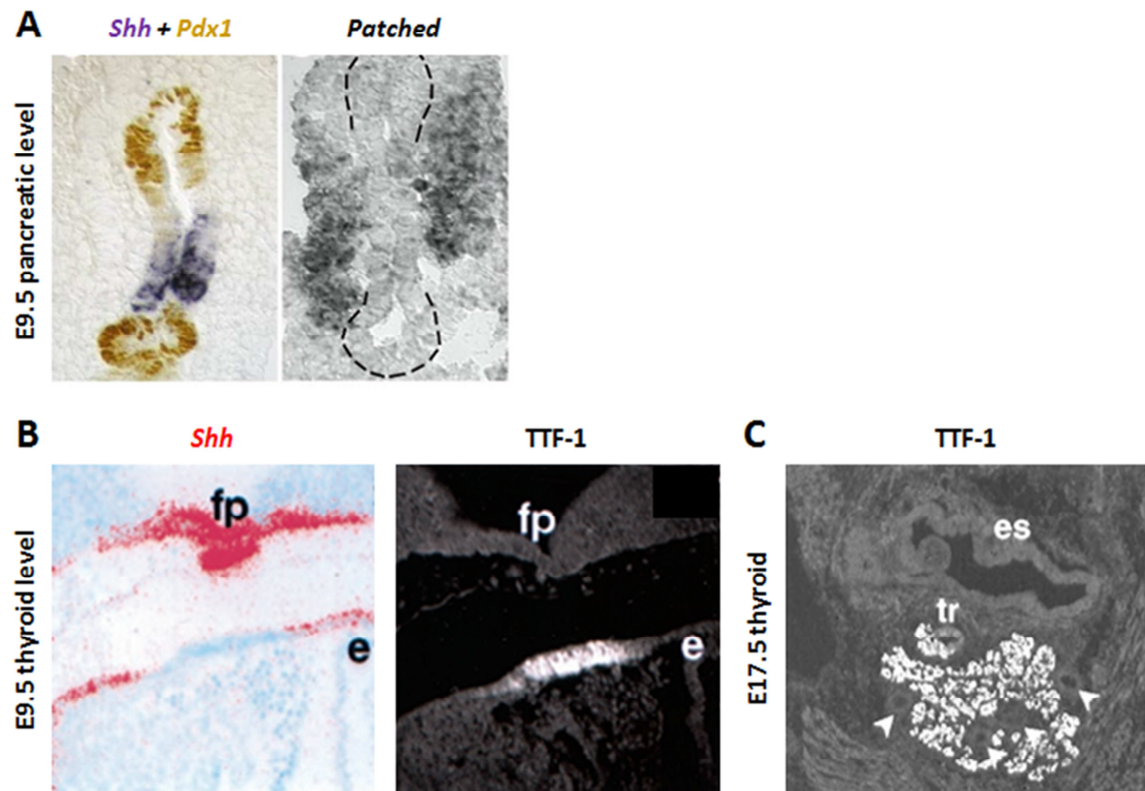


Figure 14 *Shh* is excluded from the pancreas and thyroid primordia. *Shh* suppression perturbs thyroid gland development. A) *Shh* expression is excluded from the developing pancreas anlagen. Immunolabeling of Pdx1 (brown) combined with *in situ* hybridization for *Shh* (dark blue) or *Patched* (black) on transverse sections of an e9.5 wild type embryo (Apelqvist *et al.*, 1997). B) *Shh* expression is excluded from the developing thyroid anlage. *In situ* hybridization of *Shh* with immunolabeling of TTF-1 on serial sections shows the presence of *Shh* (red) in the entire foregut endoderm but in the thyroid anlage at e9.5 (Fagman *et al.*, 2004). C) Immunolabeling of TTF-1 on transverse e17.5 *Shh* knockout embryo sections shows the presence of a unique thyroid bud, displaced to the left of the trachea and pierced by large deviant vessels (arrowheads) (Fagman *et al.*, 2004).

Similarly to the pancreas, *Shh* is absent from the endodermal cells specified to give rise to the thyroid primordium, but well-expressed in the adjacent pharyngeal endoderm (Fig. 14 B). The thyroid is properly specified in *Shh* KO embryos, but budding and subsequent disconnection from the endoderm are slightly delayed. At e15.5, the thyroid of *Shh* KO embryos appears as a unique central mass, and two days later, its size is limited to that of a single wild-type lobe, often displaced to the left side of the trachea (Fig. 14 C). Despite this perturbed organogenesis, thyrocytes in this unique lobe differentiate and organize properly into follicles. Another interesting observation is that the loss of *Shh* in the pharyngeal endoderm leads to ectopic budding of thyroid follicular cells, suggesting that *Shh* expression in wild-type embryos could restrict the site of thyroid development (Fagman *et al.*, 2004).

3.3.4 SDF-1 signaling

Stromal cell-derived factor-1 (SDF-1) signaling is important for many developmental processes (see below), but its role in mouse pancreas and submandibular glands development was unknown at the onset of this work. We determined the importance of this signaling pathway during the first part of this thesis (Hick *et al.*, 2009). For this reason, we will now look at this signaling pathway in details.

