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**Stepwise differentiation of pancreatic acinar cells
from mES cells
by manipulating signaling pathways**

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*A la mémoire de Solange Delaspre
1959-1999.*



*Pour l'amour que tu m'as donné...
Pour la patience et la persévérance que tu m'as
inculqué...
Merci à toi, maman.*

Tu es partie trop tôt...

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LIST OF ABBREVIATIONS

A

A/P: Anterior-Posterior
ACh: Acetylcholine
Afp: Alfa Foeto Protein
Amyl: Amylase
Ang II: Angiotensin II
AVE: Anterior Visceral Endoderm

B

bHLH: Basic Helix-Loop-Helix
BMP: Bone Morphogenetic Proteins
Bry: Brachyury

C

CCK: Cholecystokinin
Cel: Carboxyl-Ester Lipase
Chymo: Chymotrypsinogen 1B;
CP: Chronic Pancreatitis
Cpa1: Carboxypeptidase A1
Cyc: Cycloamine

D

D/V: Dorsal-Ventral
DAG: Diacyl glycerol
DAPI: 4',6'-diamidino-2-phénylindole
DE: Definitive Endoderm
Dex: Dexamethasone
DM: Dorsomorphin
Dox: Doxycycline

E

EB: Embryoid Body
ECM: Extracellular matrice
Ela1: Elastase 1,
EMT: Epithelial-to-mesenchymal transition
ESC: Embryonic Stem Cells.
ExE: Extra-embryonic ectoderm
ExEn: Extra-embryonic endoderm

F

FBS: Foetal Bovine Serum
FCS: Foetal Calf Serum
FGF: Fibroblast growth factor
FGFR2B: FGF receptor 2B

Fol: Follistatine

G

Gys2: Glycogen synthase 2
GMEM: Glasgow's modified Eagle's medium
GR: Glucocorticoid Receptor
Gsc: Goosecoid

H

h: Human
Hh: Hedgehog
Hlxb: Homeobox gene HB
HNF: Hepatocyte Nuclear Factor

I

ICM: Inner Cell Mass
IF: Immuno Fluorescence
IGF: Insulin-like growth factor
IGFBP: Insulin growth factor binding protein
ILV: Indolactam V
IP3: Inositol triphosphate
IPC: Insulin Producing Cell

K

KSR: Knockout Replacement Serum

L

LIF: Leukemia Inhibitory Factor

M

m: Mouse
Mat: Matrigel

N

Ngn3: Neurogenin 3

P

PAC: Pancreatic acinar cell
PanIN: Preneoplastic lesion
PDAC: Pancreatic ductal adenocarcinoma
PDGF-B: Platelet-derived growth factor
B

Pdx1: Pancreatic duodenal homeobox factor 1

PE: Pancreatic Endoderm

PEI-EBs: Pancreatic Endoderm

Induced-EBs

PFA: Paraformaldehyde

PI3K: Phosphatidylinositol 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

Pla2g6: Phospholipase A2 group 6

PP: Pancreatic Polypeptide

Prss3: Trypsin 3

PS: Primitive streak

PTF: Pancreas-specific transcription factor 1

Puro: Puromycine

R

RA : Retinoic Acid

RT: Room Temperature

S

Shh: Sonic Hedgehog

SNAREs: Soluble N-ethylmaleimide sensitive fusion protein attachment protein receptors

T

t-SNARE: Target membrane SNARE

T3: 3-3-5 triiodo-L-thyronine

TF: Transcription factor

TGFβ: Transforming growth factor-beta

TR: Thyroid hormone Receptor

Ttr: Transthyretin

V

v-SNARE: Vesicle membrane SNARE

VIP: Vasoactive intestinal peptide

3D: Three Dimensions

SUMMARY

Despite known involvement of pancreatic acinar cells in exocrine pathologies (pancreatitis and pancreatic cancer), the lack of normal cell-based models has limited the study of the alterations that occur in the acinar differentiation program. We have previously shown that mESC (murine embryonic stem cells), which are pluripotent, can acquire an acinar phenotype *in vitro*. This was achieved, in part, by a combination of signals provided by the culture of foetal pancreases which was, however, no specific for the acinar lineage. The aim of this work was to develop a protocol selective for the acinar lineage based on the sequential activation of signaling pathways that recapitulate pancreatic development *in vivo*, through the definitive endoderm formation, the pancreatic and acinar specification and the expansion/differentiation of acinar progenitors. Treatment of embryoid bodies with Activin A enhanced the expression of endodermal genes as previously described. Subsequent treatment with Retinoic acid, FGF10 and Cyclopamine, an inhibitor of the Hedgehog pathway, resulted in the enhancement of pancreatic progenitor markers Pdx1, Ptf1a and Cpa1 but also of those expressed in the hepatic lineage, which were reduced by BMPs inhibition. Cells were further cultured in Matrigel using a 3D culture system in the presence of follistatin, dexamethasone, and KGF leading to a significant enhancement of the mRNA and protein levels of acinar markers while decreasing the expression of endocrine ones. Moreover, active Amyl was released into the medium. These data indicate that the selective activation of the acinar differentiation program in ES cells can be achieved by stepwise induction

of signaling pathways involved in pancreatic exocrine development providing a potential tool for studying pancreatic differentiation and pancreas-related diseases.

INTRODUCTION

I. The Pancreas

Histology

The mammalian pancreas, etymologically “all meat” (from Greek: Πανχρεας, pan: all; kréas: meat), is retroperitoneal and is associated with the alimentary tract. It lies beneath the stomach and is connected to the small intestine at the duodenum (*Figure 1.1.A-B*).

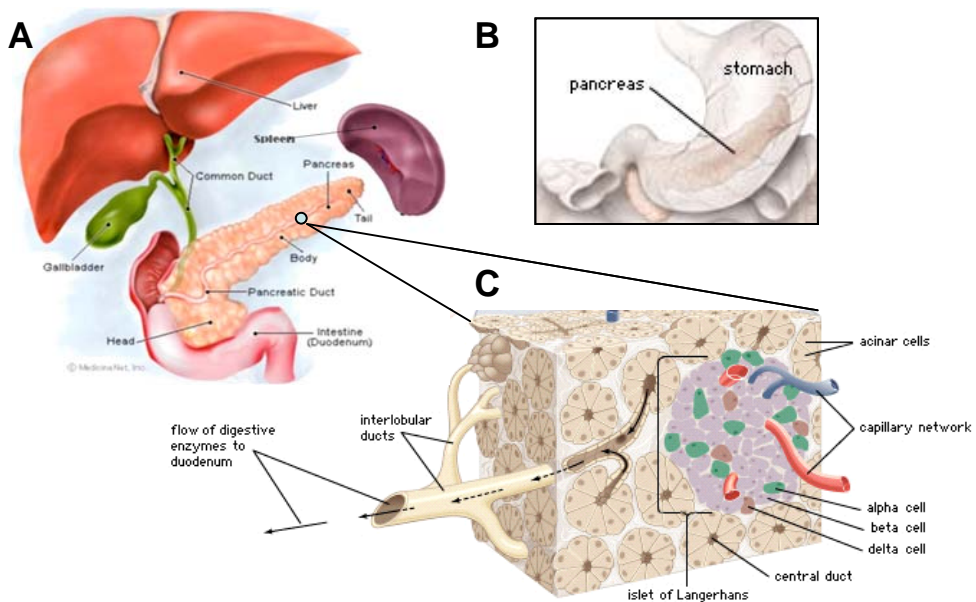


Figure 1.1. Schematic views of the pancreas. Anatomic view of the pancreas and its surrounding organs are shown in (A). To clarify the pancreas situation, the stomach was missed but it is shown in (B). The pancreas is an elongated organ from the duodenum to the spleen. Macroscopically, it is a pinkish tan organ that appears distinctly lobulated to the unaided eye. The investing connective tissue of the pancreas provides the septation to produce the macroscopic lobules. In (C), a schematic view at the cellular level of the exocrine and endocrine pancreas.

Adapted from www.cancersociety.org/pancreaticcancer.html.

The mammalian pancreas is a heterotypic gland, composed by an exocrine and endocrine compartment, playing a central role in foodstuff digestion and glucose homeostasis, respectively.

The Endocrine Pancreas

Within the mammalian pancreas, the endocrine compartment is organised in islets of Langerhans scattered throughout the exocrine tissue, which represent only 1-2% of the total volume. They are constituted by five distinct hormone-expressing cell types: α -cells, β -cells, δ -cells, Pancreatic Polypeptide (PP, or ϕ) cells and ϵ -cells, which produce glucagon, insulin, somatostatin, PP and ghrelin, respectively, and serve as acute regulators of blood glucose concentration (*Figure 1.1.C*).

The Exocrine Pancreas

The exocrine pancreas (*Figure 1.1.C*) consists of acinar cells organized in acini, which produce and secrete digestive enzymes, and ductal cells, which secrete mucus, chloride and bicarbonate, and compose the complex tubular system that drains acinar secretions to the gut. The centroacinar cells are the terminal compartment of the ductal systems and remain poorly characterized although they are thought to be important in pancreatic homeostasis [1].

➤ The Pancreatic Acinar Cells

The pancreatic acinar cell (PAC) is the functional unit of the exocrine pancreas which comprises about 80% of the organ. PACs are responsible for the synthesis, storage, and secretion of the digestive enzymes. Therefore, they are designed for an optimal digestive enzyme hyper production as polarized cells with the nucleus occupying a basal position, opposite to the lumen of the acini, which is the site of high translation of the mRNAs, and with a highly developed rough endoplasmic reticulum allowing their synthesis at high rates.

1. Synthesis and storage of the digestive enzymes

The expression of the genes coding for the digestive enzymes is under the control of a heterotrimeric transcriptional regulator complex called pancreas-specific transcription factor 1 (PTF1), which binds specifically to conserved sequences in their promoters [2, 3]. PTF1 is composed by one ubiquitous class A transcription factors (HEB or E2A), by the tissue-specific bHLH Ptf1a/p48 and another protein, RBPJ or RBPL. The composition of the complex varies during embryonic pancreatic development reflecting different functions of Ptf1a and will be addressed in more detail in the next sections (See chapter III - 2.4).

The produced digestive enzymes are classified as α -amylase, lipases and proteases according to the substrates they are responsible for the hydrolysis (i.e. carbohydrates, fats and proteins, respectively). In addition, RNAses and DNAses form apart of the enzymatic arsenal of the acinar cells. Examples of selected digestive enzymes studied in the context of

pancreatic differentiation include Carboxypeptidase A1 (Cpa1); Chymotrypsinogen 1B (Chymo); Amylase (Amyl), Elastase 1 (Ela1), Carboxyl-Ester Lipase (Cel) and Trypsin 3 (Prss3), among others, which can be used as markers of acinar differentiation due to their high specificity of tissue expression.

Once the digestive enzymes are synthesized in large amounts in the endoplasmic reticulum and in their catalytically inactive form, they are subsequently translocated to the Golgi apparatus. In this organelle, they are segregated into secretory vesicles that still contain immature granules (*Figure 1.2*). After the maturation process - consisting mostly in an acidification of the granules - the vesicles containing the pro-enzymes called zymogen granules are stored (Storage vesicles) near the apical membrane until they are exocytosed in response to secretagogues.

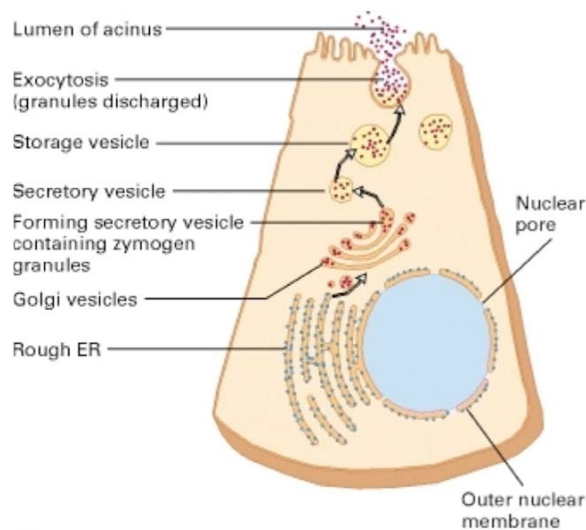


Figure 1.2. Schematic view of a PAC and its ultrastructure. Adapted from <http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=mcb&part=A1182>.

2. Regulated secretion of digestive enzymes

Secretion of digestive enzymes is a regulated process. In response to food ingestion, several neurohormonal regulators (including Cholecystikinin (CCK), secretin, acetylcholine...) are released. Upon binding of these secretagogues to their respective receptors on the basolateral membrane of the PAC, various types of signal transduction pathways are evoked (*Figure 1.3*), but the principal character of these pathways is the intracellular Ca^{++} release, which constitutes the major secretion effector. Furthermore, a cell-to-cell communication between PACs using gap junctions allows the propagation of the stimulation.

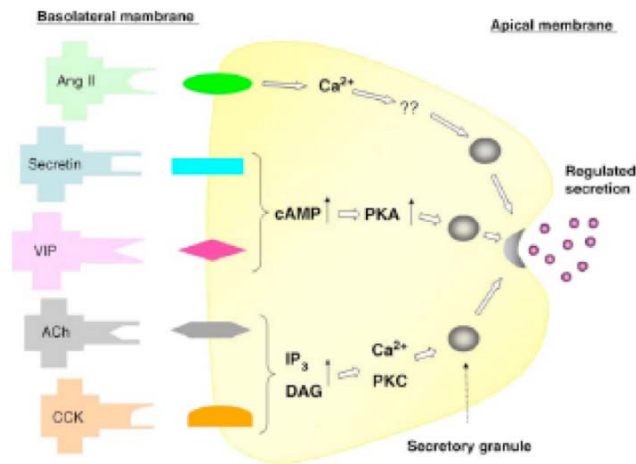


Figure 1.3. Regulated secretion of digestive enzymes by the PAC. Upon stimulation by a host of agonists such as (CCK, secretin, vasoactive intestinal peptide (VIP), acetylcholine (ACh) and angiotensin II (Ang II), signal transduction pathways are evoked. ACh and CCK stimulate acinar cell secretion by activating inositol triphosphate (IP_3)/diacyl glycerol (DAG) signalling pathways, thus leading to increased cytosolic Ca^{2+} and protein kinase C (PKC). Secretin and VIP stimulate secretion by elevating intracellular cAMP and thereby activating protein kinase A (PKA). These intracellular

mediators change the phosphorylation status of structural and regulatory proteins, finally resulting in zymogen secretion into the lumen of acini. Adapted from Leung et al. [4].

The ultimate character of the secretion is the cytoskeleton that is responsible for the close localisation of the zymogene granules to the apical membrane with microtubules, and the proteins involved in the membrane fusion, known as soluble N-ethylmaleimide sensitive fusion protein attachment protein receptors (SNAREs) that form tight complexes between the two interacting membranes: in this case, from the zymogen granule and the apical cell surface. In the PAC, Syntaxin 2, an apical membrane t-SNARE (target membrane SNARE) and VAMP8, the zymogen granule membrane v-SNARE (vesicle membrane SNARE), are the main SNARE actors of the exocytosis of the pro-enzymes into the lumen of the exocrine pancreas. After this step, the retrieval of the vesicular membranes follows.

Once in the intestinal lumen, zymogen activation is performed by several activator enzymes, including trypsin and enterokinase that initiate the digestive process.

II. Mouse pancreatic development: from the egg to the organ

For a better study of the acinar differentiation program in normal and pathologic situations it is important to first understand the fundamental processes that underlie pancreatic acinar development *in vivo*. In the case of the generation of *in vitro* models from Embryonic stem cells (ESC), the information obtained starting at the stage from which ESC are derived is also basic. Most of the knowledge about pancreatic organogenesis comes from studies in model organisms, including mouse, chicken, zebrafish, which is highly conserved among these species.

1. From the epiblast to the pancreatic endoderm

After fertilization, the egg will give rise by mitosis to the morula which forms the blastocyst (*Figure 1.4*). From the Inner cell mass (ICM) of the blastocyst will develop the epiblast which forms the entire proper embryo. The embryonic epiblast undergoes gastrulation and forms the three principal germ layers: ectoderm, mesoderm and endoderm.

All pancreatic endocrine and exocrine cells are derived from the endodermal embryonic epithelium through successive steps involving its patterning, growth and morphogenesis as well as cell specification and differentiation.

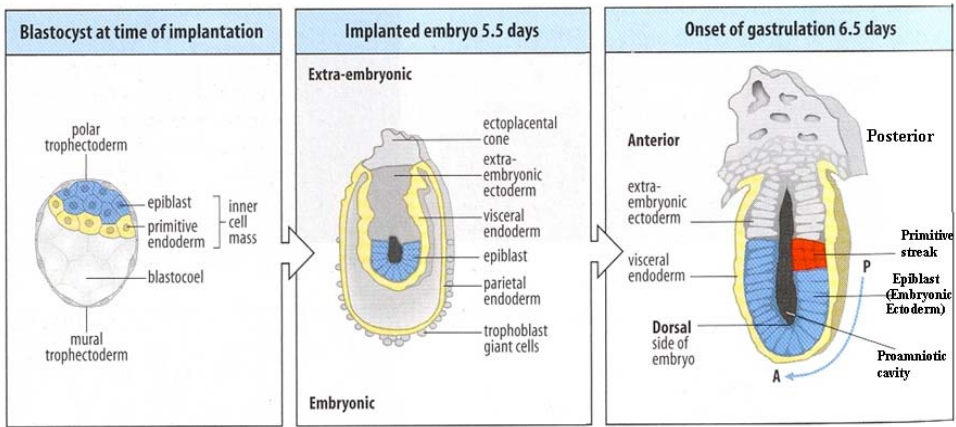


Figure 1.4. Scheme of the early steps of the mouse embryogenesis. In the first panel, the blastocyst is composed by 1) the trophoblast which delimits the blastocyst and will give rise to the placenta, 2) the ICM and 3) a fluid filled cavity called the blastocoel. In the second panel, the trophoblast invades the maternal endometrium as the ICM divides into two layers: the epiblast and primitive endoderm. The latter spreads out and covers the blastocoel to form the yolk sac, an extra-embryonic tissue that produces blood cells. The epiblast further divides into two more layers. The amnion layer (or extra-embryonic ectoderm, ExE) forms the fluid filled cavity to surround and protect the embryo during pregnancy and the epiblast (embryonic ectoderm). In the third panel, gastrulation takes place with formation of the primitive streak. Adapted from Murry et al. [5].

1.1. Gastrulation

The first sign of gastrulation is the formation of the primitive streak (PS) at day E5.5. It is characterized as a furrow in the midline of the embryonic disk at the future posterior end of the embryo. This furrow is formed by the ingression of epiblast cells which will go on to form the mesoderm and endoderm undergoing an epithelial-to-mesenchymal transition (EMT) [6]. There are increasing evidences from studies in fish,

frogs and mice, that the mesoderm and endoderm share a common progenitor referred as mesendoderm. Cell lineage analyses of early to mid-stage mouse gastrula indicate that progenitor cells are concentrated at the anterior PS near the node and that they adopt either a mesoderm or endoderm fate as they migrate through the PS and incorporate into their respective germ layers [7, 8]. The node is the principal source of **Nodal**, a member of the transforming growth factor-beta (TGF β) family of growth factors. The Nodal-related growth factor signalling pathway is required for establishing the anterior–posterior axis by restricting gastrulation to the posterior of the embryo (for review [9]). Nodal subsequently plays a role in promoting mesoderm and endoderm formation (for review [10, 11]). Studies in different species indicate that the dose of Nodal signalling directs a mesendoderm cell into either the endoderm or mesoderm lineage. For example, the use of hypomorphic *nodal* alleles in mouse revealed that high levels of Nodal signalling are required for an endoderm fate whereas lower levels promote a mesoderm fate [12-14]. Given that Nodal activity is additionally regulated through proteolytic processing, secreted antagonists, and by the presence or absence of receptor complexes [15-19], it is clear that establishing the correct dose of Nodal is a critical step in specifying the endoderm.

There is also evidence that the **Wnt** signalling pathway is involved in the mesoderm versus endoderm cell fate choice. Embryos lacking β -catenin, a key effector of the canonical Wnt pathway, have ectopic mesoderm cells forming in the endoderm germ layer [20].