SDF-1/CXCL12 is a secreted cytokine belonging to the CXC family that binds to two 7 transmembrane CXC receptors, CXCR4 and CXCR7. Its affinity is 10 times higher for CXCR7, as compared to CXCR4. While the only natural CXCR4 ligand is SDF-1, CXCR7 also binds CXCL11, though with a lower affinity than SDF-1. SDF-1, CXCR4 and CXCR7 are remarkably conserved among species (Kryczek *et al.*, 2007). Moreover, during embryogenesis, *Sdf1* and *Cxcr4* display complementary expression, *Cxcr4* being expressed in ectoderm or endoderm derivatives, surrounded by *Sdf1* positive mesenchyme, hence its name (Fig. 15) (McGrath *et al.*, 1999). Using ligand binding assays, it was demonstrated that *Cxcr7* is transiently expressed in some organs during embryogenesis (Burns *et al.*, 2006). Moreover, *Sdf1*, *Cxcr4* and *Cxcr7* are highly expressed in numerous cancers, and *Cxcr7* is frequently observed in the vasculature associated with tumors (Kryczek *et al.*, 2007; Wang *et al.*, 2008).

Sdf1, *Cxcr4* and *Cxcr7* KO mouse embryos all die at birth. While *Sdf1* and *Cxcr4* knockouts show severe nervous and hematopoietic defects (Ma *et al.*, 1998), *Cxcr7* knockout embryos exhibit lethal cardiac abnormalities. Moreover, endothelial-specific deletion of *Cxcr7* recapitulates the phenotype of *Cxcr7*^{-/-} embryos (Sierro *et al.*, 2007). In developmental and tumorigenic processes, SDF-1 signaling promotes cell survival and proliferation, supports vascularization and modifies cell adhesion and migration through its ability to degrade extracellular matrix and to reorganize actin cytoskeleton (Kryczek *et al.*, 2007).

SDF-1 signaling has been well-studied in chick pancreas specification. Before pancreas specification, *Sdf1* is expressed in the endoderm and is necessary for the recruitment of *Cxcr4*-expressing angioblasts. Angioblasts signal back to the endoderm to induce *Pdx1* expression, allowing pancreas specification (Katsumoto and Kume, 2011). Later in development and in the adult pancreas, *Cxcr4* is expressed in endocrine and a few duct cells while *Sdf1* is expressed in endocrine cells only (Kayali *et al.*, 2003). Since *Cxcr4* and *Sdf1* knockout embryos display multiple defects, the role of SDF-1 signaling in islets neogenesis is hard to study. Nevertheless, using a model of pancreas regeneration (IFN γ under the control of insulin promoter), it has been shown that SDF-1/CXCR4

signaling is important for proliferation, survival and migration of pancreatic duct cells (Kayali *et al.*, 2003).

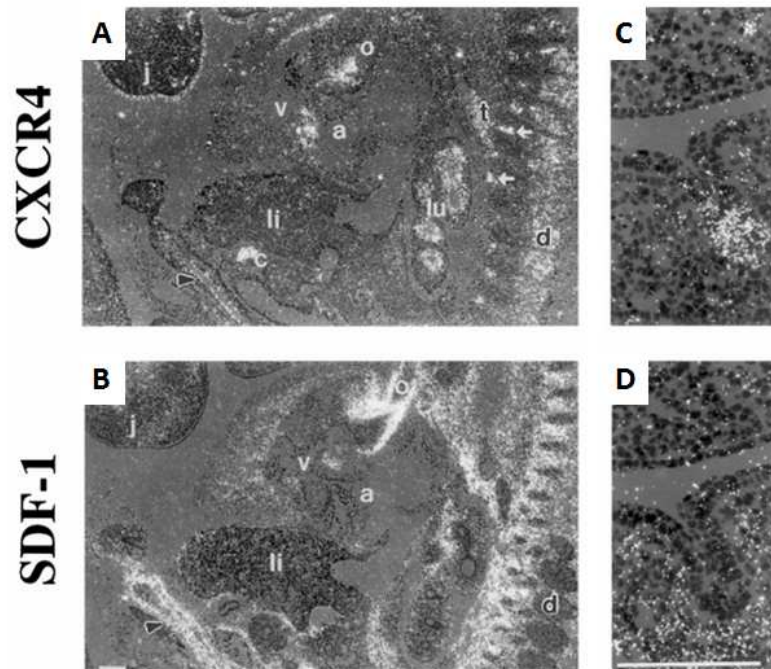


Figure 15 CXCR4 and SDF1 expression in midregion. (A, B; global comparison at e12.5) *Cxcr4* is expressed in the dorsal (d) & thoracic (t) sympathetic ganglia and spinal nerves (white arrows). Conversely, *Sdf1* is expressed in dorsal mesenchyme, but excluded from ganglia and vertebrae. *Cxcr4* is also expressed in lung epithelium (lu) and the cystic duct (c) under the liver (li). (v) ventricle, (a) atrium, & (o) outflow tract. (C-D; focus on a budding structure, at e11.5) *Cxcr4* is expressed within a budding structure invading the surrounding *Sdf1*+ mesenchyme, and annotated as the thyroid primordium. Notice pattern complementarity (Adapted from McGrath *et al.*, 1999).

A recent gene expression profiling indicates that *Sdf1* is expressed in the thyroid bud and the surrounding mesenchyme adjacent to the developing heart at e10.5 (Fagman *et al.*, 2011). It would thus be interesting to investigate *Cxcr4* and *Cxcr7* expression patterns at this stage and to test the role of *Sdf1* signaling in early thyroid development.

3.4 Cell types

3.4.1 Fibroblasts

The most abundant population of the stroma is the fibroblast (commonly referred to as “mesenchymal cell”). As detailed in the previous sections, these are not only the source of numerous signaling molecules (FGF, TGF β , SDF-1, etc.) but are also able to respond to the epithelial signals *via* the expression of specific receptors such as Ptc. A recent study reported the specific elimination of mesenchymal cells in the developing pancreas. The authors specifically induced apoptosis in these cells by crossing a mouse line expressing the Cre recombinase in the pancreatic mesenchyme (*Nkx3.2-Cre*) with a transgenic mice carrying an inducible diphtheria toxin receptor (DTR). Pregnant mothers were then injected with diphtheria toxin at different stages of embryonic development. Internalization of the toxin by DTR induces apoptosis in these cells by 24 hours. Early deletion of pancreatic fibroblasts (e9.5) impaired dorsal and ventral pancreatic budding and later development (Fig. 16). Moreover, deletion of pancreatic fibroblasts at later stages (from e11.5-e16.5 to e18.5) induced a reduction of pancreas size and branching, suggesting that fibroblasts are crucial throughout pancreatic development to provide the epithelium with signals for budding and sustained proliferation. Both endocrine and exocrine compartments were reduced, but this was due to the reduced proliferative capacity of epithelial progenitor cells in the absence of fibroblasts. Of note, endothelial and nerve cells were still present in DT-treated pancreas (Landsman *et al.*, 2011).

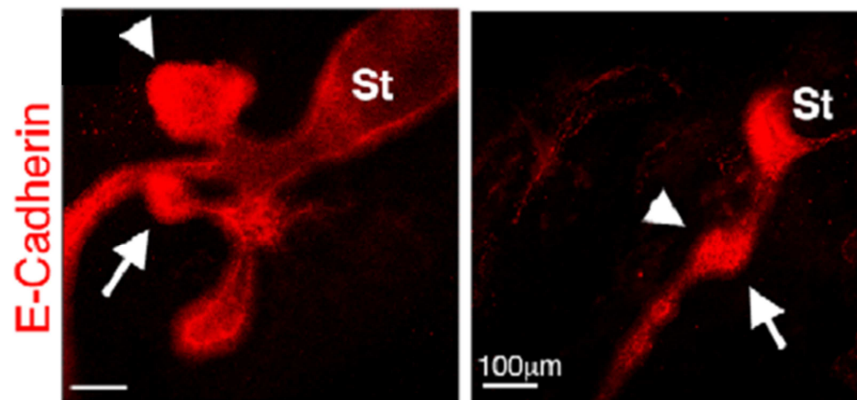


Figure 16 Pancreatic buds are smaller in transgenic *Nkx3.2-DTR* embryos. Whole-mount immunolabeling of E-cadherin on e10.5 pancreatic bud expressing the diphtheria toxin receptor driven by *Nkx3.2*, then exposed to the toxin. Both dorsal (arrowhead) and ventral (arrow) pancreatic buds are smaller in transgenic embryos (from Landsman *et al.*, 2011).

3.4.2 Nerves

Nerve cells are a small yet significant cell population of the stroma. Nerves, like blood vessels, are usually considered as a neutral network of cells, simply developing concomitantly to the epithelium, and without morphogenic properties.

However, a recent study has beautifully demonstrated a key role of parasympathetic submandibular ganglia (PSG) during submandibular glands development (Knox *et al.*, 2010). Ganglion cells expressing the neurotransmitter acetyl-choline (ACh) partially surround the initial SMG bud at e12 and, as the epithelium branches, they extend along the epithelial basement membrane without invading the mesenchyme. This study found that mechanical removal of neuronal bodies of the PSG - or inhibition of ACh/M1 signaling (muscarinic receptor M1 is the major receptor of ACh in embryonic SMG epithelium) - reduces the number of end buds, associated with the loss of cytokeratin-5 (K5) progenitor cell population. Rescue experiment with carbachol (CCh), an analog of ACh, and heparin-binding EGF (HBEGF, a known effector of ACh/M1) increased K5 staining, epithelial proliferation and budding, in an EGFR-dependent manner. In rescue experiments, addition of CCh to PSG-free explants promoted epithelial budding and K5 expression, as compared to PSG-free explants, and this effect was EGFR-dependent, since it was inhibited by an EGFR antagonist. This important study demonstrates the importance of parasympathetic innervation during SMG morphogenesis. Furthermore, since many organs expressing K5 are innervated by parasympathetic nervous system, it is likely that neurotransmitter-based paracrine communication drives multiple organs development.

3.4.3 Reciprocal interactions between epithelial cells

Although this chapter essentially deals with the paracrine interactions controlling epithelial morphogenesis, we think it would be incomplete without Ephrins signaling molecules, which have recently been shown to control the organization of the different epithelial cell types in pancreas and thyroid glands.

The ligands ephrins and their dedicated tyrosine kinase receptors, named Eph, are part of a large family expressed in a wide range of embryonic tissues, including neurons, blood vessels and epithelia. Being both membrane-bound proteins, ephrins and Ephs display cell contact-dependent signaling, like Delta/Notch. Upon ephrin:Eph engagement, two signals are generated: classical forward signaling by Ephs, and reverse signaling by ephrins (for a review, Miao *et al.*, 2009).

Two Eph receptors, EphB2 and EphB3, have been recently studied during pancreas development. Both are found on the epithelial cells but two of their ligands are specifically

expressed in the mesenchyme (ephrinB1) or in blood vessels (ephrin B2). Double *EphB2*^{-/-}*EphB3*^{-/-} KO embryos show defects in pancreas branching, decreased exocrine and endocrine masses, and initial narrower lumina, which fail to remodel into the characteristic pancreatic network found (Fig. 17) (Villasenor *et al.*, 2010).