The transcription factors that act downstream of the Nodal and Wnt signals to direct endoderm formation are also remarkably conserved across vertebrate species and include Mix-like homeodomain proteins,

Gata zinc finger factors, Sox HMG (high mobility group) domain factors, and Fox forkhead domain factors. In mouse, the **Mixl1** [21], **Foxa2** [22], **Gooseoid** (Gsc) [23], **Gata4** [24] and **Sox17** [25] were shown to be involved in endoderm formation. Also expression of Brachyury (**Bry**) marks the mesoendodermal cell population but then is directly involved in the early events of mesoderm formation and in the morphogenesis of the notochord [26]. The function of other genes shown to be regulated along the endoderm development, like **Gata3** [27] and **CXCR4** [28] is depicted in *Table 1.1*.

However, many of these transcription factors are not strictly specific of the definitive endoderm (DE) lineage. For example, Sox17, Gata4 and Gsc are also expressed in primitive/visceral/parietal endoderm whereas Foxa2 is also expressed in axial mesoderm [29]. In addition, Gata3 is implicated in epidermal formation [30]. In this sense, current works in the field are devoted to identify new markers of true DE [27].

The epiblast cells that do not migrate through the PS develop into the neuroectoderm and the ectoderm and transcription factors as **Sox1**, **Sox2** and **Zic1** mark specifically those cells [31-33]. In the mean time, Anterior Visceral Endoderm (AVE) is specified in the distal part of the hypoblast and then migrates to an anterior position. The transcription factor **Sox7** marks this extraembryonic endoderm but it is excluded from DE [25].

TF	Onset of expression	Phenotype in knockout mouse
Mix11	E5.5-6.5	Embryonic lethal (e8.5); absence of heart tube and gut [21]
HNF3 β (FoxA2)	E5.5-6.5	Embryonic lethal (e11); lack of foregut formation [34]
Gsc	E5.5	does not display any gastrulation phenotype [35, 36]
Sox17	E6.5	Deficient in gut endoderm formation [25]
Cxcr4	E7.5	Disrupted endoderm migration and gut-tube duplications [37, 38]

Table 1.1. List of selected key transcription factors (TF) involved in endoderm development and their corresponding phenotype in mutant mice.

1.2. Endoderm patterning

During gastrulation, the endoderm is composed of multipotent cells organized as a flat sheet on the outside of the mouse embryo. At the completion of gastrulation, endodermal cells undergo morphogenetic movements to generate a primitive gut tube (E8.5 in mouse). This tube is divided in three parts along the anterior-posterior (A/P) axes: the foregut, the midgut and the hindgut.

The dynamic nature of foregut morphogenesis brings the endoderm into proximity with several mesodermal tissues that provide patterning signals to establish the presumptive organ domains within the foregut according to their position along the A/P and dorsal-ventral (D/V) axes [39-41] (*Figure 1.5*). Then, evaginations arising from these different domains of the foregut will give rise to various differentiated organs, such as the thyroid, lung, liver and pancreas [42, 43]. In fact, the signals that

control regionalization are inductive signals, and the ability to respond to them is referred to as competence.

Fibroblast growth factor (FGF) [44], **Wnt** [45], **Hedgehog** (Hh) [46] and **Retinoic acid** (RA) [47-49] signalling pathways have been implicated in formation and patterning of the foregut, and are known to regulate the expression of key transcription factors, including Fox/HNF, ParaHox, and Hox factors, which are important mediators of cell fate [50]. The signals that pattern the endoderm during gastrulation and formation of the primitive gut tube generate a “pre-pancreatic endoderm” competent to respond to later pancreatic inductive cues.

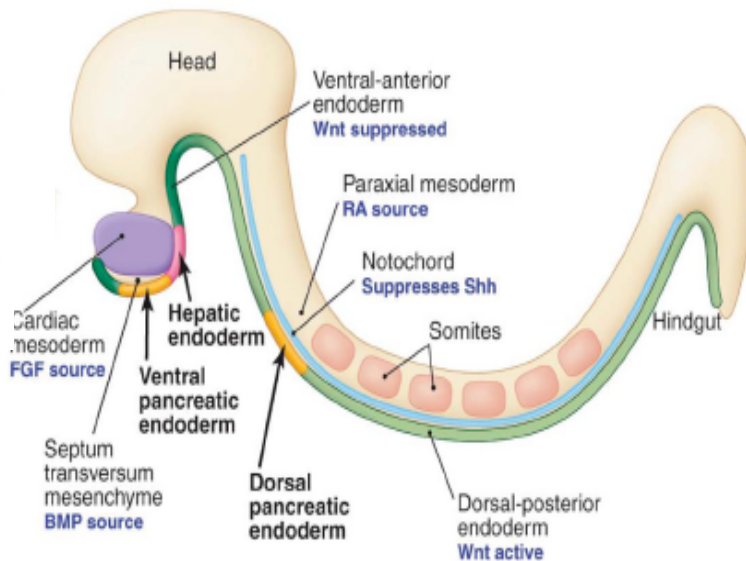


Figure 1.5. Schematic view of a mouse embryo showing the positions of the newly specified pancreas tissue domains. Signals and cell sources that pattern the endoderm are shown. Adapted from Zaret and Grompe [51].

2. Pancreatic development

2.1. Specification of the pancreatic endoderm

As mentioned above, thanks to surrounding inductive tissues the endodermal layer becomes sensitized to be specified into the pancreatic field [52]. In embryos lacking active RA signalling due to absence of the enzyme retinaldehyde dehydrogenase 2 [47], there is a lack of the dorsal pancreatic bud (*Figure 1.5*), due in part to the absence of *Pancreatic duodenal homeobox factor 1 (Pdx1)* (E8,5-E9), which is expressed in this domain even before pancreatic morphogenesis. It was shown recently that the direct binding of *Foxa2*, a marker of the foregut, to the promoter of *Pdx1*, drives its expression [53]. In addition, coincident with the start of pancreas development, *Sonic Hedgehog (Shh)* which is broadly expressed by the endoderm along the A-P axis is specifically repressed in the dorsal and ventral pancreatic buds (*Figure 1.5*). The repression of *Shh* in the dorsal bud depends on signals from the notochord that was brought close to the prepancreatic endoderm by the morphogenetic movements of the forming endoderm gut. Those signals include FGF2 and activin [54]. Deletion of the notochord results in ectopic expression of *Shh* in the dorsal pancreatic bud and loss of pancreatic gene expression [55]. Furthermore inhibition of Hh signalling with the alkaloid cyclopamine (Cyc) causes ectopic pancreatic buds to form in cultured gut tube explants [56]. In the ventral foregut, **Bone morphogenetic proteins (BMPs)** and FGFs promote liver development while concomitantly suppressing pancreatic specification (*Figure 1.5*). Actually, in murine tissue explants containing ventral foregut endoderm, septum transversum mesenchyme

and cardiac mesoderm, noggin treatment, a well known inhibitor of BMPs, blocked hepatic differentiation and induced the pancreatic progenitor marker *Pdx1* [57].

In addition to *Pdx1*, which is necessary for proper pancreas development [58, 59], there are several other transcription factors involved in pancreatic foregut patterning and early pancreas development, including *HNF6* (*onecut1*), *HNF1 β* , *Hlxb9*, and *Ptf1a/p48* (for review, see [60, 61]). The epithelial transcription factor mutants for *Pdx1*, *Ptf1a*, and *Hlxb9* all result in impaired pancreas formation [58, 59, 62-65]. Then, co-expression of *Pdx1*, *Hlxb9*, *Ptf1a*, *Nkx6-1*, and *Nkx2-2* [66] defines the common pancreatic progenitor cells in the epithelium together with *Nkx6-2* and *Sox9* [67, 68]. A summary of the role played by those genes in pancreatic development is presented in [Table 1.2](#). Interestingly, recent work suggests that the final size of the pancreas is determined by the original number of progenitor cells present already at this early stage [69]. Correct specification of the pancreatic epithelium is a prerequisite for pancreas morphogenesis.

2.2. Morphogenesis of the pancreas

Pancreas originates early in development, at E8.75 to E9.5 by the growing of two buds (ventral and dorsal) of cells from a specialized pre-patterned endodermal epithelium located in the region of the foregut marked by co-expression of *Ptf1a* and *Pdx1* ([Figure 1.6](#)).

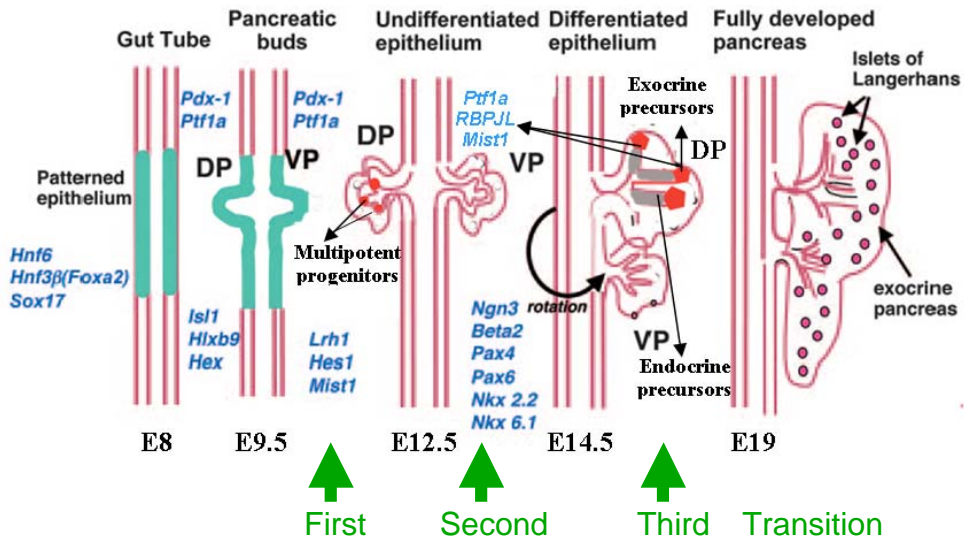


Figure 1.6. Schematic diagram of pancreatic development in the mouse showing the major steps during organogenesis. The representative transcription factors expressed during the program of development are indicated in *blue*. Adapted from Habener et al [70].

By E10.5, the partially differentiated epithelium of the two buds undergoes branching morphogenesis into a ductal tree that by E12.5 results in the formation of two primordial pancreas organs, consisting predominantly of an undifferentiated ductal epithelium (First developmental transition). Zhou et al. proposed that one type of multipotent pancreatic progenitor resides specifically at the branching tips of the growing pancreatic tree [71]. This multiprogenitor is able to give rise to the three cell types of the pancreas: exocrine, endocrine and ductal cells and can be recognized by a combination of markers: $Pdx1^+$, $Ptf1a^+$, $cMyc^{\text{high}}$ and $Cpa1^+$ (*Figure 1.7*).

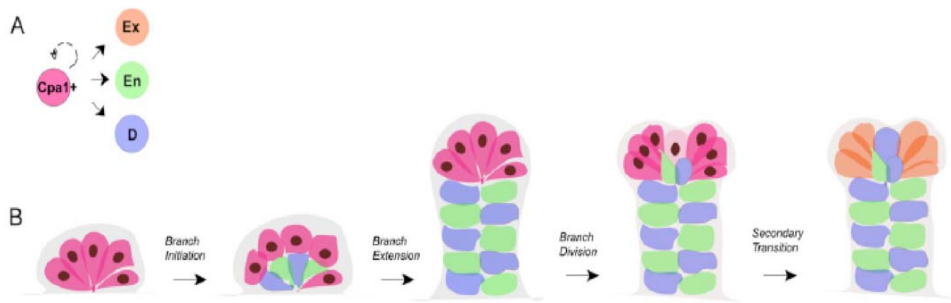


Figure 1.7. Multipotent Progenitors Guide Pancreatic Organogenesis. (A) $Cpa1^+$ multipotent progenitors give rise to exocrine, endocrine, and duct cells *in vivo* and may undergo limited self-renewal. (B) Early pancreatic buds are composed primarily of multipotent progenitors. At the onset of branching morphogenesis (E12), continued fast proliferation and differentiation of these progenitors into endocrine and duct cells generate the trunk of the branches. When the branching tip divides, $Cpa1$ down-regulates in the cleft region. Around E14, $Cpa1^+$ tip cells restrict to exocrine fate during the secondary transition. From Zhou et al. 2007 [71].

Between E13 and E15, the dorsal and ventral pancreases rotate and fuse into a single organ. During this time, which is named secondary transition (*Figure 1.6*), the $Pdx1^+$, $Ptf1a^+$, $cMyc^{high}$ and $Cpa1^+$ tips cells lose their “multiprogenitor” ability and are only able to give rise to exocrine cells and a wave of exocrine and endocrine cell differentiation begins. On E15.5, acini are histologically clearly discernible from ducts. In time, the expression of digestive enzymes starts to increase but is not regulated as a single genetic module, thus displaying specific regulatory patterns among the diverse enzymes [72, 73]. In the meantime, endocrine progenitors detach from the epithelial trunk. On E16, the endocrine cells begin to organize into islet-like clusters. The islets are not fully formed until shortly before birth on E18–E19 and undergo additional remodelling and maturation for 2–3 weeks after birth. On the other compartment, the

acinar cells follow their cytodifferentiation and proliferation even if the rate of proliferation decreases steadily from E13 to birth [74]. Thus, they increase their capacity for secretory enzyme synthesis, the amount of rough endoplasmic reticulum and Golgi apparatus, and the accumulation of zymogen granules (Third transition / *Figure 1.6*). These changes continue throughout foetal life as well as after birth once suckling begins.

2.3. Signalling pathways involved in pancreatic development

Associated to these morphogenetic changes within the pancreatic epithelium is the activation of specific signalling pathways that have in many cases multiple roles during pancreatogenesis. It remains still largely unknown how these routes cooperate and interact to regulate in proper time organ formation. Some of the signaling routes, which are known to play important roles in several steps of pancreatic development, are indicated in the following paragraphs:

During bud formation, **FGF10** is expressed in the mesenchyme, and FGF10-deficient mice exhibit severe growth retardation of the pancreas [75], whereas ectopic FGF10 expression controlled by the Pdx1 promoter results in sustained proliferation at the expense of differentiation [76, 77]. Apparently, the effect of FGF10 signalling on the epithelium is at least partly mediated by **Notch** pathway activation [76-78]. Mice deficient in various Notch signalling components all show accelerated differentiation at the expense of proliferation, resulting in pancreas hypoplasia [79-82]. Conversely, overexpression of a constitutively activated form of Notch1 in the pancreas epithelium prevents endocrine and exocrine differentiation [83-86]. Scharfmann's group also studied the role of FGF's in pancreatic mesenchyme-to-epithelium signalling during

branching morphogenesis. They found that in addition to FGF10, FGF1 and 7 forms were expressed in the pancreatic mesenchyme, whereas FGF receptor 2B (FGFR2B), a specific receptor isoform that binds all three of those FGF ligands, was expressed in pancreatic epithelium [87]. They also showed that in mesenchyme-free cultures of E11.5 rat pancreatic epithelium FGF-1, FGF-7 (KGF), and FGF-10 stimulate the growth, morphogenesis, and cytodifferentiation of the exocrine cells. Furthermore, FGF7 provided to E13.5 pancreatic explants enhanced epithelial growth with suppressed endocrine differentiation [88].

In addition, to its early role in regulating epithelium proliferation, the **mesenchyme** was found to be critical for exocrine development, as briefly mentioned above. Indeed, the pancreatic epithelium is contained within a continuous sheath of basement membrane that creates the epithelial mesenchymal interface [89], which was found to be required for the development of ducts [90]. Furthermore, the absence or depletion of mesenchyme revealed that there was a “default” differentiation of pancreatic epithelium toward islets [90, 91]. Further studies revealed that the age and location of the mesenchyme had a primary role in determining pancreatic epithelial fate [92], suggesting a change of soluble or insoluble signals during pancreatic embryogenesis. Li et al. then showed that there were several separable components to the mesenchymal effect [93]. Proximity or contact of epithelial cells to mesenchyme led to exclusively acinar/exocrine differentiation whereas the opposite led to only endocrine differentiation.

In the last years, few signalling pathways have been identified as the mesenchyme mediators guiding specifically acinar specification and/or differentiation. Consequently, limited information is currently

available regarding the molecular mechanisms involved in these decisions.

Among them, **laminin-1**, as a component of the extracellular milieu, has been shown to play a role in pancreatic duct formation [94]. In line with this, Matrigel, whose major constituent is laminin-1, was also found to induce duct formation in isolated E11 mouse pancreatic epithelium [90]. Later on, Li et al., showed that laminin-1 was a key mediator of the pro-acinar effects of the mesenchyme [93]. Nonetheless, laminin-1 has also been shown to have a role in β -cell differentiation slightly later in gestation [95].

Another piece of the mesenchymal arsenal for regulating pancreas formation is **folliculin** (Fol). The activin-folliculin system plays an important role in the intrinsic regulation of pancreatic branching morphogenesis and for the determination between exocrine and endocrine balance. Activins are expressed in early gut endoderm, and also in early pancreatic rudiment and then localize to the developing endocrine pancreas [96]. Inversely, Fol, an activin-binding protein that acts as an antagonist, is expressed by early pancreatic mesenchyme [91]. Exogenous activin profoundly alters epithelial branching morphogenesis; it causes severe disruption of normal lobulation patterns of the embryonic epithelium *in vitro* whereas Fol counteracts its effect [97]. When supplied to E12.5 rat pancreatic rudiment cultures (without mesenchyme), Fol is able to replace the pro-exocrine/anti-endocrine effects of the mesenchyme [91].

Glucocorticoids belong to another family of molecules involved in exocrine/endocrine differentiation. Early studies found that these hormones increase the protein to DNA ratio, the specific activities of

Amyl, Chymo and Cpa1, and the volume density of zymogens granules when E13 rat foetal pancreatic rudiments were incubated with dexamethasone (Dex) for 7 days [98, 99]. Furthermore, Van Nest et al. demonstrated that Dex enhances Amyl levels and suppresses insulin ones in E14 rat pancreas [100], being later supported by the observation that Dex has similar pro-exocrine action on rat acinar AR42J cells [101]. More recently, Breant's group confirmed that the *in vitro* treatment of embryonic rat pancreas with Dex increases the differentiated acinar cell area and decreases the number of differentiated β -cells, without affecting the number of precursor cells [102]. Lastly, using different transgenic mice mutants of the Glucocorticoid Receptor (GR) they showed that this pathway is not required for early steps of pancreatic development before E15.5 but is important for exocrine versus endocrine cell fate choices and after birth for modulating pancreatic exocrine maturation [103-111]. Moreover, it was suggested that glucocorticoids and **thyroid** hormones have a cooperative action on acinar development [112]. In this sense, administration of both **3-3-5 triiodo-L-thyronine** (T3) and corticosterone (another glucocorticoid) to adrenalectomised suckling rats elevates enzyme activities to a greater extent than either treatment alone. In addition, several groups demonstrated the role of T3 on the proliferation rate of post-natal acinar cells [113, 114] through the TR [113].

2.4. Transcription factors involved in pancreatic development

Signals provided by surrounding tissues in turn initiate the expression of defined transcription factors (*Figure 1.9*), which form regulatory gene networks that conduct embryonic development and differentiation. In these sense, many of them serve dual functions in

determining early specification and later in maintaining the differentiated phenotype, so they display dynamic patterns of expression.

Pdx1: Lack of *Pdx1* results in pancreatic agenesis although a rudimentary dorsal bud persists, which shows that *Pdx1* is not required for initial specification of the pancreatic domain [59]. Nonetheless, all the pancreatic epithelial cells are derived from *Pdx1*-expressing progenitor cells as shown by lineage tracing experiments [115, 116]. It was also found that *Pdx1* makes the pancreatic epithelium able to respond to the mesenchymal growth-promoting signals [117]. In the early pancreas, *Pdx1* is expressed throughout the epithelium, but then is suppressed in cells as they commit to the endocrine lineage [118], or to ducts [116, 119], but it is necessary for pancreatic exocrine formation [120]. When endocrine cells begin to differentiate toward the insulin-positive β -cell lineage, *Pdx1* reappears, and is known to be necessary for proper glucose responsive regulation of insulin synthesis in β -cells [121, 122]. Low *Pdx1* expression persists in other endocrine cell types, acinar cells and ductal cells [123, 124]. In acinar cells, *Pdx1* cooperates also with the PTF1 complex in the regulation of acinar gene expression [125].

On the other hand, a subset of *Pdx1*⁺ cells express *Ngn3*, which likely marks endocrine progenitors [116, 126]. These cells do not express the endocrine hormones until they are competent to express *Pax6*, being therefore fully committed to the endocrine lineage [127] (*Figure 1.9*). Other downstream transcription factors involved in islet cell development are also shown in *Figure 1.9*.