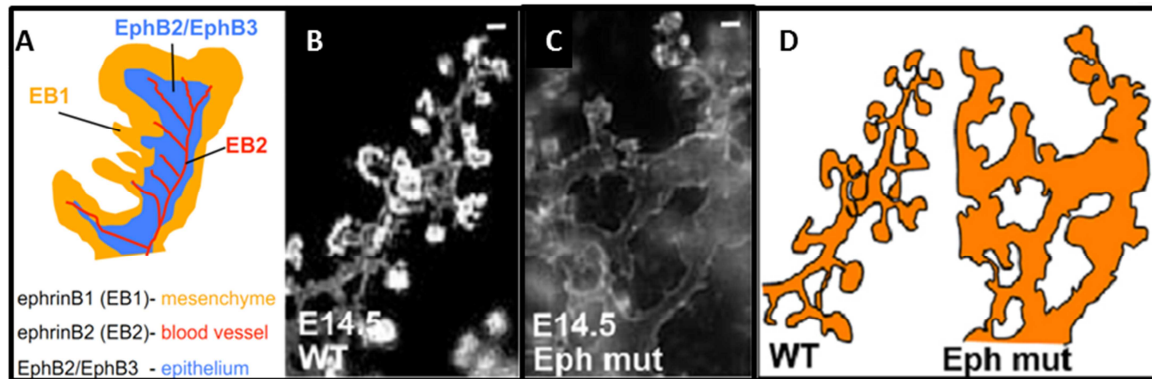


Figure 17 Pancreatic branching defects in EphB2 and EphB3 double knockouts. A) Schematic representation of the expression pattern of two ephrins ligands and their receptors. B-D) As compared to WT (B), whole-mount immunolabeling of mucin-1 at e14.5 shows a more primitive ductal plexus with dilated lumen in Eph mutants (C). This is better seen in the comparative drawing at D (adapted from Villasenor *et al.*, 2010).

Being ubiquitously is expressed in the foregut endoderm at e9.5, EphA4 is thus present in thyroid precursor cells. Thyroid cells maintain EphA4 expression during embryogenesis and even adult life. On the contrary, ultimobranchial bodies only express EphA4 at early stages. EphA4 expression is lost before fusion with the median anlage and remains absent in the C cells. Surprisingly, EphA4 mutant embryos display normal folliculogenesis and expression of thyroglobulin; EphA4 thus is thought to be dispensable for thyroid embryonic development. However, adult KO mice often display flattened thyrocytes and smaller follicles. Interestingly, functional impairment of EphA4 by replacing the intracellular receptor domain by EGFP (Knock-in; KI), thereby impairing forward signaling, results in a different phenotype, with larger follicles than in the wild type gland. Also, number and distribution of C-cells were comparable between wild type and EphA4 KO glands, but in EphA4 KI glands, these were reduced by more than 50% and mainly distributed in the center of the lobes. Therefore, during postnatal period, EphA4 modulates follicles maturation by forward and reverse signaling in the thyrocytes, and controls C-cells generation by indirect forward signaling or direct reverse signaling (Fig. 18) (Andersson *et al.*, 2011).

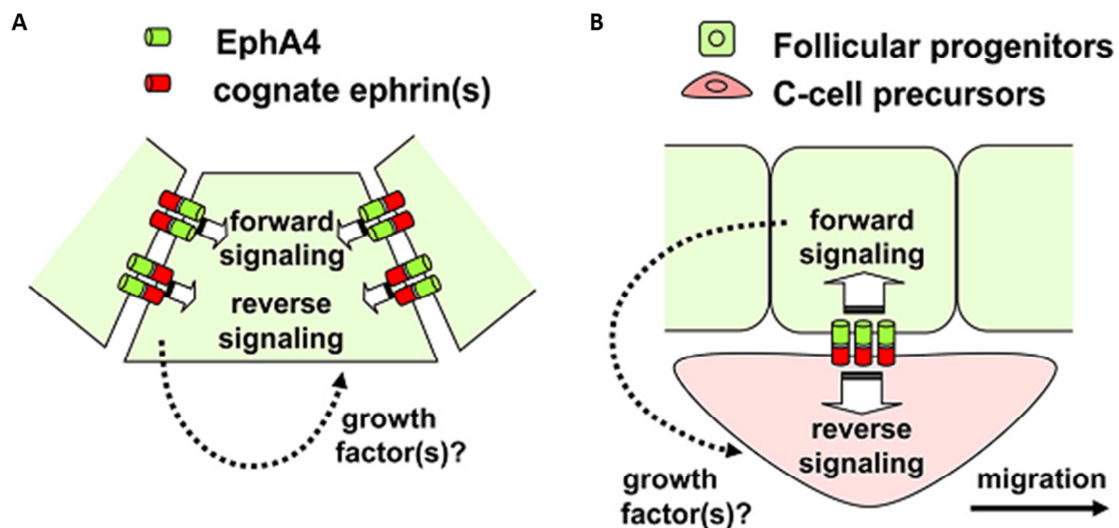


Figure 18 Model of EphA4 signaling in the thyroid. A) EphA4 and uncharacterized ephrins modulate follicles maturation by forward and reverse signaling in the thyrocytes. B) C-cells ephrins bind to follicular EphA4. EphA4 forward signaling promotes C cells proliferation and invasion of the thyroid mass. A putative role of ephrin reverse signaling is presently unknown. In both cases, EphA4 activation may induce the release of growth factors (adapted from Andersson *et al.*, 2011).

3.4.4 Endothelial cells

Blood vessels have long been viewed as a static network of tubes delivering nutrients and oxygen to organs, developing roughly at the same time as the epithelium to insure a correct association between the two cell types in the mature organ (supportive role). Nowadays, blood vessels are further considered as fundamental factors governing embryo development, organ specification, morphogenesis and differentiation, principally through paracrine interactions (instructive role; Crivellato, 2010). The coordinated growth of blood vessels with epithelia implies reciprocal communications between the epithelium and the endothelium. Paracrine communications thus include not only instructions (i) from the developing organs to ensure endothelial development (see section 3.4.4.1), but also (ii) from blood vessels to govern organ morphogenesis (see section 3.4.4.2) (Cleaver and Melton, 2003, Lu and Werb, 2008). Adequate understanding of organ morphogenesis should therefore include the fine analysis of epithelial and endothelial development, and the identification of exchanged signaling molecules.

Below, we will study in more detail the role of blood vessels, starting from the recruitment of endothelial cells by epithelial organs. Then we will analyze how blood vessels impact on the epithelium. This fascinating subject has been at the centre of the second part of my thesis, during pancreas development (Pierreux *et al.*, 2010) and thyroid folliculogenesis (Hick *et al.*, submitted).

3.4.4.1 Blood vessels recruitment by epithelia

3.4.4.1.1 VEGFs and their receptors

Vascular endothelial growth factors (VEGFs) are secreted dimeric glycoproteins belonging to the platelet-derived growth factor (PDGF)/VEGF superfamily. Five VEGF members have been described in mammals: VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). VEGF-E and VEGF-F are two homologues produced by Orf viruses and snake, respectively. These ligands bind with different specificities to three tyrosine kinase receptors VEGFR1 (Flt-1), VEGFR2 (Flk-1/KDR) and VEGFR3 (Flt-4). Their co-receptors are the neuropilins 1 and 2, previously termed semaphorin receptors (Lohela *et al.*, 2009).

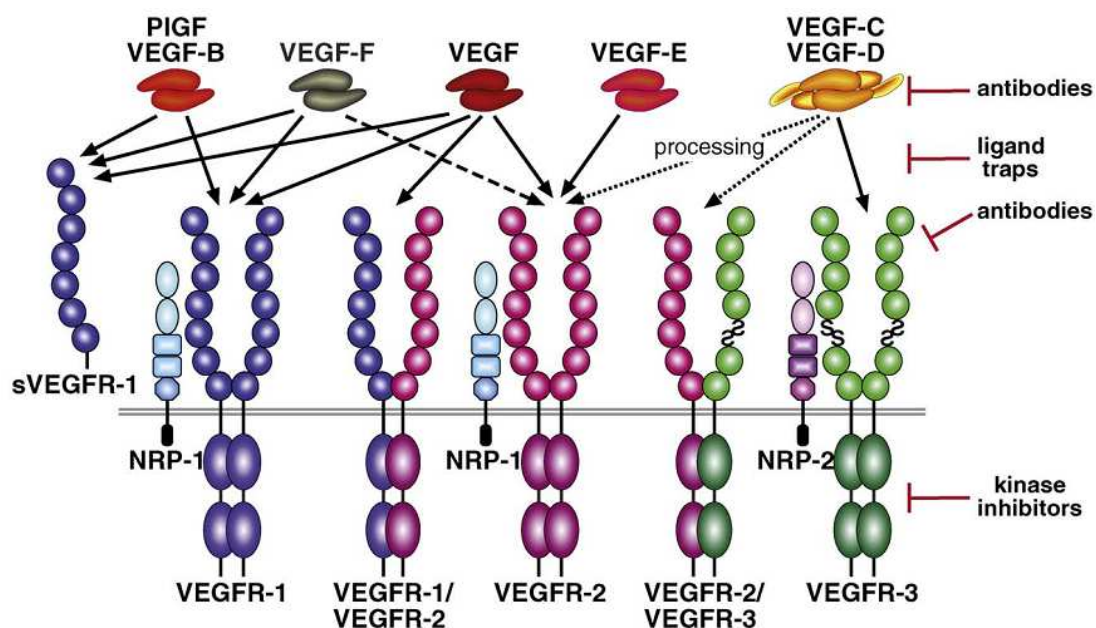


Figure 19 VEGF ligands and their (co)-receptors; at right, targets for pharmaceutical intervention (from Lohela *et al.*, 2009)

VEGF-A, a key regulator of angiogenesis, interacts with both VEGFR-1 and -2 (Fig. 19). Three major isoforms of VEGF-A are found in mice: VEGF-A120, VEGF-A164 and VEGF-A188; in humans, they bear an additional amino acid residue (VEGF-A121, 165 and 189). Other less frequent isoforms have been described in humans. VEGF-A isoforms differ in their solubility and capacity to bind heparin, and thus the heparan sulfate proteoglycan in the ECM and on the cell surface. This binding ability, encoded by the exons 6 and 7 of VEGF-A, is found in VEGF188/189 isoform and accounts for its accumulation in the extracellular matrix. In contrast, VEGF120 lacks domains encoded by exons 6 and 7 and is therefore more diffusible and accessible for interaction with other distant cells. As an

intermediate form, VEGF₁₆₄ contains the domain encoded by exon 7, but not exon 6, and thus 50-70% of this isoform remains associated with the cell surface and ECM. Several factors are known to induce *Vegfa* expression: hypoxia-inducible factors (HIF1A and HIF1B), insulin-like growth factor (IGF1), FGF2 (basic FGF), FGF7, PDGF, TGF α and TGF β , as well as cytokines and hormones. *Vegfa* is mainly expressed during embryonic development at sites of blood vessels formation and in the yolk sac. In the adult, *Vegfa* expression is restricted to hypervascularized organs, like the kidney, during menstruation or wound healing, but also in pathological conditions, such as cancers (Haigh, 2008; Karamysheva 2008). Tumor angiogenesis has attracted major attention but is beyond the scope of my thesis.