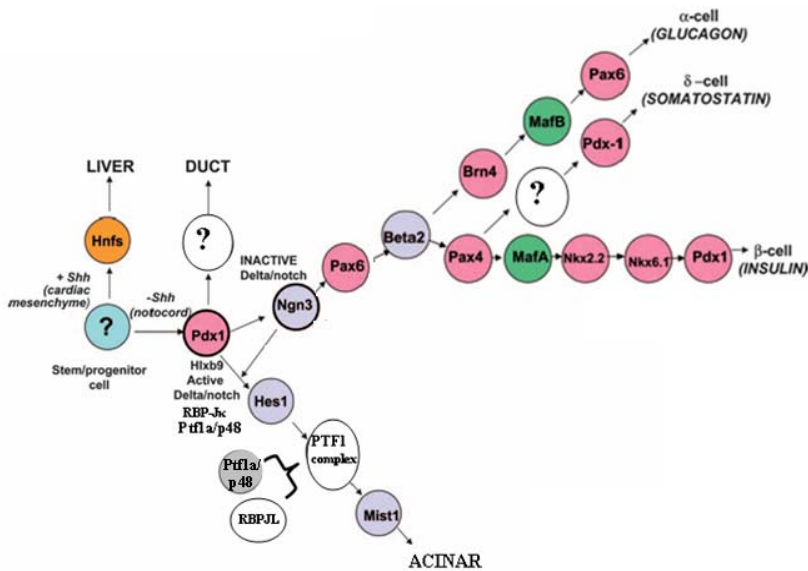


Figure 1.9. Cascade of transcription factors during pancreatic development. The colour scheme designates the type (class) of transcription factors involved in the proposed hierarchical cascade: pink, homeodomain proteins; grey, bHLH; green, Maf proteins; orange, Hnfs. Adapted from Habener et al. [70].

***Ptf1a/p48*:** *Ptf1a* is required for pancreas formation as in its absence there is a compromised exocrine as well as endocrine cell formation and redirection of the fate of the pancreatic progenitor cells to become duodenal epithelium [65, 128]. In addition, Fukuda et al. demonstrated with an *ptf1a* hypomorphic mutant mouse that the correct dosage of *ptf1a* is critical for endocrine versus exocrine determination and that low levels of *Ptf1a* are necessary for endocrine specification unless high levels specify exocrine fate [129]. Thus, it appears to play an important role in early specification of pancreatic progenitor cells and later, in regulating exocrine differentiation.

Indeed, Masui et al. showed that early in pancreatic development and until E13.5, active Ptf1a requires interaction with RBPJ κ , the vertebrate Suppressor of Hairless, within the stable trimeric complex PTF1 [130]. Furthermore, a single amino acid change in Ptf1a that eliminates its ability to bind RBPJ κ causes pancreatic development to truncate at an immature stage, without the formation of acini or islets. These results indicate that the interaction between Ptf1a and RBPJ κ is required for the early stage of pancreatic growth, morphogenesis, and lineage fate decisions. The defects in pancreatic development phenocopy those of Ptf1a-null embryos. Notably, RBPJ κ action in the PTF1 complex is independent of its role in Notch signalling.

Between E13.5 and E16.5, Rbpjk is swapped for RbpjL, the constitutively active, pancreas-restricted paralog of Rbpjk. In fact, the *Rbpjl* gene is a direct target of the PTF1 complex. At early stage of pancreatic development, the PTF1 complex contains RBPJ κ and it binds to the *Rbpjl* promoter, inducing *Rbpjl* gene expression [130]. As development proceeds, RBPJL gradually replaces RBPJ κ in the PTF1 complex which in turn binds to *Rbpjl* and appears on the binding sites for the complex in the promoters of other acinar-specific genes, including those for the secretory digestive enzymes. Thus, the strong induction in expression of digestive enzymes like *Chymo*, *Amyl* and *Ela1* during the secondary transition is due in part to the switch of Rbpjk to RbpjL in the PTF1 complex, participating therefore in the maturation of the acini [73]. It is interesting to notice that these events are concomitant with the lost of multipotency of the Cpa1⁺ tip cells suggesting that the switch of Rbpj partner into the PTF1 complex may play a role in this specification into a single lineage. On day E15.5 the levels of RBJL and Ptf1a increase

continually until E18.5, this new PTF1 complex is supposed to be the main regulator of late acinar differentiation and is proposed to be automaintained [131]. In addition, Ptf1a is required for exocrine tissue homeostasis by displaying a strong anti-proliferative activity [132].

Mist1: This bHLH factor is expressed in a wide array of secretory tissues [133] and, in the adult pancreas, is detected only in PAC. During pancreatic embryonic development its expression starts at E10 before acinar cell differentiation. However, it is not required for early exocrine formation but for proper cell polarization and maintenance of acinar identity. Indeed *Mist1* inactivation or inhibition of Mist1 function results in a severe impairment of acinar organization, including loss of gap junctions, structural alterations of secretory granules, and acinar-ductal metaplasia [134-136]. Mist1 is also involved in the regulation of acinar cell proliferation [137]. In this way, Mist1 has a dual role in the development of the exocrine pancreas by controlling cell proliferation and by promoting terminal differentiation.

TF	Onset of expression	Pancreatic phenotype in knockout mouse
Hlxb9	E8	Failure of dorsal bud development; 65% reduction in β -cells in the remaining pancreas [62, 63]
HNF1 β	E8	Ventral pancreas agenesis; atrophic dorsal pancreas [138]
HNF6	E8	Severely reduced field of pancreas-specific endoderm [139]
Pdx1	E8.5	Agenesis of pancreas; initial dorsal bud formation [58, 59, 117]
Ptf1a/p48	E9.5	Complete absence of exocrine pancreatic tissue; pancreatic progenitors assume an intestinal fate [64, 65]
Sox9	E9.5	Conditional inactivation in pancreatic Pdx1+ progenitors impairs organ growth from e11.5; defective differentiation of the exocrine and absence of endocrine [68]
Mist1	E10.5	Severe distortion of acinar cell architecture and polarity; decrease in number and size of ZG; acinar-to- ductal transdifferentiation [134-136]
Ngn3	E9-9.5	Complete absence of endocrine cells and endocrine precursors [126]
Nkx6.1	E9-9.5	Specification of endocrine fate [140] - Complete absence of β -cells [141]
Nkx6.2	E10.5	Pancreas develops normally; coinactivation of <i>Nkx6.2</i> and <i>Nkx6.1</i> reveals <i>Nkx6.2</i> requirement for the normal proliferation of β - and α -cells [142]

Table 1.2. List of selected key TFs involved in pancreas development and their corresponding pancreatic phenotype in mutant mice.

III. Pancreatic Exocrine diseases

1. Pancreatitis

Pancreatitis is an acute or chronic inflammation of the pancreas.

Acute pancreatitis

It is a disease with high incidence (10-20 cases per 100.000 people/year in western countries) defined as an acute condition typically presenting with abdominal pain and usually associated with raised pancreatic enzymes in blood or urine. Thus, it is characterized by pancreatic tissue oedema, acinar cell necrosis, haemorrhage and inflammation of the damaged gland. The major etiologic factors are gallstones and alcoholism. Disordered secretion, including inhibition of apical secretion and enhanced basolateral exocytosis are early features of acute pancreatitis and may be central to disease pathogenesis. Its treatment is mainly based on the care of the pain with analgesic drugs and blockade of gastric acid secretion.

Chronic pancreatitis (CP)

CP is defined as a continuing inflammatory disease of the pancreas characterised by irreversible morphological change and typically causing pain and/or permanent impairment of function. It is characterized by chronic inflammation, progressive fibrosis, pain and loss of exocrine and endocrine function. Histologically, acinar cells are reduced in number and size and replaced by ductal complexes. The major etiologic factor is the alcohol. This disease represents an increased risk to develop pancreatic cancer [143-146].

2. Pancreatic cancer

The vast majority of pancreatic cancers are classified as pancreatic ductal adenocarcinomas (PDACs) [147] which is a devastating disease that results in the 5th cause of mortality due to cancer in western world. It carries a very poor prognosis due to its extreme aggressiveness and the 5-years survival rate is less than 5% [148]. Main risks factors are the tobacco and chronic pancreatitis. PDAC is a genetic disease, being activating mutations in the K-RAS2 oncogene and functional inactivation of tumor suppressor genes such as *CDKN2A (p16)*, *SMAD4* and *P53*, the best characterized genetic alterations. The ductal morphology of PDAC led to postulate that ductal cells were at the origin of transformation. Supporting this hypothesis, PDAC occurs with high frequency in association with dysplastic and hyperplastic ductal lesions [149, 150]. However, this hypothesis has been surprisingly difficult to prove, as direct targeting of oncogenic *K-Ras* to mature ductal cells using the cytokeratin 19 promoter fails to induce preneoplastic lesions (PanIN) or PDAC in mice [151]. On the other hand, the frequent cases of acinar-ductal metaplasia in human [152] and in mouse models using acinar specific promoters suggests also an acinar origin [153-158]. Indeed, activation of *K-Ras(G12V)* in acinar cells can lead to PanIN and PDAC through an acinar-ductal transdifferentiation process, in particular during embryonic development when acinar cells are still immature [158]. Moreover, in the adult acinar cells, *Kras(G12V)* oncogene is unable to promote the full spectrum of PaIN lesions and PAC unless an experimental inflammation (CP-like) is induced [158]. In addition, in chemically induced tumors in mice, an extensive acinar-ductal metaplasia

occurs, further supporting a role of cell plasticity and transdifferentiation in PDAC generation.

IV. In vitro models of pancreatic acinar differentiation

Taking into account that many exocrine diseases are associated to alterations in the acinar differentiation program, the generation of relevant *in vitro* models of exocrine development/differentiation is required for a better understanding of these processes. In addition, these systems will be valuable for drug/toxicological screenings. In this way, several groups tried to set up *in vitro* conditions to culture purified acini, but in few days these cells undergo an acinar to ductal transdifferentiation, losing their functional properties [159]. In addition, they exhibit a limited ability to proliferate thus making difficult to obtain a significant number of cells.

Nevertheless, few established cell lines displaying features of an acinar phenotype were obtained from rodent tumours.

For instance, AR42J cells were derived from azaserine-induced malignant nodules in the rat pancreas [160]. They differ from normal acinar cells in at least three reasons: 1) they proliferate rapidly; 2) they synthesize, store, and secrete digestive enzymes but the regulation of their exocrine function is abnormal, from the emergence of atypical receptors to unusual inositol phosphate metabolism and cytoskeleton disorganization resulting in high constitutive secretion rate and 3) they possess an added neuroendocrine-regulated pathway characterized by voltage-sensitive ionic currents, post-translational processing of peptidic prohormones, and the release of small neurotransmitters.

The 266-6 is a murine acinar pancreatic cell line derived from a tumor induced with an elastase I/SV-40 T antigen fusion gene [161]. These cells retain a partially differentiated phenotype and express detectable levels of a number of digestive enzyme mRNAs.

To face the lack of relevant normal physiological *in vitro* models, our laboratory is currently working on the generation of PACs displaying functional and differentiated features from murine embryonic stem cells, taking the advantage that these cells display the ability to differentiate *in vitro* into many cell types.

V. Embryonic stem cells as a model of pancreatic acinar differentiation

ESCs were established as permanent cell lines from pluripotent undifferentiated cells of mouse [162] and human [163] ICM of the early developing embryo. ESCs show *in vitro* self renewal properties i.e. ability of almost unlimited proliferation, and pluripotency i.e. properties of differentiation into diverse cell types of the organism [164]. High serum composition medium and the presence of the Leukemia Inhibitory Factor (LIF) are the most common method to maintain the undifferentiated state and the self renewal ability of the mES cells in culture [165, 166].

During *in vitro* differentiation, both murine (m) and human (h) ESCs recapitulate early stages of embryonic development [5, 162]. mESCs and hESCs can be differentiated in monolayer, on adhering cell culture dish or in suspension. In this last case, ESCc will form embryoids

bodies (EBs). EBs correspond to a spherical arrangement of ES cells destined to differentiate into precursors of the three germinal lineages: ectoderm, mesoderm and endoderm recapitulating the earliest events occurring during gastrulation. These properties qualify ES cells as unique model to study early embryonic development of mammalian cells *in vitro*. Actually, ESCs spontaneously differentiate *in vitro* in a large panel of cell types resulting in a low percentage of fully differentiated cells in a specific cell type. Consequently, different strategies were used separately or conjugally to enhance differentiation of ESCs to a specific cell type. Those strategies include 1) exogenous expression of cell type specific transcription factors, 2) use of growth factor cocktails to enforce signalling pathways implicated in formation of the cell type *in vivo*, and 3) selection of specific cell type marker expressing cells.

Embryology has offered important insights into key pathways regulating ESC differentiation, resulting in advances in modelling gastrulation in culture and in the efficient induction of endoderm, mesoderm and ectoderm and in many of their downstream derivatives.

In the last decades several protocols using those strategies were established in order to obtain a new source of insulin producing cells (IPCs) that could be used in the treatment of type I diabetes but none of them really succeed yet. Actually previous experiments aiming to differentiate ESCs to IPCs were initiated by complex culture media, and the addition of several growth factors, such as insulin, laminin, nicotinamide and Foetal Calf Serum (FCS) [167-178]. Most representative studies of these approaches are shown in [Table 1.3](#).

Publication	Type of ESCs	Strategy used
Lumelsky et al. [172]	mES	Nestin ⁺ selection
Blyszczuk et al. [179]	mES	Pax4 overexpression + 20%FCS
Blyszczuk et al. [180]	mES	Nestin ⁺ selection+Pax4 overexpression
Vaca et al. [181]	mES	SN+Selection of <i>pInsulin-neomycin</i> ^r
Lavon et al. [182]	hES	Pdx1 and Foxa2 overexpression+ SD
Treff et al. [183]	mES	Doxycycline-inducible Ngn3+ SD

Table 1.3. Summary of some studies using ES cells to generate insulin-producing cells. SN: Supernatant obtained from the culture of E16.5 foetal pancreases. SD: Spontaneous Differentiation.

Nevertheless, in 2006, Beatge and colleagues proposed a new form of protocol for differentiation of hESCs into IPCs that relied on the *in vitro* recapitulation of the different steps that lead to the IPCs *in vivo* through endoderm formation, pancreatic specification and endocrine differentiation (*Figure 1.10*). As endocrine and exocrine cells arise from a common pancreatic multiprogenitor *in vivo*, reproducing first key steps of the protocol for IPC derivation *in vitro* could be a perfect basis to build a differentiation method to direct ESCs into pancreatic acinar like-cells (PAC-like).

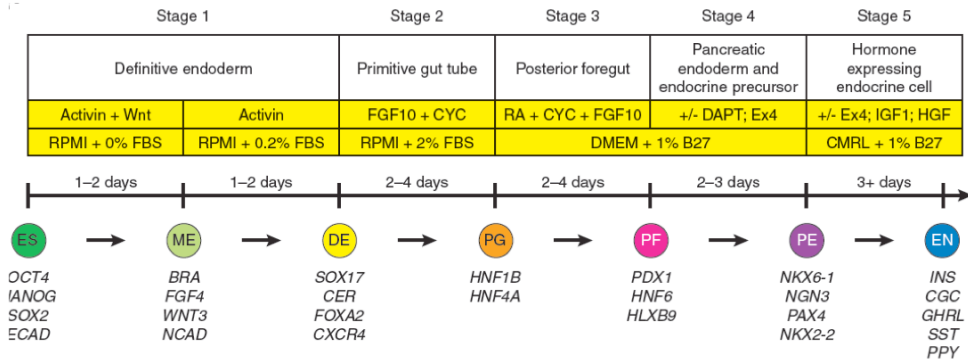


Figure 1.10. Scheme of differentiation procedure of ES *in vitro* and gene expression for some key markers of pancreatic differentiation. The differentiation protocol is divided into five stages and the growth factors, medium and range of duration for each stage are shown. This protocol orchestrates differentiation through five identifiable endodermal intermediates in route to production of hormone expressing endocrine cells. Several markers characteristic of each cell population are listed. CYC, KAAD-cyclopamine; RA, all-trans retinoic acid; DAPT, γ -secretase inhibitor; Ex4, exendin-4; ES, hES cell; ME, mesendoderm; DE, definitive endoderm; PG, primitive gut tube; PF, posterior foregut endoderm; PE, pancreatic endoderm and endocrine precursor; EN, hormone-expressing endocrine cells. Adapted from d'Amour et al. [184].

Endoderm induction

In vivo, BMP4, Wnt and Nodal signalling were shown to be involved in both PS and mesendoderm formation. In ESC cultures, BMP4 and Wnt signalling induced the formation of a PS-like population, monitored by the induction of Bry expression, as well as the subsequent development of a Flk1⁺ mesoderm cell population [185-188]. Then, incubation with **Activin A** -a member of the TGF β superfamily - induced a PS-like population and, depending on the strength of the signal, the subsequent formation of endoderm or mesoderm, as it occurs *in vivo*

through Nodal signalling [189], thus showing that the same pathways are involved in the differentiation of ESCs to endoderm *in vivo* and *in vitro*.

Nevertheless, successful formation of DE by Activin A treatment is significantly affected by different parameters, such as contaminating constituents in culture media during Activin A application, Activin A concentration, time and duration of treatment, and differentiation models used. Actually Kubo et al. demonstrated that FCS at high level negatively influences endoderm induction by Activin A [189]. Since then, Activin A treatment has been performed in chemically defined medium [190], or in low FCS medium [184, 191-193], or with knockout replacement serum (KSR) [189, 194]. Later on, Mclean et al. demonstrated that insulin / insulin-like growth factor (IGF) contained in FCS or KSR, respectively, were inhibiting the Activin A-dependent DE induction through activation of the phosphatidylinositol 3-kinase (PI3K) pathway [194]. They showed that inhibition of the PI3K pathway by the LY 294002 compound led to up-regulation of mesendoderm (*Bry*, *Mixl1*) and endoderm (*Gsc*, *Sox17*).

As we commented previously, the concentration of Activin A is a key parameter in DE induction. In fact it was established that concentrations higher than 30 ng/ml and up to 100 ng/ml are sufficient to induce DE in mESCs [189] and hESCs [195], and can be monitored by a decrease in neural specific *Sox1* and an increase in endoderm-specific *Gsc*, *Foxa2* and *Mixl1* transcript levels [195]. Consequently, in most of the recent studies high level of Activin A (100 ng/ml) was used to differentiate ESCs into DE [184, 191, 192, 194, 196-199].

Also, the time of Activin A application during *in vitro* differentiation was found to be critical for induction of ESCs into DE. While Activin A application starting from the beginning of *in vitro*

differentiation in *monolayer* was successful for hESCs to differentiate into DE cells [184, 191, 192, 194, 198, 199], the time of initiation/incubation in the case of mESCs was more complicated to establish. Indeed, in these differentiation studies the time and duration of Activin A application vary substantially and depend on the culture system used, EB versus monolayer.

Nevertheless, in mESC cultures, DE induction through EB formation is widely carried out by starting application time of Activin A varying from 1 [27], 2 [193, 200] or up to 4 or 5 [201] days after EB formation. In addition, supplementation is performed at 50 [200, 201] or 100 [196] ng/ml for 1 [196], 2 [200], 3 [197] and up to 7 days [27]. However, mESC was also successfully derived into DE in monolayer with 10 ng/ml Activin A [190, 202].

Furthermore, the extent of DE induction by Activin A can be affected by additional parameters such as exogenous signalling molecules.

For instance, in many assays, **Wnt3a** was supplemented within the first days of Activin A treatment in order to increase the efficiency of mesendoderm specification and the synchrony of DE formation [184]. Despite that BMPs were shown *in vivo* to induce first a PS-like population and afterwards a mesoderm population, it was found to be detrimental in Activin A mediated DE formation *in vitro* [203] while their inhibition with Noggin during Activin A treatment led to the opposite effect [203]. In addition, sodium butyrate, an activator of histone hyperacetylation involved in induction of differentiation and induction or repression of gene expression, enhances Activin A-mediated DE formation but treatment with this drug alone has any effect [204].

Additionally, endogenous signals produced by the different lineages emerging from the EBs can modulate the response to Activin A by target cell populations.

Thus, it has been demonstrated that in human EBs, cells from the extraembryonic endoderm (ExEn) produce platelet-derived growth factor B (PDGF-B) that negatively affects the mesendoderm lineage because it selectively expresses the PDGFRA receptor [205]. Therefore, any culture conditions or signalling molecules that would favour ExEn formation could have a negative effect on endoderm specification by inhibiting the mesendoderm population.