3.4.4.1.2 *How do VEGFs control blood vessels development?*

In the embryo, blood vessels formation begins by the differentiation of angioblasts, i.e. endothelial precursors of mesodermal origin, into endothelial cells; and their organization into a primitive vascular network. This initial step is called *vasculogenesis*. Sprouting of endothelial cells from pre-existing vessels is referred to as *angiogenesis*. During angiogenesis, sprouting is triggered by pro-angiogenic signals (typically a VEGF gradient) that attract endothelial cells and renders them more motile and invasive. During endothelial cells activation, tip and stalk cells are selected. Tip cells protrude filopodia and crawl towards the source of VEGF, while stalk cells proliferate to maintain the contact between the tip cell and the existing vessel. Mutual inhibition between tip and stalk cells is achieved *via* VEGF/VEGFRs, Dll4/Notch and BMP signaling. Stalk cells then form an intracellular lumen by cell or cord hollowing (see section 1.2). Pericytes and smooth muscle cells recruitment, controlled by PDGFR β , confers stability and modulates perfusion (Potente *et al.*, 2011, Moya *et al.*, 2012).

Gene inactivation studies have demonstrated the importance of VEGF signaling. The loss of a single *Vegfa* allele is embryonic lethal, indicating that VEGF-A concentration is important for endothelial development and embryogenesis (Carmeliet *et al.*, 1996). VEGFR2-deficient mice die around e8.5 – e9.5 due to defects of hematopoietic and endothelial cells development (Shalaby *et al.*, 1995). By contrast, VEGFR1-deficient mice die at the same age due to excessive growth and disorganization of blood vessels (Fong *et al.*, 1995). This observation, together with the 10 times higher affinity of VEGF-A for VEGFR1 than for VEGFR2, suggests that VEGFR1 could act as a decoy receptor for VEGF-A.

3.4.4.2 Role of blood vessels in epithelial morphogenesis

3.4.4.2.1 Pancreas

At the onset of this work, the role of endothelial cells in pancreas development was known for the early (i.e. budding) and late (endocrine differentiation) steps.

Early mouse pancreas development was shown to be dependent on endothelial signaling from the adjacent big vessels. Histologically, around e8.75-e9, the nascent dorsal pancreatic bud is located near the aorta and the ventral pancreatic buds are adjacent to the vitelline veins (Fig. 20).

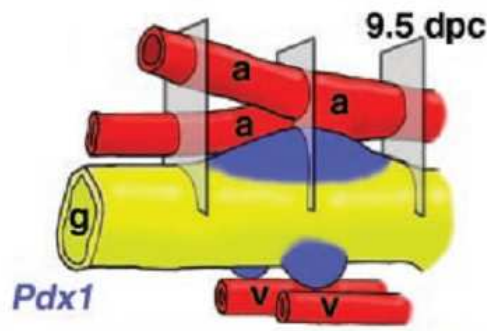


Figure 20 Schematic view of blood vessels (red), surrounding the gut endoderm (yellow) at the site of pancreatic budding (endoderm expressing Pdx1, blue). (a) aorta, (g) gut tube and (v) vitelline veins are marked (from Lammert *et al.*, 2001).

Removal of the dorsal aorta from *Xenopus* embryo abrogates insulin expression. *In vitro* experiments with mouse embryo tissue showed that aortal cells are responsible of the induction of *insulin* expression when recombined with isolated endoderm (Lammert *et al.*, 2001). Moreover, *Vegfr2* KO embryos, which are devoid of endothelial cells and aorta, lack dorsal pancreatic budding and *Ptf1a* expression. Again, aortal cells induce *Ptf1a* expression when recombined with isolated *Vegfr2* KO endoderm (Yoshitomi and Zaret, 2004). Later on, it was proposed that aortal cells also promote the survival of dorsal mesenchyme, which in turn, *via* FGF10 signaling, maintains *Ptf1a* expression and therefore promotes dorsal pancreatic differentiation (Jacquemin *et al.*, 2006).

Later in pancreatic development, blood vessels regulate endocrine differentiation. Overexpression of *Vegfa-164* in the pancreatic region, under the control of the *Pdx1* promoter, induces hypervascularization and islet hyperplasia (Lammert *et al.*, 2001). However, when epithelial *Vegfa* is suppressed late in development (*Pdx1*-Cre; *Vegfa*^{fl/fl} embryos), islet vascularization is reduced but islets display normal morphology and glucose metabolism is weakly perturbed (Lammert

et al., 2003). This suggests that (i) vascularization promotes endocrine differentiation (Lammert *et al.*, 2001) and, (ii) in turn, islets cells induce endocrine capillary development via VEGF-A production (Lammert *et al.*, 2003). It was proposed that recruited endothelial cells organize in capillaries within the islets and generate a laminin-411 rich extracellular matrix. Laminin interacts with $\alpha 6 \beta 1$ integrin on beta-cells to promote their proliferation and insulin expression (Nikolova *et al.*, 2006). Endothelial cells infiltrating islets also express Netrin-4 during pancreas development. In a model of human fetal pancreatic cell culture, netrin-4 promoted epithelial cell adhesion and endocrine differentiation through epithelial integrins ($\alpha 2 \beta 1$ and $\alpha 3 \beta 1$), suggesting again a role for blood vessels in matrix deposition and islet development (Yebra *et al.*, 2011).

In conclusion, endothelial cells promote the early expression of pancreatic specific genes (Ptf1a) and later stimulate beta-cell proliferation and insulin expression. How and when endothelial cells are recruited to the pancreas as well as their role in exocrine differentiation was unknown at the beginning of this work. We will see in the results section that endothelial cells are recruited specifically near the trunk cells, at a distance from the tip cells, by the localized expression of VEGF-A in the trunk cells. In turn, endothelial cells limit the exocrine differentiation of the epithelium to the tip cells (Pierreux *et al.*, 2010).

3.4.4.2.2 *Thyroid*

Throughout its development, the thyroid gland is in close association with large blood vessels. First, thickening of the endoderm that gives rise to the thyroid primordium develops in close proximity with the aortic sac, without mesenchymal cell interpositioned (Fagman *et al.*, 2006). Then, lateral expansion of the thyroid primordium coincides with the path of the paired third pharyngeal arch arteries (paa in Fig. 10). These arch arteries will notably give rise to the carotid arteries that are positioned in the immediate vicinity of the two mature thyroid lobes (ca in Fig. 10). The close interactions between these two large vessels and the thyroid primordium have been recently documented (Fagman *et al.*, 2006; Alt *et al.*, 2006). However, the role of the numerous blood vessels and capillaries that invade the thyroid lobes has not been explored yet.

In zebrafish, the thyroid gland develops from a small mass of cells along the anteroposterior axis to generate a strand of follicular tissue in the midline. Expansion is intimately associated with the emergent ventral aorta. In the absence of *Scl*, *Vegf* or *Kdr* (*Vegfr2*), ventral aorta fails to develop and the thyroid fails to elongate along the anteroposterior axis. Moreover, insertion of ectopic endothelial cells in the mesenchyme contiguous to the thyroid primordium induces the protrusion of thyroid epithelial cells towards the grafted cells. These results suggest that endothelial cells may

guide zebrafish thyroid primordium (Alt *et al.*, 2006). Using fluorescent proteins, Opitz *et al.* have recently illustrated the coordination between thyroid and cardiovascular development (Opitz *et al.*, 2012).

As mentioned above, *Shh* KO mouse embryos have a normal thyroid specification and budding, but a single lobe is formed on one side of the trachea (section 3.3.3 and Fig. 14). At the time of specification and budding, the relationship between the thyroid primordium and the aortic sac is maintained. Later in development, the carotid arteries in *Shh* KO develop asymmetrically, which could explain bilobation failure (Fagman *et al.*, 2004; Alt *et al.*, 2006). Indeed, the unique thyroid epithelial lobe is constantly located adjacent to the mislocated carotid arteries. Since *Shh* is not expressed in the thyroid, or in the surrounding mesenchyme, it is likely that the lack of *Shh* around the thyroid region induces vascular defects that impact on thyroid morphology. Furthermore, in *Shh*^{-/-} *Gli3*^{-/-} double KO, both carotid arteries and thyroid are located symmetrically on both sides of the trachea (Alt *et al.*, 2006). So, it has been proposed that arch arteries could guide thyroid progenitors while they migrate towards the ultimobranchial bodies (Fagman *et al.*, 2006, Alt *et al.*, 2006). Specific deletion of carotid arteries would be useful to better understand their role in thyroid bilobation.

Clinical observations support this link, as cardiac anomalies represent the most recurrent birth defects associated with thyroid dysgenesis (Olivieri *et al.*, 2002). In Di Georges's syndrome, although the main malformations concern the aortic arch and outflow tract heart, associated hypothyroidism is extremely frequent (Liao *et al.*, 2004). Deletion of the *Tbx1* gene in mice, that has been associated with Di George's syndrome, also causes aortic arch and outflow tract heart defects. *Tbx1* KO thyroids are correctly specified but subsequent bilobation fails and ultimobranchial bodies do not develop (Liao *et al.*, 2004). Association of cardiac and thyroid gland malformations could be due to a common defect in the developmental process. Alternatively a signaling molecule coming from one structure could be necessary for proper development of the other (Fagman *et al.*, 2009).

Altogether, these observations suggest that primitive blood vessels might provide paracrine signals for thyroid migration and positioning. Whether endothelial cells play a role in other steps of thyroid development remains to be elucidated.

3.4.4.2.3 Other organs

Recent studies focused on epithelial-endothelial communications during lung morphogenesis. Unlike the glands we have studied, lung airways develop from the foregut monolayer by iterative bifid branching, i.e. cells always maintains epithelial polarity and lumen

continuity. Iterative bifid branching is highly stereotyped and requires 3 modes: domain branching (i.e. definition of domain specificity and periodicity), planar bifurcation and orthogonal bifurcation (requiring a rotation) (Metzger *et al.*, 2008). Remarkably, lung branching is tightly associated with vasculature branching and requires its own mesenchyme. When added on lung explants, VEGF-A164 promotes branching, but it has no effect on mesenchyme-free cultured lungs. Conversely, impaired *Vegfr2* expression inhibits lung branching (Del Moral *et al.*, 2006). It was then shown that decreased vascular density *in vivo* and elimination of endothelial cells *ex vivo* perturbed orthogonal bifurcations during lung branching. Importantly, these effects were independent of blood perfusion, pointing to an instructive effect. Moreover, addition of HUVEC-conditioned medium on endothelial cells-depleted explants partially rescued branching, indicating a contact-independent, i.e. paracrine mechanism. The loss of blood vessels likely perturbs mesenchymal FGF10 expression pattern, a key mediator of lung branching morphogenesis (Lazarus *et al.*, 2011).