In a different approach, some studies try, in one hand, to identify new molecules that could enhance DE formation and in another, to find out new specific markers of DE using reporter cell lines. As an example, a mESC line was genetically modified and harbours an *Gsc::GFP/Sox17::cd25* construct [190]. This cell line expresses GFP under the control of the *Gsc* promoter and *cd25* (transmembrane protein used as target of fluorescent antibody) under the control of the *Sox17* promoter and was used to sort DE cells (GFP^+ and $cd25^+$ cells) after Activin A treatment, allowing the identification of specific extracellular markers for DE, like CXCR4, that could be useful to purify endodermal populations from genetically unmanipulated ES cells. Using a similar reporter cell line (*Sox17::DsRed*) for DE formation and a high-throughput screening of small molecules, two compounds were identified which displayed higher activity than Activin A and Nodal [206]. As small molecules are usually more stable and easier to produce than protein components, it is expected to ensure a better reproducibility in the production of desired ESC derivatives in a less expensive way.

In alternative ways to enhance DE formation, some studies used successfully the overexpression of specific transcription factors implicated in *in vivo* DE formation demonstrating that the same transcription factors are involved in DE formation *in vivo* than *in vitro*. For example, *Mixl1* was found to play an important role in mesoderm and endoderm formation and/or specification during embryogenesis. Similarly, it was found that overexpression of *Mixl1* in hEBs enhances DE formation at the expense of mesoderm [207]. Moreover, in this model, *Mixl1* regulated the expression of *Gsc* and *Sox17* supporting *in vivo* data that *Mixl1* has a direct role in DE formation.

Actually, overexpression of *Sox17* also enhances DE formation in hESCs [208] but in mESCs it was found to promote ExEn in a monolayer model [209]. Also, it has been suggested that mESCs differentiate in DE more efficiently in the EB model than in monolayer [27]; therefore, it is not known what *Sox17* could promote in the mEB model. This difference in the response of mouse and human ESCs to *Sox17* could be explained by the recent observation that hESCs seem more reminiscent of cells within the epiblast than cells within the ICM [210, 211]. Then, if we consider that hESCs are a step ahead of the mESCs into embryonic differentiation it could explain the differences in the protocols of differentiation into DE between both of them and also could let us think that the DE-induced gene profile could also differ. Moreover, it has been observed that different hESC lines show different ability toward differentiation, some lines showing clear propensity to differentiate into specific lineages [212]. Such clear differences were not yet reported for mESC lines but it is probably also the case, even if mESCs are derived

from established strains and the variability between cell lines should be restrained.

Pancreatic specification

As we described in chapter III-2.1, the correct regulation of the Shh and RA signalling pathways is crucial for pancreatic specification and early pancreas development, being the first one inhibited and the other activated at appropriate time. Later on, FGF signalling is implicated in the proliferation of the pancreatic progenitors at the expenses of differentiation.

Accordingly, d'Amour et al. applied successfully in hESC a combination of Cyc with FGF10 and RA to direct DE induced by Activin A into the pancreatic lineages, showing once again that *in vivo* data can be reproduced *in vitro*. Combined supplementation of Cyc and FGF10 resulted in a clear increase in insulin mRNA levels at the end of the protocol in comparison to the same differentiation procedure without this treatment, pointing to the necessity to recapitulate sequentially the major steps of pancreatic embryogenesis (ie. mimic pancreas specification before endocrine differentiation). Likewise, treatment with RA alone led to lower insulin mRNA levels compared to Cyc/RA- treated variants [184, 191], but absence of RA at this step of the protocol led to no appreciable *Ngn3*, *Insulin* or *Glucagon* expression at later stages. Nevertheless, several studies only use Activin A and RA for pancreatic endoderm formation [196, 213].

After this pioneer study, the combination of RA/Cyc/FGF10 has been extensively applied in pancreatic differentiation protocols using

variable concentrations of these molecules. For instance, FGF10 is usually used at 10 to 50 ng/ml, Cyc at 0.25 to 2.5 μ M and RA, between 0.1 and 2 μ M. In addition, the timing for the pancreatic specification step varies between 2 [195, 214], 3 [191], 4 [215, 216] or 6 [184, 217] days. In general, these studies do not provide data on how the optimal concentration and timing of individual soluble factors were selected. Nevertheless, Johannesson et al. provided data on the regulation of RA receptor gene expression during DE induction by Activin A in hESCs to validate the application time of RA [213].

In a quest for new small molecules inducing pancreatic specification, Melton's group identified Indolactam V (ILV) using a reporter *Pdx1*-ESC line differentiated with Activin A in a high-content chemical screening [218]. ILV seems to act through RA signalling.

Furthermore, FGF signalling has been involved in the A/P patterning of the endodermal gut *in vivo*; high concentrations of **FGF4** promote a posterior/intestinal endoderm cell fate, whereas lower FGF4 levels induce a more anterior/pancreas-duodenal cell fate [219]. However, *in vitro*, FGF4 did not exhibit the same activity on Activin A-derived DE cells as it was unable to induce *Pdx1*⁺ cells [213]. Nevertheless, in combination with RA, FGF4 promoted *Pdx1*⁺ cell survival.

Finally, we have already addressed that pancreas and liver arise from the ventral foregut endoderm and that FGFs and BMPs signalings are responsible for the right specification of both (See III-2.1). Consequently, Noggin was used to differentiate hESCs by combined application of EGF and bFGF, which induced the differentiation of DE into pancreatic exocrine and endocrine cells [204]. Also, Kroon et al. substituted FGF10 (in d'Amour protocol, see [Figure 1.10](#)) by Noggin together with RA and

Cyc to differentiate primitive gut-like cells into posterior foregut-like derivatives [191]. In a similar approach, Mfopou et al demonstrated both the combined functions of endogenous BMPs and supplemented FGF in inducing differentiation of hepatocytes from hES cells and the ability to shift developmental pathways from hepatic to pancreatic cell differentiation as *in vivo* using BMPs inhibitors [220].

Endocrine differentiation

The large majority of the multistep protocols published for pancreatic cells are focused in endocrine differentiation. The most successful protocols commonly include Activin A during the initial stage, but vary widely at the subsequent stages. In order to obtain endocrine cells, and generally IPCs, several signalling molecules have been used, being Exendin 4 [184, 216, 217, 221, 222], DAPT (γ -secretase inhibitor) [184, 217], β -cellulin [217, 221] and B27 [184, 191, 204, 217, 222, 223], the most commonly used.

Moreover, the function of specific endocrine transcription factors on cytokine-induced competent cells has been approached *in vitro*. For instance, *Ngn3* is transiently expressed during pancreatic endocrine differentiation (*Figure 1.9*). To model this fact, Serafimidis et al proposed a multistep protocol which was composed successively by an Activin A-induced DE formation, a FGF10-Cyc-RA pancreatic specification, and then *Ngn3* overexpression by tetracycline (tet-on/tet-off)-inducible systems [214]. Addition of doxycycline (Dox), a tetracycline family member, induces the overexpression of the gene of interest, here *Ngn3*. Thus, the “tet-on/tet-off”-inducible system permits relatively stringent,

reversible (on \leftrightarrow off), quantitative, temporal and spatial regulation of transgene expression [224]. In this study, the proceeding enhanced mESC differentiation towards the pancreas endocrine lineage. This is shown by strong upregulation of endocrine lineage terminal differentiation markers and strong expression of the glucagon, somatostatin and insulin hormones. Then it looks like that the Activin A induced DE formation protocol is not enough to reach the *in vivo* maturation level as overexpression of *Ngn3* is still necessary to enhance some features. Even though, the maturation is not complete meaning that the *in vitro* conditions used to mimic the *in vivo* development are not sufficient and some signals are missing in order to reach the optimal conditions.

Others protocols incorporate an aggregation step via growth in suspension to form multicellular clusters [195, 221] and mimic endocrine cell clustering *in vivo*, but it is unclear whether the IPCs have to reside in 3D structures for their proper functioning and survival after transplantation. Even though independent groups have shown repeatedly that IPCs cells can be derived from hESCs *in vitro*, none of these end-stage products responded adequately to exogenous glucose stimuli to meet the requirement for surrogate β -cells. Based on the observed expression of multiple endocrine hormones per cell, it has been argued that these cells may represent immature endocrine cells that might develop eventually into fully functional glucose-responsive cells.

One successful approach to the treatment of experimental diabetes with hESC-derived cells did not use fully differentiated insulin-producing cells, but a heterogeneous population of cells containing pancreatic endoderm-like tissue [191]. The additional weeks of development *in vivo* that were required before endocrine cells fully differentiated and

normoglycemia achieved in these grafts suggest that the final differentiation process may take longer than allowed for the *in vitro* protocols. Thus, final maturation may simply depend on time.

Alternatively, other undefined factors that are difficult to reproduce *in vitro*, such as vascularisation, and the interaction with adjacent tissues, may contribute to the final steps of maturation of functional endocrine cells. The source of such signals remains unknown, although they do not appear to depend on the normal pancreatic location, since the epididymal fat pad appeared apt at providing those signals [191]. It also remains possible that other undefined hESC derived cells included with the engrafted pancreatic endoderm tissue have assisted the differentiation of progenitors into mature endocrine cells. Further progress on defining these signals may aid efforts to complete the final differentiation steps of mature β -cells *in vitro*.

The different strategies developed to derive β -cell-like cells from hESCs described thus far have yielded relatively small numbers of pancreatic endocrine cells, and the heterogeneous nature of the resulting cell populations could prove problematic. We do not know whether co-transplanting such non-endocrine cells or undifferentiated cells represents a risk to patients. Actually, teratomas can arise in recipient mice that receive unpurified hESC grafts, indicating that the inclusion of a purification step prior to transplantation is desired to dispose of potentially hazardous mitotically active cells.

Exocrine differentiation

Recently, the first Activin A-induced DE formation protocol for exocrine pancreatic differentiation of mESCs was published. It consists in a 4 days-EB formation step in high FCS medium content, followed by EB adhesion on gelatin-coated plates and sequential incubation with Activin A (25 ng/ml), sodium butyrate, and Dex during 2, 5 and 7 days, respectively. At the end of the 18 days-culture period, 6% of Amyl positive cells are obtained after spontaneously differentiation and 17% after the Activin A-induced DE formation protocol [225]. Nevertheless, it is not demonstrated that 1) the protocol induces DE formation and pancreatic specification, 2) the generation of other endodermal lineages whose specification is also regulated by some of these signals, 3) the extent of exocrine differentiation (expression of transcription factors, secretory proteins, etc), as their analysis is basically made by qRT-PCR.

Our group has previously shown that ES cells can express pancreatic acinar markers in a process that recapitulates many aspects of early embryonic pancreatic development [226, 227]. We have demonstrated that ES cells respond to single signals involved in pancreatic development [167, 227] and that the viral mediated co-expression of two key transcription factors, p48 and Pdx1, leads to a selective increase in the proportion of cells adopting an acinar phenotype [228]. To further increase the efficiency of differentiation we have developed several strategies based on i) the optimization of the cell culture conditions consisting in conditioned media (CM) of cultured foetal pancreatic rudiments, ii) the adenoviral-mediated co-expression of p48/Ptf1a and Mist1 and iii) the genetic selection of cells that have

activated an acinar differentiation program via the expression of a puromycin resistance gene under the control of the elastase I promoter. Using this multiple step approach based on the isolation of cells committed to the exocrine lineage, we have developed an *in vitro* system allowing us the purification of cell populations exhibiting distinct transcriptional signatures, proliferative abilities and functional properties [72]. Importantly, these cells do not express an intermediate acinar-ductal phenotype. We have therefore set up for the first time an *in vitro* experimental model suitable for studies of acinar cell differentiation. Nevertheless, the generated cells have not completed their terminal differentiation program and lack cell polarity. In particular, cells co-expressing Ptf1a and Mist1 were found to highly increase the expression of digestive enzymes only expressed early during embryonic development but modestly those expressed at later stages (i.e Amyl, ela2.). In addition, the highest expression of these markers was in general found in cells forming aggregates (not shown), suggesting that 2-D cultures could be a limiting factor for optimal differentiation.

Extracellular matrices (ECM) are a key regulator of normal homeostasis and tissue phenotype [229, 230]. Important signals are lost when cells are cultured *ex vivo* on two-dimensional plastic substrata. Many of these crucial microenvironmental cues may be restored using three-dimensional (3-D) cultures of laminin-rich ECM [231]. Then we hypothesised that using Activin A-induced DE formation protocol followed by the application of adequate soluble factor implicated in exocrine differentiation within a 3D model culture we could direct mESCs into PAC-like.

MATERIALS & METHODS

Statistical analysis

Statistical significance of the experimental data was determined with paired Student *t* test. We used * $p < 0.1$; ** $p < 0.05$ and *** $p < 0.005$.

Culture conditions

ES cell culture and differentiation

CGR8 cells, a feeder independent ES cell line, and its derived Ela-Pur-CGR8, harboring a pEla1-Puro-ires-LacZ [72] construct were routinely cultured at 37°C in a 7.5% CO₂ atmosphere on 0,1% gelatine in ES medium composed of Glasgow's modified Eagle's medium (GMEM, Gibco) supplemented with 10% foetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids (Gibco), 2 mM glutamine, 1% penicillin-streptomycin, and 1000 units/ml leukemia inhibitory factor (LIF; produced in our laboratory). Culture media was changed every day and cultures passaged every two days.

Cell Differentiation was performed by allowing ES cells to aggregate in bacterial Petri dishes ($3,3 \times 10^4$ cells/mL) in GMEM medium supplemented with 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids (Gibco), 2 mM glutamine, 1% penicillin-streptomycin and 3% Knockout Serum Replacement (KSR; Invitrogen) or in 3% fetal bovine serum (Gibco) without LIF. Medium was renewed by EBs sedimentation, discarding the supernatant and replacing by fresh medium. After 7 days, 30–50 EBs were plated in gelatin-coated or

Matrigel-coated 24-wells culture dishes for 12 additional days in GMEM-1% KSR (some variation in the plating have been tested and are commented in the results). Medium was changed every two days.

During the study different soluble factors were used: **Activin A** (100ng/ml; Sigma), **Cyclopamine** (Cyc; 2.5 μ M; Sigma), **Dexamethasone** (Dex; 10⁻⁷M; Sigma), **FGF4** (10ng/ml; R&D), **FGF10** (50ng/ml; Sigma), **Follistatin** (Fol; 200nM; Sigma), **Growth Factor Reduced – Matrigel** (BD), **Insulin Growth Factor-II** (IGF2; 50mg/ml; Sigma), **KGF** (10ng/ml; Sigma), **Retinoic Acid** (RA; 1-10 μ M; Sigma), **3-3-5 triiodo-l-thyronine** (T3; 100nM; Sigma), **(-)-Indolactam V** (ILV; 300nM; Alexis Biochemicals), **mWnt3a** (25ng/ml; R&D).

ExtraCellular Matrix (ECM) Preparation

At confluency, producing matrix cells were incubated 30-45 minutes with 20mM EGTA, 10mM EDTA, 20mM HEPES in HBSS without CaCl₂ and without MgCl₂ (Gibco). After extensive washes with PBS, cells were released in the SN. Subsequently, EBs were plated directly on the decellularised ECM with medium.

Semi quantitative RT-PCR

For semi-quantitative RT-PCR, RNA (0.1-0.2 μ g) was reverse transcribed and cDNAs were amplified using the one step RT-PCR kit (Qiagen) according to the manufacturer's instructions and conditions described in [Table 2.1](#). The sequence of primers is shown in [Table 2.2](#). Products were visualized by ethidium bromide staining after 2 % agarose gel electrophoresis.

Steps	Time	Temperature
Reverse transcription	30 min	50°C
Activation of HotStarTaq DNA polymerase	15 min	95°C
Denaturation	30 s	94°C
Annealing	30 s	55-60°C
Extension	45 s	72°C
Final extension	10 min	72°C
Keep	∞	4°C

35- 40 cycles*

Table 2.1. Semi-quantitative RT-PCR conditions.* For Hprt primers, PCR was done using 25 cycles.

Quantitative RT-PCR

RNA was prepared using the GenElute mammalian total RNA kit (Sigma, St. Louis, MO). Retro-Transcription was performed using the TaqMan RT reagents for retrotranscription. Quantitative PCR was performed in triplicate on an ABI Prism 7900HT Sequence Detector using the quantitative SYBR Green PCR kit (Applied Biosystems, Foster City, CA), and the primers referenced in [Table 2.2](#). The data were processed using SDS 2.1 software and results were normalized to Hprt mRNA levels. After normalization, the samples were plotted relative to the first sample in the data set and the standard deviation of the N independent gene expression measurements (experiments) +/- SEM is reported.

Name	Sequence
<i>Afp</i>	F - AGC CAA AGT GGA GTG GAA AGA C
	R - AAC TCT CGG CAG GTT CTG GAA
<i>Amylase</i>	F - TGG CGT CAA ATC AGG AAC ATG
	R - AAA GTG GCT GAC AAA GCC CAG
<i>Bry</i>	F - CAT TAC ACA CCA CTG ACG CAC A
	R - AGA AGA CGA GGA CGT GGC AG
<i>Cdx2</i>	F - CGA GCC CTT GAG TCC TGT GA
	R - AAC CCC AGG GAC AGA ACC A
<i>CEL</i>	F - CCT ACA AGT TGC CCG TGA AAA
	R - ACA GGG ATG AAG GCC AGG TA
<i>Chymo</i>	F - GCA AGA CCA AAT ACA ATG CCC
	R - TGC GCA GAT CAT CAC ATC G
<i>Cpa1</i>	F - GCC ACG GTA AGT TTC TGA GCA
	R - ACA CCC ACA AAA CGA ATC GC
<i>Cxcr4</i>	F - ATC AGC CTG GAC CGG TAC CT
	R - GGA TCC AGA CGC CCA CAT AG
<i>Cx32</i>	F - CCC ACC GAG AAA ACC GTC TT
	R - AGG CCC GGA TGA TGA GGT A
<i>Ela 1</i>	F - GCT CCA GCT CCT CTT ACT G
	R - GCT TCC TGG CGA CAT TAC
<i>Ela 2</i>	F - AGA CCT ACC GAG TGC TGC TG
	R - GAG GCA AGC TGT CTG GAT GTT
<i>Foxa2</i>	F-GAACTCCATCCGCCACTCTCT
	R-TGGGTGCAGGGTCCAGAA
<i>Gata3</i>	F - TTC GCA GGA GCA GTA TCA TGA
	R - CCA CCT CGA GCT CCT TTG AA
<i>Gata4</i>	F - TGG CGG GAC AGT CAT GAT AG
	R - GGG TGA TGA GGA CAA GGA AGAA
<i>Glucagon</i>	F - CCA CTC ACA GGG CAC ATT CA
	R - CCG GTT CCT CTT GGT GTT CA
<i>Gsc</i>	F-AGAACC GCCGAGCCAAGT
	R- TCCGGCGAGGCTTTTGA
<i>Hnf1β</i>	F - GCC TGC TGC GGA ATG GT
	R - TTG TTC GAT GAT GGG TTG CA
<i>Hnf4α</i>	F - CGG AGC CCC TGC AAA GT
	R - CTA TCC AGT CTC ACA GCC CAT TC
<i>Ins 2</i>	F - CCC TGC TGG CCC TGC TCT T
	R - CCC TGC TGG CCC TGC TCT T
<i>Mist1</i>	F - GAA CAC CCA TGC AGG ACA CAG
	R - CCT GGA AGG CAT TGT TGA G
<i>Myf5</i>	F - CCA ACT CAC AGC CTG CTA CCC
	R - TGG CAA GAC AGT ATT TAC AAC
<i>Ngn3</i>	F - ACA GGC CCA AGA GCG AGT T
	R - GCC GAG TTG AGG TTG TGC AT

<i>Nkx6.1</i>	F – TGT TGG ACA AAG ATG GGA AGA GA
	R – GCC AAG TAC TTC GTT TGT TCG A
<i>Pdx1</i>	F - AAATCCACCAAAGCTCACGC
	R - CGGTCAAGTTCAACATCACTGC
<i>Ptf1a</i>	F - ACA AGC CGC TAA TGT GCG AGA
	R - TTG GAG AGG CGC TTT TCG T
<i>Pla2g6</i>	F – GGG AGA CTC CTG CAT TGA TAG C
	R – TCG AGA GAT GGG CAT GAG ATC
<i>Prss3</i>	F - CCC TAC CAG GTG TCC CTG AA
	R - TGC GGG TTT TGT AGC AGT GA
<i>Rbpjl</i>	F - ACT CCG GTG CCT CTC ATC AG
	R - CTA CGC ACA CCA AGG AAC GA
<i>Rbpjk</i>	F – TGC CAC CTT CGT TCC TGA A
	R – GTG TTC CTC AGC AAG CGG ATA
<i>Sox1</i>	F-CAGACTGGCCTCTTAGACTGAACTT
	R-TCCGAAGCCGAGAAAACG
<i>Sox7</i>	F – CCT GCT GCC AAG TCT GAT GA
	R – CAC GTG CAT TTG GAA GTC AGA A
<i>Sox9</i>	F – CCA CCC CGA TTA CAA GTA CCA
	R – TAG CAT TAG GAG AGA TGT GAG TCT GTT C
<i>Sox17</i>	F – GAA ACT GCA GAC CAG AAG CTA TCA
	R – ACA TGC TGA GGT TTT CCT GTA TTA TTT
<i>Ttr</i>	F – CAG AGT GGA CCA ACC GTT CA
	R – CTG TGC ATC TAC AGC CCT TCA G
<i>Zic1</i>	F – CAG ATG CGG CTA GGT TTC TC
	R – AGC TCT TGC TTG ATG GGT TG

Table 2.2. Primers used in this study for qRT-PCR analysis.

Extraction of AR42J-ECM

After decellularisation of AR42J-ECM, the remaining ECM was extract with 6M Urea, 2%SDS, 10% 2-mercaptoethanol in 62,8mM Tris pH 6.8. The proteins were precipitated with acetone (Over night) and sent to proteomic facility.

Immunocytochemistry (Immunofluorescence (IF))

Cells were fixed with 4% paraformaldehyde (PFA) for 20 min, washed with TBS and incubated with 50 mM NH₄CL for 5 min. After washing

with TBS, cells were incubated with 0.1% saponin, 0.25% TX-100/TBS for 15 min at room temperature (RT) for permeabilization. After TBS washing, cells were incubated with 5% FBS/TBS for blocking non-specific interactions for 45 minutes at RT. Subsequently, cells were incubated with primary antibodies (*Table 2.3*) diluted in 2% FBS/TBS for 1h30 under a humid chamber. After several washes in TBS, cells were incubated with appropriate secondary antibodies (*Table 2.3*) for 45 min in 2% FBS/TBS. Cells were washed again and incubated with To-pro3 (1 μ M) or DAPI (1:2000) during 5 min for nuclear staining. Stained cells were visualized under a Leica (Houston, TX) TCS-SP2 confocal microscope. To estimate the efficiency of differentiation at the cell level, clusters were randomly selected, and the numbers of double positive cells (Chymo-Cpa1 or Chymo-Amyl) were counted at EB7P12. The total cell number was determined by DAPI nuclear staining. Data are given as mean +/- SEM, and were obtained from 2 independent experiments (and from 3 independent coverslips).

Name (target)	Specie	Origin	I.C.C. dilution
Primary antibodies			
Amyl	Rabbit (polyclonal)	SIGMA	10mg/ml
Cpa1	Rabbit (polyclonal)	Biogenesis	1/1000
Chymo	Mouse (monoclonal)	Biogenesis	1/1000
Insulin	Guinea Pig (polyclonal)	Dako	1/20
Glucagon	Rabbit (polyclonal)	Dako	1/20
Rbpjl	Rabbit (polyclonal)	D ^f R. MacDonald	1/100

Pdx1	Mouse	[228]	1/20
Foxa2	Goat (polyclonal)	Santa Cruz (Kind gift Dr. FX Real)	1/100
Afp	Goat (polyclonal)	Santa Cruz	1/1000
Gys2 (Liver)	Rabbit (polyclonal)	Genosys	1/1000
Secondary antibodies			
Mouse-Ig	Donkey IgG- Alexa 488	Invitrogen	1/300
Mouse-Ig	Donkey IgG- Alexa 555	Invitrogen	1/300
Rabbit-Ig	Donkey IgG- Alexa 488	Jackson	1/300
Rabbit-Ig	Donkey IgG- Alexa 555	Jackson	1/300
Guinea- Pig-Ig	Goat IgG Alexa 546	Invitrogen	1/300
Goat-Ig	Chicken IgG Alexa 488	Invitrogen	1/300

Table 2.3. Listing of the antibodies used in the study.

Amylase Secretion assay

Amyl activity was measured using the Infinity™ Amylase Liquid Stable Reagent (Termo Electron Corporation). To detect Amyl secretion, the culture medium of differentiated and control cells were collected and preserved at 4°C, as basal Amyl activity level. Then, cells were washed twice with PBS and incubated with fresh medium supplemented or not with Carbachol (5×10^{-6} M) or with CCK (10^{-12} M) for 45 min at 37 °C.

Then, supernatants were collected; the cells were washed twice with PBS and lysed in Krebs lysis buffer (*Table 2.4*).

Components of Krebs buffer	Final concentration
Hepes	24.5 mM
NaCl	115 mM
Glucose	5 mM
Glutamine	2 mM
Essential Amino acids	1X
Non-Essential Amino acids	1X
KCl	4.8 mM
CaCl ₂ (2H ₂ O)	0.5 mM
MgSO ₄ (7H ₂ O)	4.8 mM
KH ₂ PO ₄	1.2 mM
STI (Trypsin inhibitor)	0.01%
BSA	0.25%

Table 2.4. Composition of Krebs buffer.

To measure the Amyl activity, 25 μ l of supernatants or cell lysates were mixed with 25 μ l of kit reagent and incubated for 30 min at 37 °C. The absorbance was determined at 420 nm and the activity was calculated according to kit instructions. The activity in supernatants shows the activity of secreted Amyl in response to secretagogues, or the constitutive secretion, whereas the activity in cell lysates is related to the Amyl content of the cells. To normalize the amount of Amyl secretion, protein concentration was measured by the Bradford (Bio-Rad) method. Experiments were performed in triplicates.

β -gal staining

Cells were fixed with 4% PFA in PBS for 20 min, then washed with PBS and incubated overnight with a PBS solution containing Potassium ferrocyanide (5 mM); Potassium ferricyanide (5mM); MgCl₂ (2 mM) and X-Gal (1 mg/ml) at 37°C.

RESULTS

I. Formation of pancreatic endoderm (PE) from mESCs

Different protocols have been applied in mESC to generate DE cell population using Activin A. As a starting point, we decided to develop the EB model versus the monolayer method for several reasons: 1) our previous expertise in generating exocrine cells from mESC was based on the EB system [227], 2) several groups have reported a better efficiency in DE formation in the EB model in response to Activin A, 3) we wanted to preserve the 3D integrity maintained during the suspension culture (EB) to directly transfer the differentiating cells in 3D-culture systems, thus avoiding to trypsinize the cells and perturbing cell-cell interactions as it would be the case in monolayer cultures. From this EB model, our aim was to recapitulate sequentially the generation of endodermal cells, the specification of pancreatic progenitors and the expansion/differentiation of acinar progenitors.

1. Activin A induces definitive endoderm formation

Kubo et al, demonstrate that application of high concentration of Activin A on mESC-derived EB culture induces development of DE through mesendoderm formation under low serum concentration [189]. Accordingly we set up a five days protocol including 4 days of Activin A treatment (*Figure 3.1*).

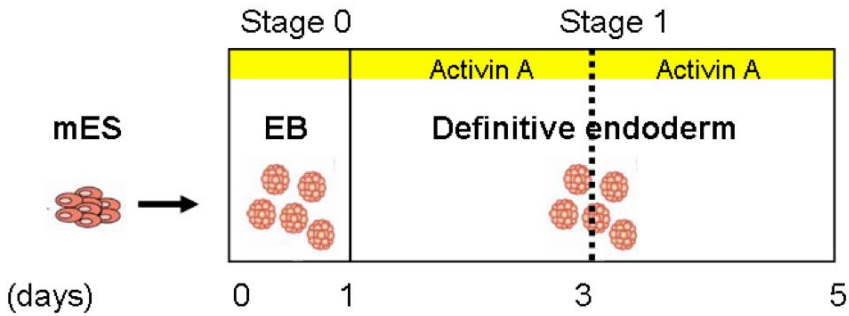


Figure 3.1. Schematic diagram of the differentiation procedure. mESCs were allowed to form EBs in suspension in low KSR medium (3% KSR is represented by the yellow band). After 24 hours, at day1, Activin A was added at 100ng/ml. Then at day3, as described in materials and methods, medium was renewed as Activin A application (dashed line).

After 3 days of culture, we observed by qRT-PCR an increased in the expression of mRNAs encoding for *Gsc* and *Bry* which are expressed in vertebrate mesendoderm in cells treated with Activin A but not in those that were not treated and underwent spontaneous differentiation (Figure 3.2). In addition, the expression of *Sox7* which is expressed in ExEn [25] did not significantly change during this period of time independently of Activin A supplementation (Figure 3.2).

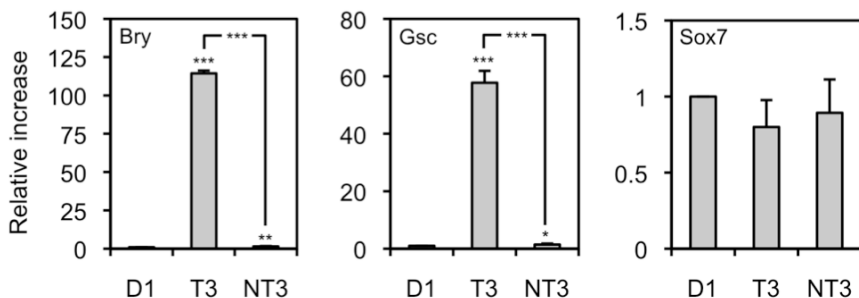


Figure 3.2. qRT-PCR analysis of mesendodermal (*Bry-Gsc*) and extraembryonic endodermal (*sox7*) marker gene expression during stage 1 of the differentiation

procedure in cells cultured during 3 days with Activin A (T3) or without (NT3). T, treated; NT, non-treated. D1 represents the first day of culture. Experiments were performed 4 times independently (N=4).

On day 3, culture medium is renewed as described in Materials and methods, and high levels of Activin A during two additional days promoted the up-regulation *Gsc*, *Foxa2*, *Cxcr4*, *Sox17*, *Gata4* and *Gata3* mRNAs, encoding for a set of markers of the DE (*Figure 3.3*).

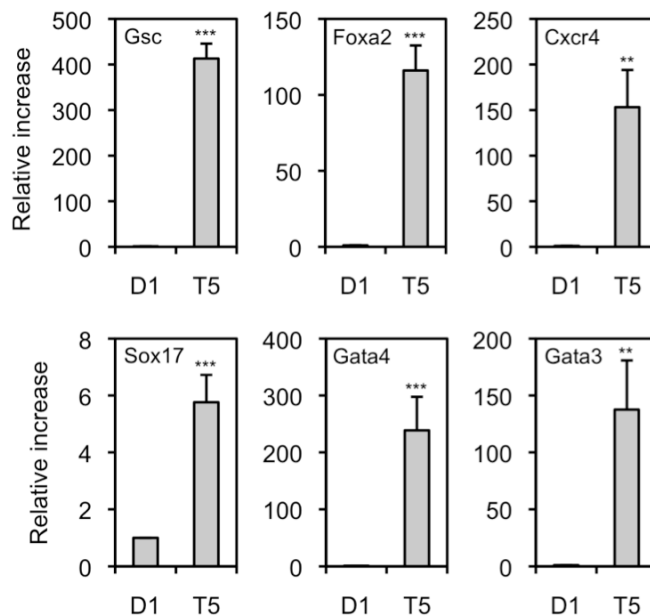


Figure 3.3. qRT-PCR analysis of endodermal marker gene at stage 1 of the differentiation procedure, in cells cultured during 5 days in the presence of Activin A. (N=4).

While other groups have reported that concomitant supplementation of Activin A and Wnt3a during a short time enhances the generation of DE [184, 232], in our hands it did not work when applied at 25 ng/ml from day 1 to day 3.

To analyze whether Activin A-induced gene expression was specific to the endodermal cell lineage we additionally studied the expression of other germ layer markers. Thus, we found now that two days later, from day 3 to day 5, there is a down-regulation of the early mesoderm marker *Bry* (Figure 3.4), which was increased at day 3, suggesting that the cells transit through a mesendoderm step. Conversely, the expression of *Myf5*, a latter mesoderm marker, increased slightly during this period, favouring the notion that the mesendodermal population is preferentially differentiated toward an endoderm fate rather than mesodermal fate.

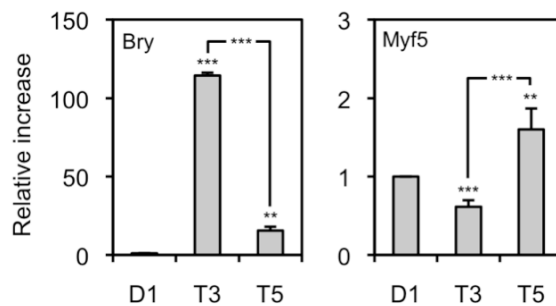


Figure 3.4. qRT-PCR analysis of mesodermal marker gene expression during stage 1 of the differentiation procedure, in cells cultured during 3 days or 5 days in the presence of Activin A. (N=4).

In addition, we found that during spontaneous differentiation (NT condition) (Figure 3.1), only the genes that mark the Neuro-Ectoderm (NE), such as *Sox1* and *Zic1* were highly increased during the 5 days of culture (Figure 3.5), suggesting that this cell population is highly enriched by default. On the contrary, treatment with Activin A lead to a strong reduction in their expression, suggesting an inhibition in the

differentiation of mESCs along the NE pathway as previously described by Vallier et al. [233], and Mclean et al. [194].

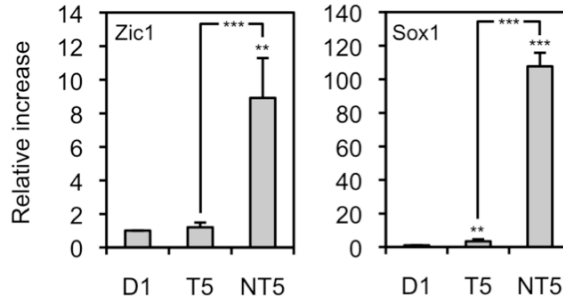


Figure 3.5. qRT-PCR analysis of NE marker gene expression at stage 1 of the differentiation procedure, in cells cultured during 5 days in the presence (T5) or absence (NT5) of Activin A. (N=4).

Consistent with the development of an endodermal cell population, a significant increase in transcripts encoding for the foregut endoderm *Pdx1* and *Hnf1 β* markers was observed between day 3 and day 5 (Figure 3.6).

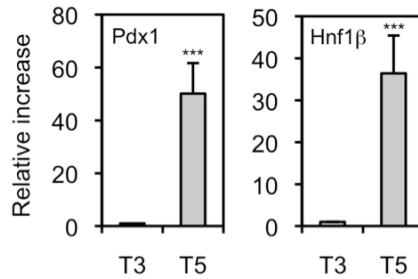


Figure 3.6. qRT-PCR analysis of PE marker gene expression during stage 1 of the differentiation procedure, in cells cultured during 3 days or 5 days in the presence of Activin A. (N=4).

Overall, these results show a specific Activin A-mediated induction of the expression of endodermal markers, leading to the enhancement of those already expressed in the pancreatic endoderm.

2. Combination of Retinoic acid, Cyclopamine, and FGF10 allows PE specification

To further enhance pancreatic endoderm specification, cultures were progressed through stage 2 directly following the stage 1. At this step, culture medium was renewed with a cocktail of factors depleted of Activin A and now supplemented with FGF10, Cyc and RA, in order to promote the specification and proliferation of the pancreatic Pdx1⁺ progenitors cells (*Figure 3.7*).

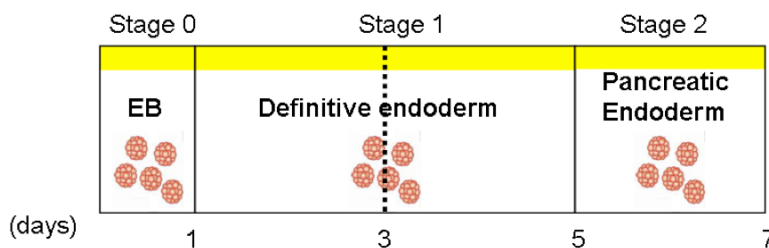


Figure 3.7. Schematic diagram of the differentiation procedure to generate pancreatic progenitors. At day 5, as described in Materials and Methods, medium (3%SR) was renewed and supplemented with 50ng/ml FGF10, 2,5 μ M Cyc and 10 μ M RA for 2 days.

In these conditions, a significant increase in the expression of mRNAs encoding for a set of PE markers such as *Pdx1*, *Ptf1a*, *Hnf1 β* , *Sox9*, *Nkx6.1* and *Cpa1* was observed after two days as shown by qRT-PCR (*Figure 3.8*). This effect was RA dose dependent. Thus, a statistically significant increase was observed for the expression of the

majority of these markers using 1 μM or 10 μM , and the effect was more pronounced at the highest concentration, particularly regarding *Hnf1 β* and *Ptf1a*. Therefore, the combination of 50 ng/ml FGF10, 2.5 μM Cyc and 10 μM RA was selected for further studies and will be referred here in as FCR mix.

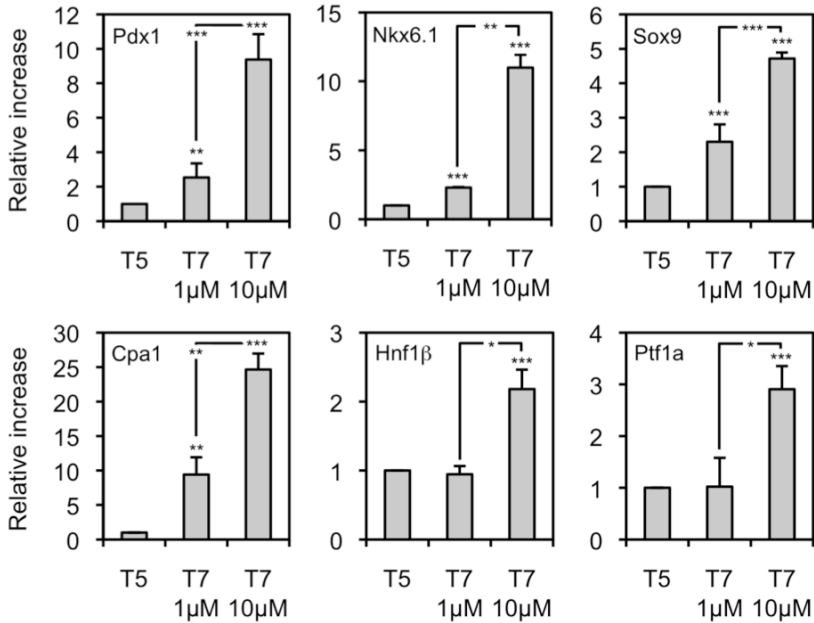


Figure 3.8. qRT-PCR analysis of PE marker gene expression at stage 2 of the differentiation procedure. Comparison of cells cultured through stages 1 and 2 in the presence of 50 ng/ml FGF10, 2.5 μM Cyc with 1 μM RA (T7-1 μM) or 10 μM RA (T7-10 μM) during 2 days was made with cells only cultured until stage 1. (N=3).

Concomitantly, *cdx2* (duodenal marker) mRNAs were barely up-regulated during stages 1 and 2, suggesting that Pdx1 up-regulation, which marks duodenum-stomach-pancreas domains, is rather due to PE formation than intestine formation (Figure 3.9). Conversely liver markers as *Afp* and *Ttr* were highly up-regulated (Figure 3.9), further supporting

that the FCR mix promotes preferentially posterior foregut formation instead of anterior midgut (intestine).

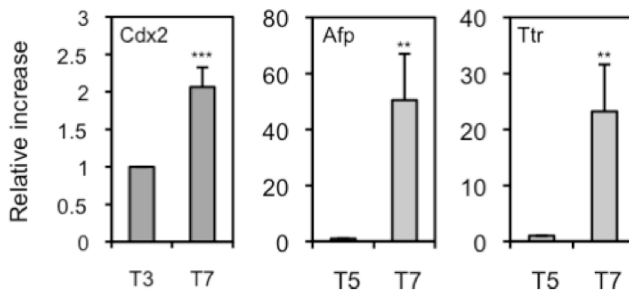


Figure 3.9. qRT-PCR analysis of duodenal and liver marker gene expression, in cells progressing through stage 1 and 2 of the protocol. (N=3).