The role of endothelial cells has also been investigated in the developing kidney. Given that glomeruli act as filtration barriers between the blood and urine, glomerular vascularization is indeed critical for kidney function. Homozygote glomerular-selective deletion of *Vegfa* in the mouse embryo (nephrin-Cre) impairs endothelial cells migration, differentiation and survival in the kidney. As a consequence, “glomeruli” are smaller, avascular, lack mesangial cells (local equivalent of pericytes), and are merely composed of multilayered podocytes. In the absence of ultra-filtration barrier, perinatal mortality is inescapable (Eremina *et al.*, 2003). Of greater interest, even heterozygosity for *Vegfa* results in renal disease by 2.5 weeks of age, indicating a dosage effect. By 9 weeks of age, glomeruli are misshaped without patent capillary loops, podocytes contain large empty cytoplasmic vesicles and dedifferentiate, and tubules are dilated. Remarkably, overexpression of VEGF-A (by over 30-fold) also leads to the collapse of glomeruli capillary loops, dilatation of proximal tubules and abnormal development of podocytes, inducing death by 5 days of age (Eremina *et al.*, 2003). This observation is consistent with an optimal dosage effect and suggests toxicity by excess signaling. In adult mice, conditional overexpression of *Vegfa* specifically into proximal tubular cells (Pax8-rtTA/(tetO)₂ VEGF) induced the formation of cystic tubules surrounded by fibrotic tissue; and enlarged glomeruli with collapsed capillaries embedded in mesangial nodules (Hakrrouch *et al.*, 2009).

These studies provide additional examples of the paracrine controls operating in the microenvironment of epithelial tissues and demonstrate the importance of VEGF-A signaling therein.

VI. AIM OF THE WORK

The pancreas and submandibular glands as well as the thyroid, are structured on polarized epithelial monolayers originally derived from the endoderm then undergoing two opposed epithelial transitions. During the first transition, endodermal epithelial cells loose polarity and proliferate so as to generate an undifferentiated three-dimensional mass of cells. In the second transition, this bud reorganizes to form polarized monolayer(s). The final epithelial architecture being open branched tubes in the exocrine organs, and closed follicles in the endocrine thyroid.

The aim of my thesis was to better understand the tissular and molecular mechanisms controlling this second epithelial transition. Deciphering the mechanisms involved in this transition is not only important for developmental biology but also for medicine, since its reversion (monolayers → mass of cells) may occur in some carcinomas.

For the three organs, we first performed a precise description of the reorganization of the epithelial mass into functional monolayer(s), i.e. organ morphogenesis, by focusing on the polarization of the epithelial cells and on their topological relationships with mesenchymal and endothelial cells. Based on this description, we analyzed how paracrine communications with/from mesenchymal or endothelial cells control this epithelial transition.

Since the laboratory had obtained preliminary data on the implication of the mesenchymal factor *Sdf1* and its epithelial receptor *Cxcr4* in pancreas development, I raised the question of the generalization of this observation. In the first part of my thesis, I thus studied if *Sdf1* and its two receptors could control the development of the submandibular glands, another branched organ, and of the thyroid.

Our descriptive study of the topological relationships between epithelial cells and their environment revealed the abundance and proximity of endothelial cells with the developing pancreatic and thyroid epithelia. Using *in vivo* and *in vitro* systems we determined whether (i) VEGFA/VEGFR2 signaling was involved in the recruitment and expansion of the endothelial cells around these developing organs, and (ii) if endothelial cells could in turn, via paracrine communications, control pancreas and thyroid gland morphogenesis and differentiation. Both hypotheses were found to be true.

VII. RESULTS

1 Mechanism of primitive duct formation in the pancreas and submandibular glands: a role for SDF-1

Anne-Christine Hick, Jonathan M van Eyll, Sabine Cordi, Céline Forez, Lara Passante, Hiroshi Kohara, Takashi Nagasawa, Pierre Vanderhaeghen, Pierre J Courtoy, Guy G Rousseau, Frédéric P Lemaigre and Christophe E Pierreux

My contribution to this work was (i) to analyze submandibular gland reorganization of the mass in monolayers, and (ii) to determine the role of SDF-1 signaling during submandibular gland development. I studied epithelial cells polarization and demonstrated that the formation of the branched epithelial monolayers from the mass occurred without cell apoptosis. Using *in situ* hybridization technique, I detected the expression of *Sdf1* in the mesenchyme and of *Cxcr4* in the epithelium of the developing submandibular glands. The second SDF-1 receptor, CXCR7, was also detected in the endothelium by immunolabeling. Based on the literature, I set up submandibular glands culture in the lab. I finally performed loss-of-functions experiments by looking at the effects of pharmacological inhibition of SDF-1 signaling in explants and of CXCR4 genetic inactivation *in vivo*.

2 Epithelial:Endothelial cross-talk regulates exocrine differentiation in developing pancreas

Christophe E. Pierreux, Sabine Cordi, Anne-Christine Hick, Younes Achouri, Carmen Ruiz de Almodovar, Pierre-Paul Prévot, Pierre J. Courtoy, Peter Carmeliet, Frédéric P. Lemaigre

My contribution to this work was to improve tissue imaging. I set up immunoperoxidase after *in situ* hybridization. This allowed us to precisely demonstrate the preferential expression of *Vegfa* in the trunk cells away from the CPA+ tip cells. Conversely, we showed the expression of Vegfr2+ endothelial cells around the trunk cells. I have extended the use of the multiphoton microscope (LSM510) to the acquisition of successive confocal images on triple immunolabeled samples, for their 3D analysis as projections or movies. This allowed the visualization of the blood vessels organization during early pancreas development. The tools I developed for this paper were extremely useful for the thyroid study.

3 Reciprocal epithelial: endothelial paracrine interactions during thyroid development govern follicular organization and C-cells differentiation

Anne-Christine Hick, Anne-Sophie Delmarcelle, Mahé Bouquet, Sabrina Klotz, Tamara Copetti, Céline Forez, Patrick Van Der Smissen, Pierre Sonveaux, Olivier Feron, Pierre J. Courtoy and Christophe E. Pierreux

This work is my main contribution. I planned and performed all the experiments and wrote the manuscript.

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