3. Dorsomorphin inhibits liver marker gene expression

Hepatic and ventral pancreatic progenitors derived from very close portions of the foregut. Actually, defects in BMP and FGF signalings promote pre-hepatic endoderm to a ventral pancreatic fate. We hypothesized that blocking BMP signaling would result in a best specificity of the protocol due to an inhibition/reduction of the hepatic fate. Previous data reported by several groups showed that using Noggin, a BMP antagonist, the hepatic cell lineage is repressed [191, 204, 220]. In our protocol we inhibited this signaling pathway by using **dorsomorphin** (DM), a soluble small molecule known to inhibit BMP signaling [234]. Consequently, as shown in [Figure 3.10](#), addition of DM to the FCR mix resulted in a strong reduction of *Afp* and *Ttr* expression but not on pancreatic markers. In line with a down-regulation of hepatic

markers *Hnf1β* and *Hnf4α* were also reduced as these genes are expressed both in hepatic and pancreatic progenitor cells.

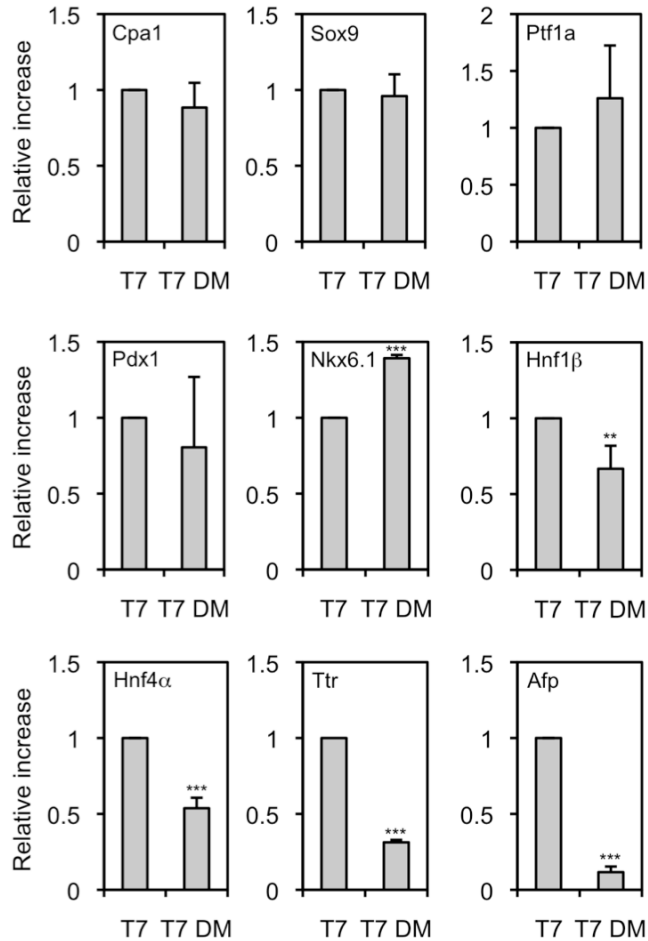


Figure 3.10. qRT-PCR analysis of PE and liver marker gene expression at stage 2 of the differentiation procedure. Comparison was made with cells cultured at stage 2 with the FCR mix (T7) or with the FCR+DM mix (T7DM). (N=3).

On the other hand, while other groups reported that additional supplementation of FGF4 at this step further promotes foregut/PE

specification [213], it did not result in such improvement at the mRNA expression level in our hands.

Overall, we show the development of a short stepwise protocol that promotes the generation of an endodermal cell population and the emergence of pancreatic progenitors while limiting the formation of liver and intestinal progenitors. In the next sections, we investigated new methods to promote the acinar differentiation of these cells. In first attempts, we analyzed the effect of the potential pro-exocrine signals in cultures in where endoderm was generated spontaneously in the presence of FCS or KSR to limit the expensive cost of the experiments using commercial Activin A. In a second time, the selected conditions were then applied in cultures progressing through stages 1 and 2 of the protocol.

II. Induction of pancreatic acinar differentiation

The Exo1 protocol developed previously in our laboratory [72] aims to produce PAC like cells and relies on the genetic selection of cells that have activated an acinar differentiation program based on their ability to express an antibiotic resistant gene (puro^r) and the β -galactosidase gene under the control of the elastase 1 promoter/enhancer, an acinar specific marker (Ela-pur ES) (*Figure 3.11.A*).

At the end of this first protocol, many of differentiated cells were not polarized and were often unorganized in histological structure (acinus). Nevertheless, we observed that clustering of PAC-like cells was frequently associated to an increase in the reporter gene activity (*Figure*

3.11.B). In this sense, the cells expanding around the EB structures on the gelatin showed less expression of acinar markers than the cells organized in a 3D manner inside the EBs (data not shown). These differences could be explained by the lack of 3D organization of the expanded cells and/or lack of ECM production by the expanded cells in monolayer. In order to investigate the role of ECM components on acinar differentiation of mESCs we developed different assays.

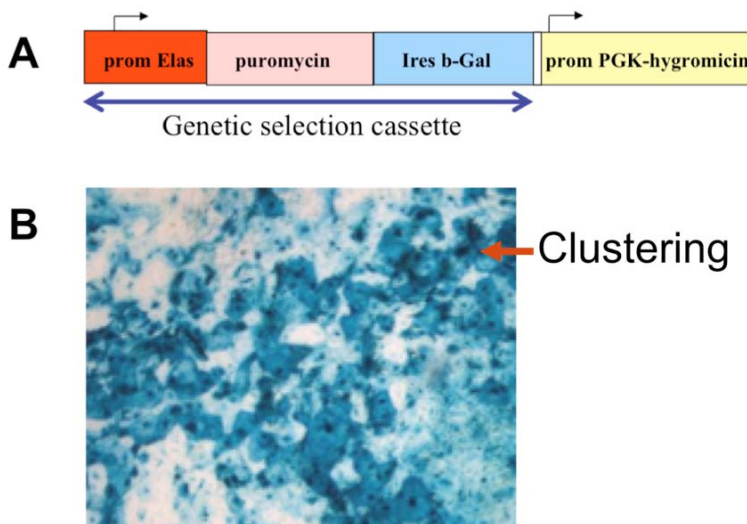


Figure 3.11. Acinar differentiation of mESC using a genetic selection strategy. (A) Schematic representation of the genetic construct of the ela-pur CRG8 mES cell line (B). Differentiated cells obtained with the Exo1 protocol after X-gal staining. B-gal expressed under the control of the elastase promoter hydrolyzes the X-gal that precipitates and turns blue. The positive cells indicate PAC-like cells.

1. Pancreatic Exocrine Matrix

It has been shown that mES cells seeded on complex ECM from human normal fibroblasts (HNF) reconstitute *in vitro* a fully differentiated skin [235]. To that end, confluent HNF culture is treated with an EDTA-EGTA solution that leads to the detachment of the cells from their own produced ECM. The result is a decellularised complex ECM template that is used for seeding ES cells (see M&M). Following this procedure on HNF-ECM, the differentiated cells express cytokeratins, basement membrane proteins and late differentiation markers of epidermis and displayed a histological organization similar to native skin (*Figure 3.12*).

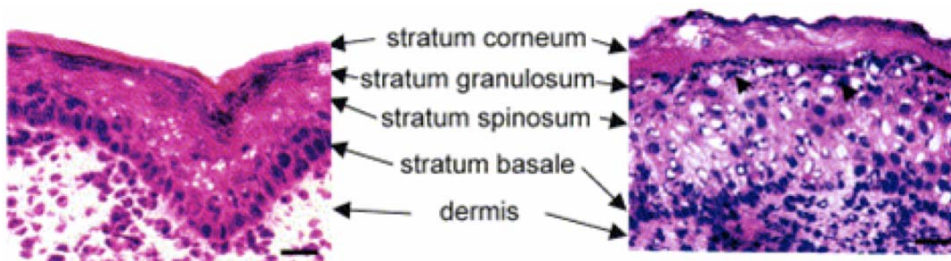


Figure 3.12. Histological aspect of *In vivo* mouse E17,5 embryonic skin (left panel) and ES-derived reconstituted skin (right panel). The 3D organization of the embryonic skin and the ES-derived cells is similar. From Coraux et al. [235].

Importantly, in these assays, the tissue cell origin for the generation of the templates was found to be a critical parameter as those produced by bladder, tongue or mammary epithelial cells were not able to promote ES-derived reconstituted skin.

Therefore we decided to investigate the ability of pancreatic acinar ECM to induce ES cell pancreatic differentiation and generated the templates from the rat acinar AR42J cell line. EB were cultured in

suspension for 7 days (EB7) and seeded on the AR42J-ECM for 7 additional days (EB7P7) in medium supplemented with 10% FBS (Figure 3.13). To ensure the lack of AR42J cells contamination on the ECM templates, they were monitored by crystal violet staining under the microscope.

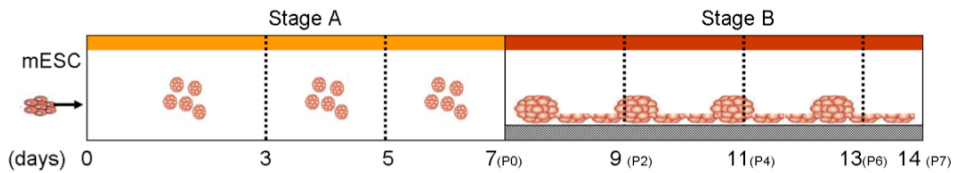


Figure 3.13. Schematic diagram of the differentiation procedure using ECM templates. During Stage A, mESCs were allowed to form EBs in suspension in 3% FBS (orange) during 7 days. Then, EBs were adhered on gelatin-coated or ECM- dishes in 10% FBS (brown). Medium is renewed every two days (dashed line). After 14 days of culture, the expression of acinar differentiation markers is analysed. Days of culture are indicated as well as the corresponding day after plating (in brackets).

Reporter activity performed on EBs plated on the different templates showed that EB seeded on AR42J-ECM expressed more *elastase* than those plated on gelatine or NIH3T3 (Fibroblast cell line), a cell line previously described to promote pancreatic differentiation on embryonic pancreas [93], or on gelatin (Figure 3.14.A). Indeed, not only the intensity of the X-gal staining was higher in the AR42J-ECM condition but also the number of positive cell clusters. In addition, semi-qRT-PCR and qRT-PCR analysis confirmed the increased expression in other pancreatic markers such as *Cpa1*, *Chymo* and *Amyl*, demonstrating that AR42J matrix contains proper signals for driving pancreatic acinar differentiation of ES cells (Figure 3.14.B-C). In line with these results, we found by Immunofluorescence (IF) the formation of large cells clusters

expressing Cpa1 and Chymo, only when EB were cultured on AR42J-ECM (*Figure 3.14.D*). To ensure lack of contamination by remaining AR42J cells after EDTA/EGTA treatment, RT-PCR assays were also conducted using specific rat-*Ptfla* primers that amplified the expected bands on AR42J RNAs but not on EB7P7 RNAs, ruling out that the inductive effect was due to an artefact (*Figure 3.14.E*).

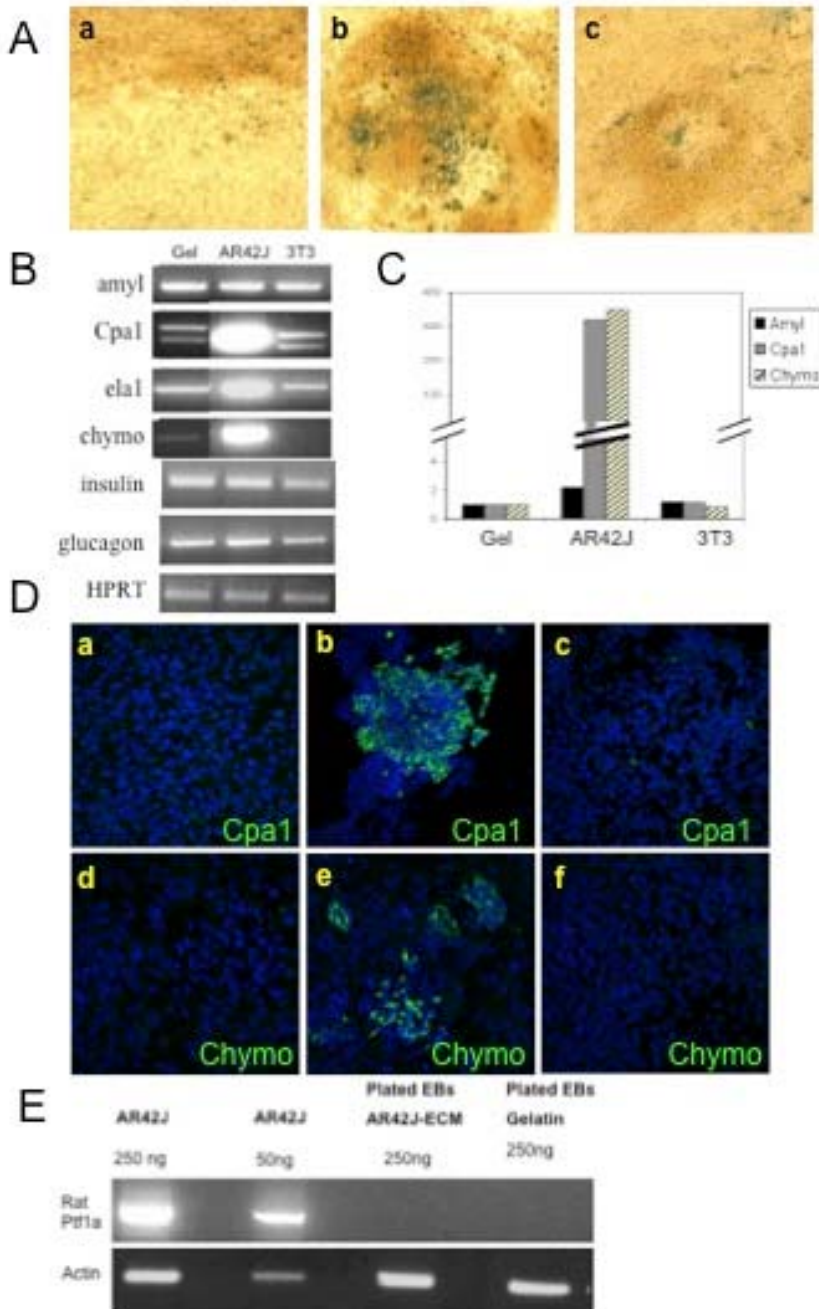


Figure 3.14. Analysis of pancreatic differentiation induced by AR42J-ECM. (A) β -gal assay on EB7P7 cultures on (a) gelatin, (b) AR42J-ECM or (c) NIH3T3-ECM. (B) semi-qRT-PCR of EB7P7 cultures on gelatin (Gel), AR42J-ECM (AR42J) or NIH3T3-ECM

(3T3) for the indicated pancreatic markers. HPRT was used for normalization. (C) q-RT-PCR of EB7P7 cultures on gelatin (Gel), AR42J-ECM (AR42J) or NIH3T3-ECM (3T3). (D) IF analysis on EB7P7 cultures on (a,d) gelatin, (b,e) AR42J-ECM or (c,f) NIH3T3-ECM. (E) semi-qRT-PCR using rat *Ptf1a* specific primers on mRNAs extracted from AR42J cells or from EB7P7 cultures on AR42J-ECM or on gelatin.

To confirm the selectivity of the inductive signals, the assays were also performed using ECM produced by the C2C12 (muscle), DSL6B (pancreatic duct), N2a (Neuron) and IEC-18 (intestine) cell lines and the results were similar to those observed using NIH3T3-ECM, demonstrating the tissue specificity of AR42J-ECM to induce acinar pancreatic differentiation ([Table 3.1](#)).

Analysis of the beta-gal activity of differentiated <i>el</i> as-βGal ES cells on complex ECM derived from different cell lines.	
Culture Conditions	Relative Amount of X-gal stained ES cells
Gelatin	±
NIH3T3-ECM	+
C2C12-ECM	+
Neuro2a-ECM	++
IEC-18-ECM	+
DSL6B-ECM	++
AR42J-ECM	+++

Table 3.1. The relative amount of X-gal stained cells has been scored by microscopy after 14 days in culture as described below: ± rare positive cells; + few positive cells; ++ significant number of positive cells; +++ many positive cells; large and abundant patches of positive cells.

Furthermore, expression of acinar markers did increase with culture time (i.e. 14 days more) ([Figure 3.15](#)), suggesting an increase in the number of acinar progenitors or/and in the differentiation state.

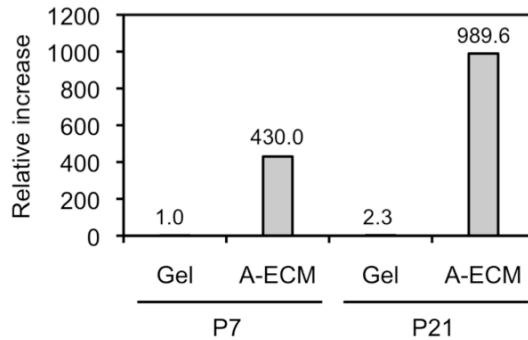


Figure 3.15. qRT-PCR analysis of the acinar marker Cpa1 on EB7EBs plated on gelatin (Gel) or on AR42J-ECM (ECM) for 7 days (P7) or 21 days (P21). Results are representative of one experiment performed twice.

Then we decided to identify the component(s) present in the AR42J-ECM able to induce the pancreatic differentiation. The composition of the AR42J-ECM is probably very complex by the nature of the components (protein, proteoglycans), the quantity of components (adhesion proteins, growth factors, cytokines/chemokines, and proteases) and the complex 3D structures formed by the interactions between those components. In a first step, we analysed the effect of single well-known ECM components on acinar ES differentiation.

2. Role of Purified ECM molecules on mESC acinar differentiation

Using similar culture conditions described in *Figure 3.13*, we investigated the ability of distinct ECM compounds to promote exocrine marker expression in the EBs differentiation model. To that end, EBs (EB7) cultured for 7 days were allowed to adhere on gelatin (0,1%) - or on purified- laminin (0.1; 0.01 and 0.001 $\mu\text{g/ml}$), Matrigel (1/200 and

1/2000), collagen I (1 and 4 $\mu\text{g/ml}$) and fibronectin (0.1 and 0.01 $\mu\text{g/ml}$)-coated culture dishes in 10% FBS medium for 7 days. As shown by the β -gal assay (*Figure 3.16*), a higher activity of the acinar reporter gene was observed in cells cultured with Matrigel and laminin, which is the main component of the Matrigel, consistent with a known role of laminin in pancreatic exocrine development/differentiation [90, 93, 94].

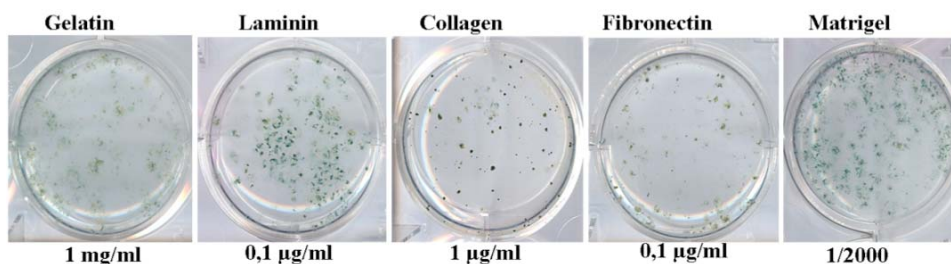


Figure 3.16. X-gal staining assay. Results of EB7P7 protocol for each ECM component are shown and are representative of two experiments performed in duplicates. The optimal concentration for acinar β -gal activity for each component is indicated.

Of note that these results (*Figure 3.16*) were obtained at concentrations favouring the generation of semi-thick gels, allowing the culture of EBs mostly on the “on top” manner with the cells penetrating in those gels (*Figure 3.17*). On the other hand it has been demonstrated that breast epithelial acinar cells cultivated in a 3D culture system form polarized acinus-like structures [236], and that exocrine branching is favoured in embryonic pancreatic epithelium when cultured in 3D conditions in Matrigel [90]. Those data suggest that improvement of 3D culture systems could optimize the pro-acinar effects of Matrigel on acinar ESC differentiation. For these reasons, EBs were cultured in 3D embedded conditions, as represented in *Figure 3.17*.

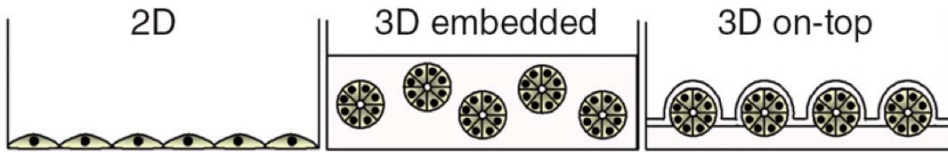


Figure 3.17. Scheme of non-malignant breast epithelial cells grown as a monolayer on tissue-culture plastic (left), in the 3D embedded assay (middle) and in the 3D on-top assay (right). Adapted from Lee et al.[237].

3D-Matrigel embedded culture with 10%FBS.

EBs (EB7) were cultured on gelatin or in Matrigel at different concentrations with 10% FBS supplemented medium for 7 days (EB7P7) and the expression of acinar markers analyzed by qRT-PCR ([Figure 3.18](#)). The 3D embedded condition in Matrigel increased the expression of *Cpa1* mRNAs in a concentration dependent manner, compared to EBs plated on gelatin. Nevertheless, only the dilution of Matrigel at 1/20 also significantly enhanced the expression *Chymo* suggesting that Matrigel has a positive effect on pancreatic progenitors (likely proliferation of $Cpa1^+$) but at 1/20 it also promotes differentiation of the pancreatic progenitors towards an acinar fate.

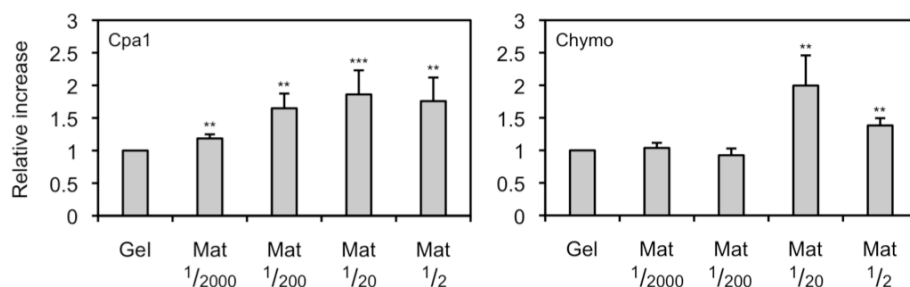


Figure 3.18. qRT-PCR analysis of acinar markers at EB7P7 in EBs plated on gelatin (gel) or Matrigel (Mat) at the indicated dilutions with 10% FBS supplemented medium. (N=3).

3D-Matrigel embedded culture with 1%KSR.

On the other hand, we modified culture medium conditions to increase the efficiency of this method. In this sense, Odorico's group has noticed that long ESC culture (EB7P28) in medium supplemented with 1% SR enhances *Amyl* expression, suggesting that low serum concentration is permissive for acinar differentiation [238]. Therefore, EBs were cultured in the same conditions as above, except that the medium was supplemented with 1% SR (EB7). As shown by qRT-PCR at EB7P7 (Figure 3.19), *Cpa1* expression was decreased at high dilutions of Matrigel while at the same conditions *Chymo* expression increased, as compared to gelatin condition, indicating that the culture in low serum concentration supports the development of acinar progenitors. Notably, at the 1/20 dilution, there was the highest increase in *Chymo* expression without a reduction in *Cpa1* expression. This increase was much more pronounced than the one in the same conditions in the presence of 10% FBS (Figure 3.18).

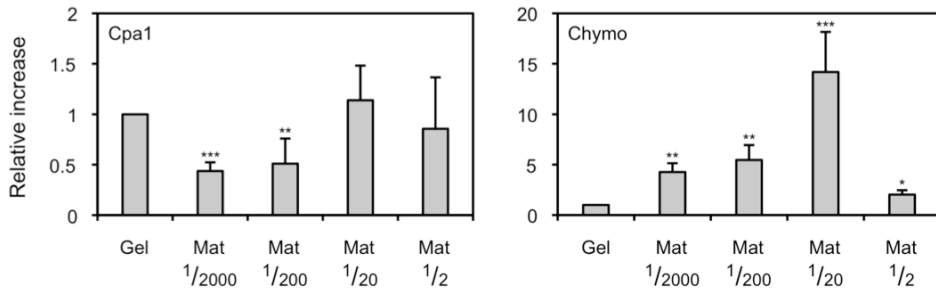


Figure 3.19. qRT-PCR analysis of acinar markers at EB7P7 in EBs plated on gelatin (gel) or Matrigel (Mat) at the indicated dilutions with 1% SR supplemented medium. (N=3).

1%SR versus 10%FBS supplemented medium.

Differential effects were observed on the pattern of exocrine marker expression in both conditions (*Figure 3.20*). For instance, in gelatin coating, Cpa1 was induced in 1% SR whereas ChymoB and Amyl were not, suggesting that 1% SR promoted mostly the proliferation/specification of pancreatic progenitors (Cpa1⁺). In any of these conditions we observed a significant increase in the expression of Amyl mRNA transcripts (*Figure 3.20*), suggesting that the acinar progenitors are in an early differentiation state.

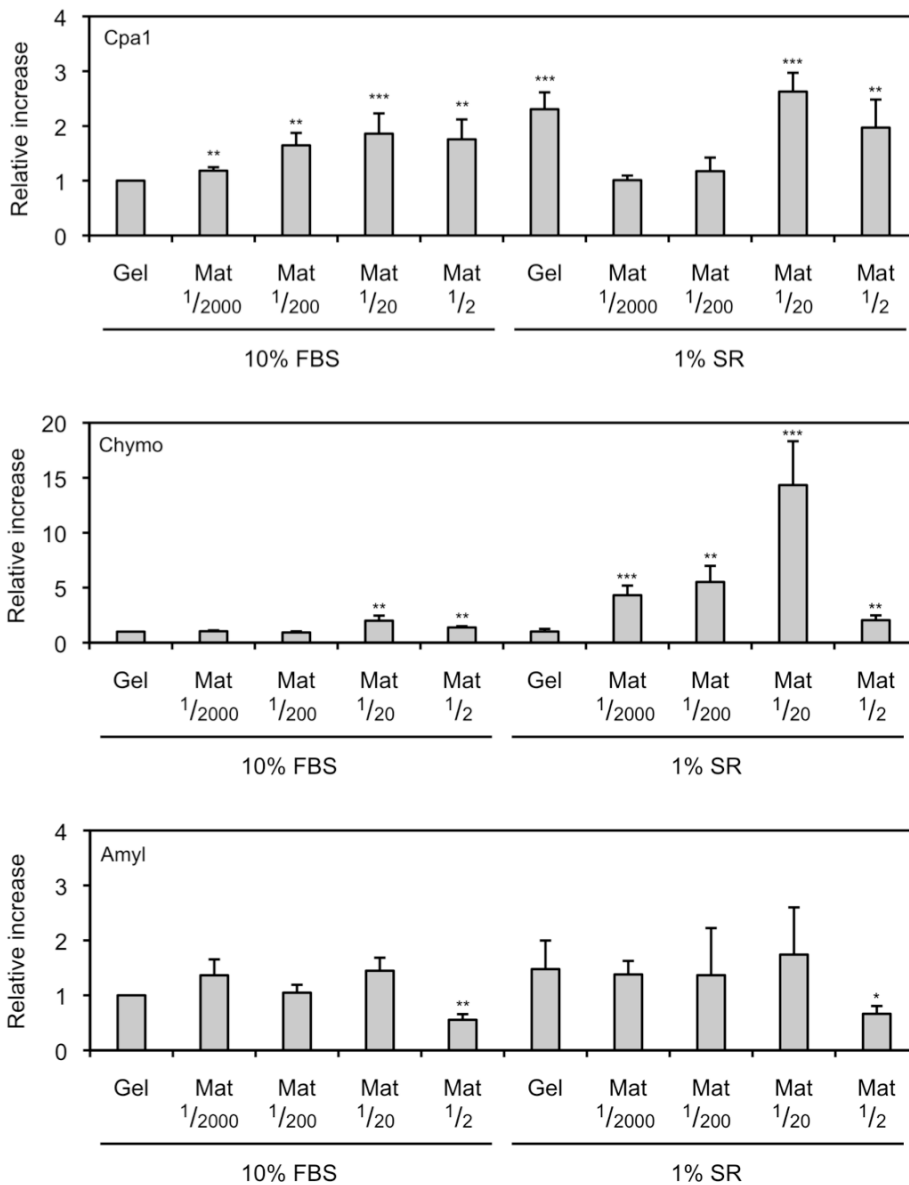


Figure 3.20. qRT-PCR analysis of *Cpa1*, *Chymo* and *Amyl* at EB7P7, in EBs plated on gelatine (gel) or in Matrigel at the indicated dilutions with 10% FBS or 1% SR supplemented medium. (N=3).

Overall, these data show that distinct ECM components (mainly Matrigel and laminin) are able to direct ESC acinar differentiation *in vitro* and that this process is highly dependent of the concentration/3D organization.

As laminin is very enriched in Matrigel, we analyzed its expression by IF both in AR42J cells and in decellularised AR42J-ECM. In AR42J cells, laminin was distributed as expected in the basal domain of the cells and was present in AR42J-ECM with a punctuate pattern (*Figure 3.21*), which was absent in the negative control (not shown). These results indicate that laminin may account in part for the inductive effect of the AR42J-ECM on the ESC acinar differentiation.

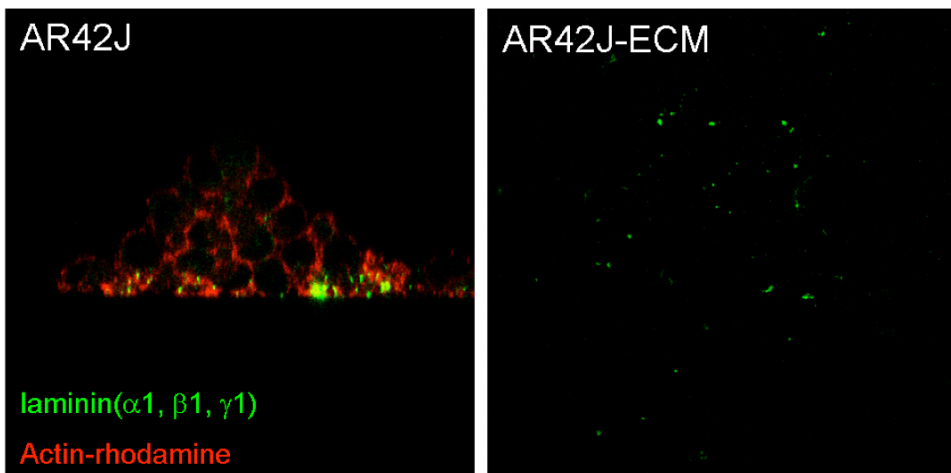


Figure 3.21. Immunofluorescence staining on AR42J cells (left panel) and AR42J-ECM (right panel) using an anti-laminin($\alpha 1$, $\beta 1$, $\gamma 1$) antibody (Green) and phalloidin-rhodamine (Red).

3. Identification of AR42J-ECM acinar inducing factors

In order to obtain some clues on the biochemical nature of the factor(s) involved in the induction of acinar differentiation by AR42J-ECM we treated those ECMs with different conditions before to use them as templates for ES differentiation studies as indicated in [Figure 3.13](#). These treatments include changes in the pH, salt concentration, detergents etc. after the ECM decellularisation as follows: NaCl (1 M); Sodium Acetate (25mM-pH 5.4 in 140 mM NaCl); Triton X-100 (0.1% in 140 mM NaCl) and as a control CaCl₂ (10 mM en Tris, pH 7.5-140 mM NaCl). Treated ECMs were then extensively washed with PBS before ESC plating. At the end of the culture (EB7P7), qRT-PCR assays were performed ([Figure 3.22](#)).

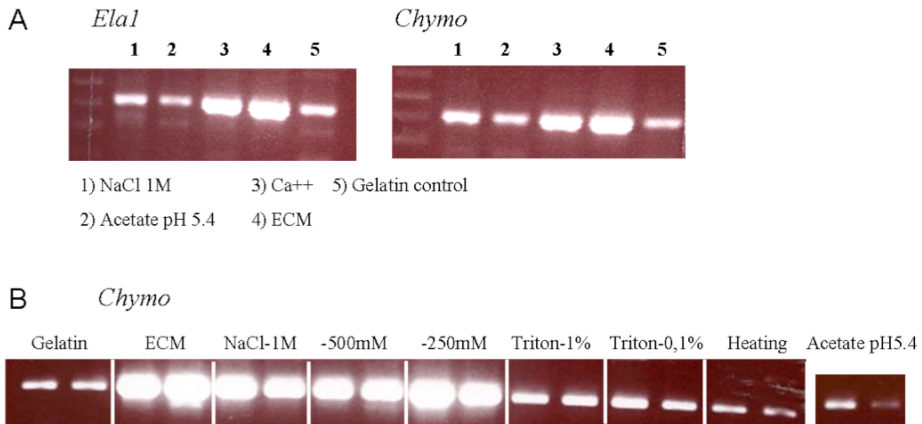


Figure 3.22. Semi qRT-PCR analysis of digestive enzyme expression in EB7P7-EBs. (A) semi qRT-PCR analysis of *Elal* and *Chymo* in EB7P7-EBs. (B) semi qRT-PCR analysis of *Chymo* in EB7P7-EBs in an independent experiment. The treatment conditions of the ECM are indicated. EBs cultured on non treated AR42J-ECM (ECM) are used as positive control. EBs cultured on gelatin are used as negative control.

As shown in *Figure 3.22.A*, AR42J-ECM highly increased the expression of acinar markers as *Ela1* and *Chymo* in comparison to gelatin-coated dishes condition. As expected, treatment with CaCl_2 (in 140 mM NaCl) did not modify the acinar inductive ability of the AR42J-ECM as for decellularizing the template it was previously incubated with an EGTA/EDTA solution, suggesting that the structure or interaction of the factor(s) is bivalent cation independent or at least not crucial; otherwise the factor will be release in the solution as the AR42J cells. Interestingly, 1 M NaCl and 25 mM sodium Acetate pH5.4 treatments abrogated this ability to levels comparable of control condition. In an independent experiment (*Figure 3.22.B*), AR42J-ECM was additionally treated with different concentrations of Triton X-100 leading to similar results. We decided to play with a range of NaCl concentrations in order to reduce the number of extracted proteins while maintaining the release of the potential inductors for an easier subsequent identification. Thus, we found that our readout (expression of acinar genes by RT-PCR) was not really affected at 250 mM NaCl but at 500 mM and 1 M the decrease was already detected (*Figure 3.20-B*), suggesting that the interactions of this factor with the complex ECM are mediated by hydrogen bonds. Nonetheless, in the second experiment, the reduction at 1 M was not as strong as in the first one, as the time was 5 minutes longer suggesting that the time of treatment could be critical. Further, pre-heating at 100° C during 10 minutes the AR42J-ECM also abrogated its inductive ability showing that the responsible factor(s) have a proteinic nature.

Taking into account these properties, we decided to analyze the proteomic profiles of the active non-treated AR42J-ECM. In order to

reduce the list of potential candidates we also planned to analyse the proteomic profiles of the NaCl treated AR42J-ECM (*Figure 3.23*).

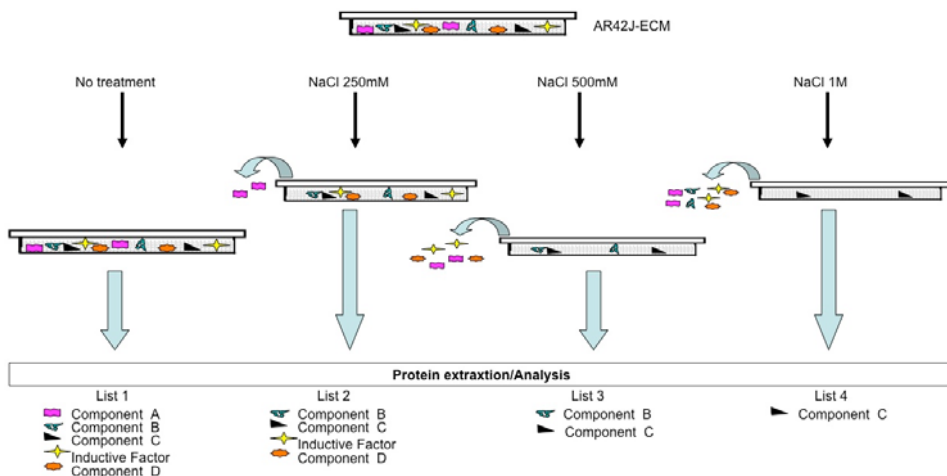


Figure 3.23. Schematic view of the protocol for hypothetical identification of the inductive factor. the proteomic profil of 250 mM NaCl treated AR42J-ECM (list 2) should hypothetically shorten the list 1 ruling out non effective protein (as 250 mM NaCl treated AR42J-ECM still can induce acinar differentiation of mESCs) from the list of potential candidate. Moreover, List 3 (500 mM NaCl treated AR42J-ECM) and List 4 (1M NaCl treated AR42J-ECM) will not content the “inducible factor” but will give us a list of potential non-candidate that could be ruled out.

Two samples from independent experiments were processed by HPLC (C18) and MS/MS techniques in preliminary assays. A summary of the list of identified proteins is provided in *Table 3.2*.

Score	Protein Name	Access Number
198	similar to Histone H2B 291B	gi 62663604
185	insulin-like growth factor binding protein 5 protease	gi 5815459
158	histone H2A	gi 817939
129	Protease, serine, 11	gi 51859442
102	IGFBP-2 gene product	gi 457880
42	Histone 1, H2aa	gi 76828190
39	Spondin 1, (f-spondin) extracellular matrix protein	gi 18088208

Table 3.2. Proteomic profile of AR42J-ECM. Only some of the significant hits are shown. Score > 37 indicate identity or extensive homology ($p < 0.05$).

Among them, several members of proteins belonging to the IGFBP (Insulin growth factor binding protein) family or associated to them were found. Indeed, IGFBP2 has been shown to directly interact with integrin $\alpha 5$ through the RGD domain in glioma cells, a property that could explain its presence in the analyzed ECM enriched extracts. In fact, various members of the IGFBP family have been described to be secreted into the extracellular milieu, including IGFBP2 [239] and IGFBP5, which is itself known to bind fibronectin, a component of the ECM [240, 241].

An unexpected result was to found in the ECM extracts various members of the Histone family (H2A; H2B). To ensure lack of nuclear contamination in the preparations we analyzed by western blotting another nuclear component, Bmi1, with negative results (data not shown), but further confirmed the presence of H2A in the extract. Indeed, the presence of Histone 1 in the ECM has been previously reported. In this compartment, H1 interacts with perlecan thus stimulating myoblast proliferation in regenerating skeletal muscle [242].

On the other hand, in a very short list of proteins, we expected to find many components of the ECM but we only identified f-spondin. This molecule has been recently shown to be expressed in embryonic pancreas from E9.5 to E15.5 where it localizes in nascent acini [243]. In addition, spondin1 can promote nerve precursor differentiation depending upon its concentration and distribution between substrate-attached and soluble forms, making this molecule a good candidate to be analyzed in the context of pancreatic differentiation [244, 245].

Unfortunately, this preliminary analysis could not be reproduced and improved because the inductive effect of the AR42J-ECM on ESC differentiation did not work anymore as described in the next section.

4. AR42J-ECM-induced acinar differentiation is lost

The loss of the induction phenotype correlates with the change of serum used in the laboratory for maintaining the AR42J in culture. Then by maintaining the AR42J in culture (passaging) before to use them in ECM assay we could recover the phenotype (*Figure 2.25*), but not systematically, then face to the loss of reproducibility we decide to focus on Matrigel embedded system and another components that could enhance acinar differentiation and especially soluble factors.

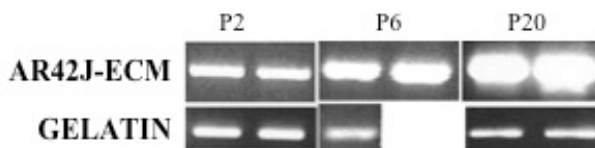


Figure 3.25. Semi qRT-PCR analysis of *Cpai* expression at EB7P7 in 3 independent experiments (P2,P6,P20). The numbers of passage of AR42J previously of ECM assay are indicated.

5. Soluble factors inducing acinar differentiation

Previous data in our group have shown that soluble factors present in conditioned medium obtained from the culture of foetal pancreases were able to activate the acinar differentiation program [227, 228]. However, this mix of factors is not selective for the acinar phenotype as endocrine differentiation is also up-regulated and the ability to do so is largely dependent on the quality of the conditioned medium. The rationale of this section was to investigate which factors could selectively direct acinar differentiation based on the knowledge acquired on embryonic pancreas development. To that end, cells were induced to differentiate as EB in 3% FBS during 7 days, plated on gelatin-coated dishes and further cultured in the presence of soluble factors for additional 7 or 14 days. The activity of these factors, including Fol, Dex, and KGF, was studied alone or in combination at both time points by qRT-PCR (*Figure 3.25*).

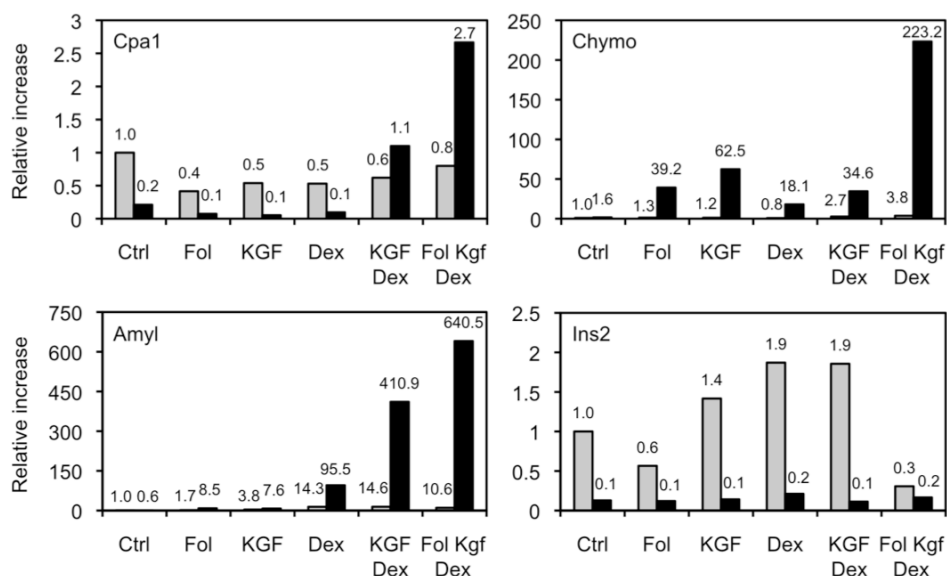


Figure 3.25. Gene expression on cultures treated with pro-exocrine soluble factors by q-RT-PCR. The analysis of pancreatic markers was carried out at EB7P7 (grey) and EB7P14 (black) time points. Spontaneously differentiated EB7-EBs were plated on 0.1% gelatin with the indicated soluble factors alone or in combination in 1% SR medium. Ctrl (control). Results are representative of one experiment performed twice.

At EB7P7, we did not find a clear increase in acinar gene expression upon supplementation with single soluble factors, independently of their nature, although an increase on Amyl expression was observed in response to Dex. However, the combination of Dex and KGF or Dex, Fol and KGF did result in such increase in comparison to single factors and control conditions, being the Dex-Fol-KGF- condition the most efficient. These data indicate a synergism between different signalling pathways known to promote exocrine differentiation *in vivo*. Moreover, Fol treatment alone or in combination with Dex and KGF resulted in the down-regulation of *Ins2* expression suggesting that β -cell differentiation is repressed by Fol. Taken together those results suggest

that these signals might be selective for the exocrine differentiation program.

However, a week later of culture, at EB7P14, we found that the expression of cytodifferentiation markers was now increased in response to single factors with specific regulation patterns. For instance, *Chymo*, an early marker of acinar cells [72] was increased by Dex, Fol and KGF whereas *Amyl*, which induction occurs slightly later during embryonic development [73], was only increased by Dex. Notably, the concurrence of the three types of signals resulted in a high rise in both markers as compared to EB7P7. On the other hand, *Cpa1* expression was decreased with single factors in comparison to EB7P7, suggesting the commitment of pancreatic progenitors into the exocrine lineage. In addition, *Ins2* expression dramatically decreased in all the conditions demonstrating that these methods are not suitable for β -cell lineage maintenance and/or differentiation.

III. Pancreatic acinar differentiation protocol through Activin A-induced endoderm

1. Stepwise differentiation protocol for acinar cells

The main objective was to optimize the culture conditions to direct the acinar differentiation program in a sequential manner by integrating previous data in a new Exo2 protocol. Therefore, cells were induced to progress through stages 1 and 2 of the protocol (*Figure 3.26*) to generate pancreatic progenitors during 7 days (EB7), embedded in 3D Matrigel at 1/20 and cultured with the pro-exocrine signals for 12 days (EB7P12) as represented in *Figure 3.26*.

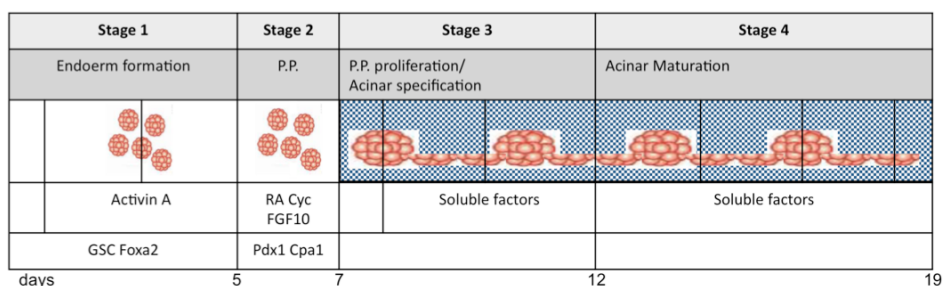


Figure 3.26. Stepwise ESC differentiation protocol for the generation of acinar cells. mESC are allowed to form EBs in 3%SR medium. Then Activin A is added to induce endoderm formation for 4 days. Followed by FCR mix treatment for 2 days, to specify pancreatic endoderm. On day 7, EBs are seeded in Matrigel 1/20 (3D embedded system). Then combinations of factors were assayed (*Table 3.3*).

In this protocol, KGF was provided only at Stage 3 as it promotes the proliferation of pancreatic cells *in vivo*, in order to facilitate, with its retrieval at Stage 4, the differentiation/ maturation of acinar progenitors. Also optimal combinations of Fox and Dex with additional soluble factors

at stages 3 and 4 were analyzed as indicated in [Table 3.3](#). In particular, we assayed by qRT-PCR whether T3 and IGFII could further support acinar gene expression at EB7P5 (Stage 3) and EB7P12 (Stage 4).

EB7	P1-P5 (Stage 3)	P5-P12 (Stage 4)
MIX-1	DEXAMETHASONE FOLLISTATIN KGF	DEXAMETHASONE FOLLISTATIN
MIX-2	DEXAMETHASONE FOLLISTATIN KGF T3 IGFII	DEXAMETHASONE FOLLISTATIN T3
MIX-3	FOLLISTATIN KGF IGFII	DEXAMETHASONE T3

Table 3.3. Composition of the different mix tested for acinar differentiation.

As shown in [Figure 3.27](#), culturing pancreatic endoderm induced -EBs (PEI-EBs) only in Matrigel increased the expression of digestive enzymes at EB7P5 as compared to gelatin condition but also of endocrine markers. Additional culture with Mix1 and Mix2 further increased acinar gene expression at similar levels and down-regulated a set of endocrine genes, including *Ins2*, *Ngn3* and *Nkx6.1*. On the contrary, Mix3 failed to follow this pattern both in acinar and endocrine gene expression, suggesting that Dex at early stages is mostly responsible of this inductive effect.

As expected, the increase on acinar gene expression was more efficient one week later at EB7P12, with the Mix1 combination being the optimal one. In addition, the down-regulation of endocrine markers was also sustained with time. In line with an up-regulation of acinar markers, *Ptf1a* and *Rbpjl* of the PTF1 complex were also raised. The fact that

with time Mix2 was not any more able to promote exocrine gene expression suggests that T3 is the factor responsible for this detrimental effect as it is also present in Mix3. As Mix1 was found to be the optimal combination we analyzed IGFII supplementation to this Mix at Stage 3 but it did not improve the results (not shown).

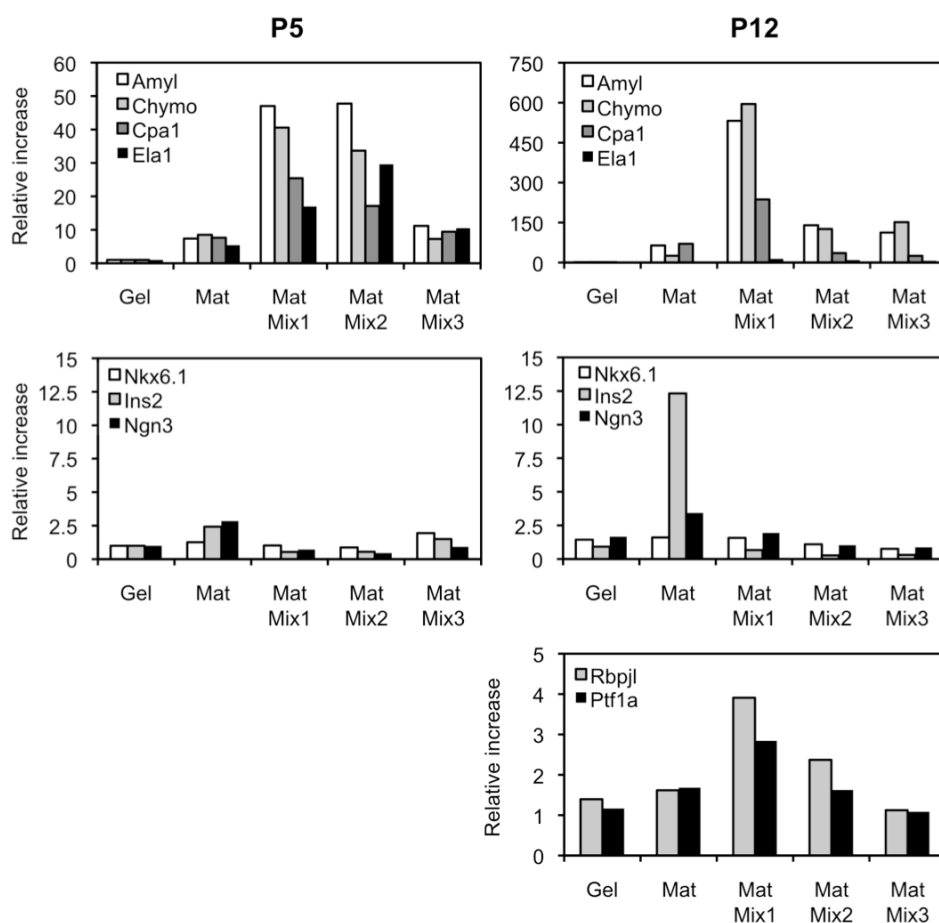


Figure 3.27. qRT-PCR analysis of the expression of pancreatic markers of EB7P5 and EB7P12. PEI-EBs were plated on gelatin (Gel) on in Matrigel 1/20 (3D-embedded system) (Mat) with or without soluble factor mix (Mix1-2-3) supplied in 1%SR medium. Results are representative of one experiment performed twice.

Therefore, the conditions for ESC acinar differentiation were established with Mix1 following the protocol described in [Figure 3.28](#).

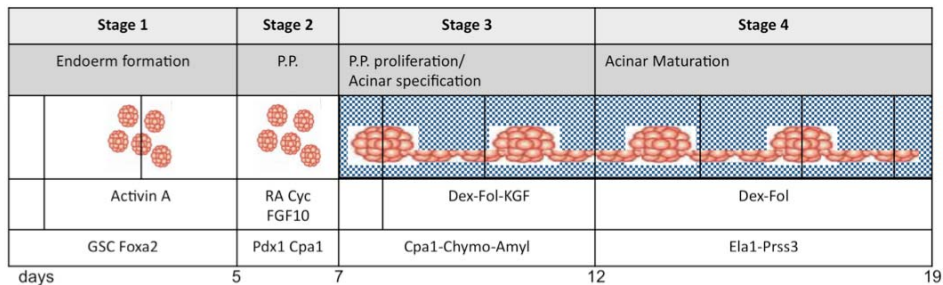


Figure 3.28. Stepwise ESC differentiation protocol for the generation of acinar cells. Following stages 1 and 2, EB7-EBs were seeded in Matrigel at 1/20. After 12hrs, Dex-Fol-KGF was supplemented for 4 days to induce proliferation and specification of acinar progenitors, then only Fol and Dex were added to enforce acinar differentiation/maturation. Medium was renewed every two days.

Role of pancreatic endoderm induction in acinar differentiation efficiency

To further investigate that the expected cell population (pancreatic progenitors) was likely the one responding to exocrine signals, the increase in acinar gene expression was also studied in cultures which did not progress through FCR mix treatment, but were exposed to Activin A and exocrine signals (Mix1). As shown in [Figure 3.29](#), the extent of acinar gene expression was near 70% much lower than cultures progressing through all the stages, showing that the acquisition of the cell competence to respond to them is acquired after progressing through stages 1 and 2 of the protocol.

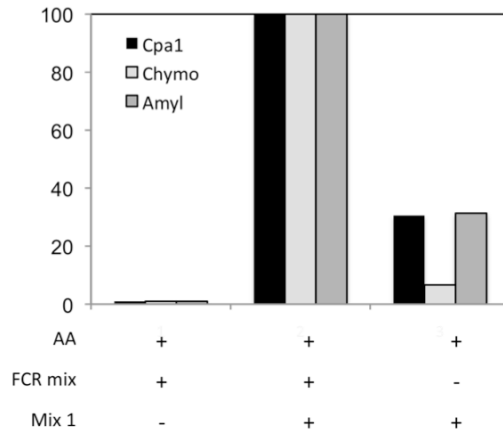


Figure 3.29. qRT-PCR analysis of digestive enzyme expression at EB7P12 in EBs cultured in different conditions. Values of cultures progressing sequentially through all the stages of the protocol were arbitrary designed as 100% of expression level: PEI-EBs that were not treated with Mix1 were arbitrary designed as 1% expression level. Results are representative of one experiment performed twice.

2. Characterization of the differentiated cells

To further characterize the generated cells, a large panel of exocrine markers was analyzed by qRT-PCR (*Figure 3.30*). Cells differentiated through the whole protocol (T19) significantly expressed high levels of *Cpa1*, *Chymo* and *Amyl* mRNAs as compared to cells progressing through stages 1 and 2 (PEI-EBs) but non-treated with soluble factors (NT19). In addition, increased levels of *Ela1*, *Ela2* and *Prss3* mRNAs were observed while other enzymes such as *CEL* and *Pla2g6* were induced at much lowest level.

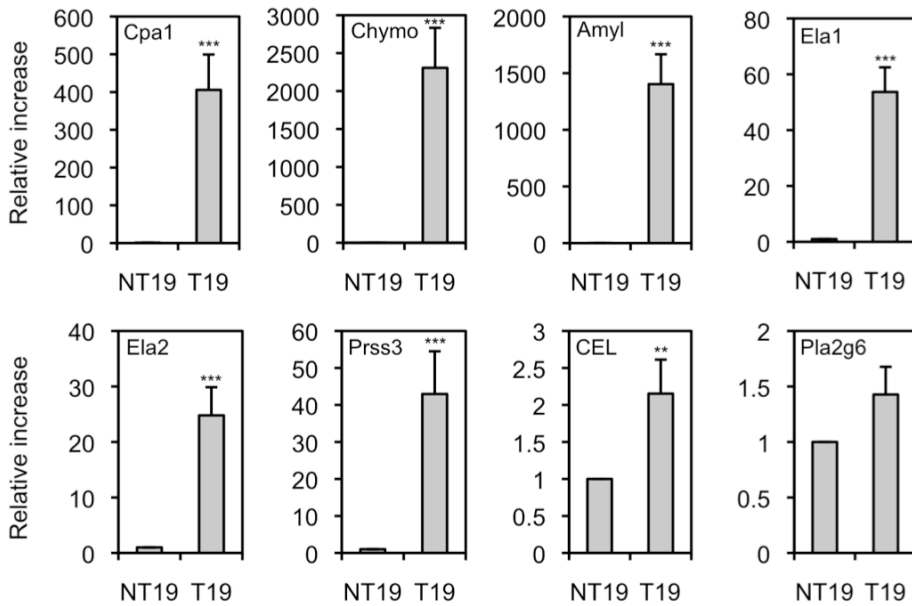


Figure 3.30. qRT-PCR analysis of digestive enzyme expression at EB7P12. NT19, PEI-EBs not treated with pro-exocrine signals; T19, PEI-EBs treated with the Mix1 of soluble factors. $N \geq 3$.

The increase in digestive enzyme gene expression paralleled with a significant increase in mRNAs encoding for transcription factors that regulate their expression, such as *Ptf1a* and *Pdx1* (Figure 3.31). In addition, *Rbpjl*, which confers the high activity of the PTF1 complex, tend to increase in treated cells although it was not significant. Interestingly, *Rbpjk*, which participates at early stages of embryonic pancreas development, was down-regulated as demonstrated *in vivo* [130], suggesting that the embryonic PTF1 complex is replaced by the PTF1 complex containing RBPJL. *Gata4*, known to regulate exocrine development, was up-regulated but not *Mist1*, involved in the terminal maturation of acinar cells. However, *Cx32*, a well known target of *Mist1* crucial for efficient acinar secretion [246] was increased.

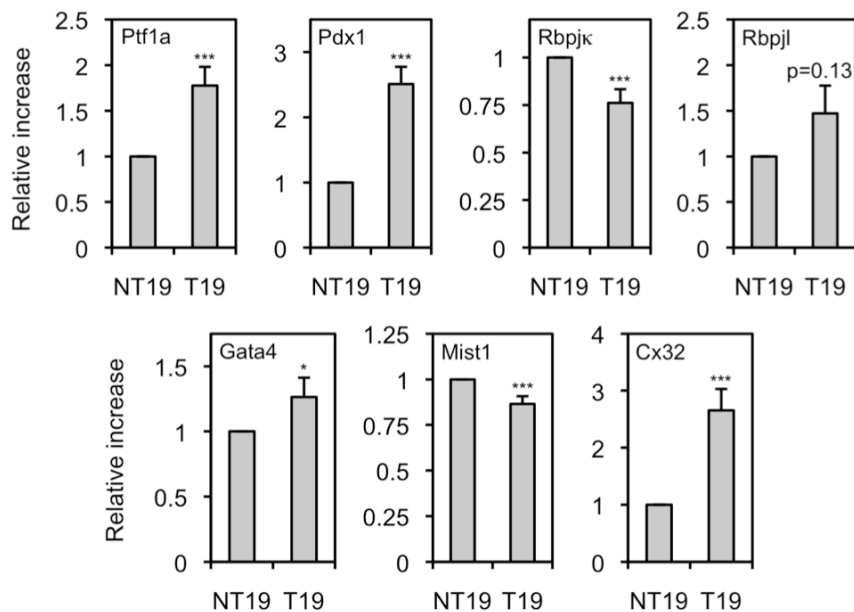


Figure 3.31. qRT-PCR analysis of pancreatic marker expression at EB7P12. NT19, PEI-EBs not treated with pro-exocrine signals; T19, PEI-EBs treated with the Mix1 of soluble factors. $N \geq 3$.

On the other hand, T19 cultures displayed a statistical significant decrease in the expression of a set of endocrine marker mRNAs ([Figure 3.32](#)), suggesting that the protocol is selective for the acinar lineage.

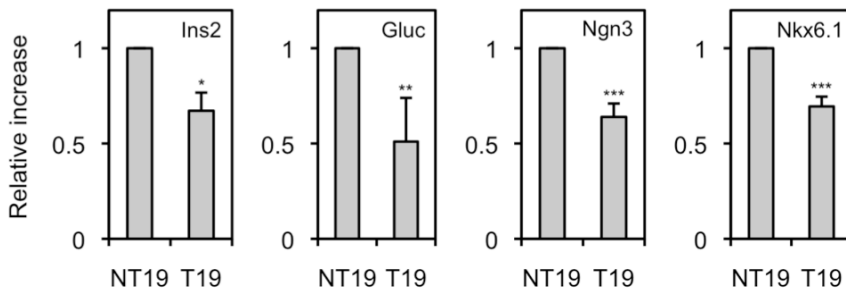


Figure 3.32. qRT-PCR analysis of endocrine marker expression at EB7P12. NT19, PEI-EBs not treated with pro-exocrine signals; T19, PEI-EBs treated with the Mix1 of soluble factors. $N \geq 3$.

To further confirm the specificity of the protocol markers of other endodermal lineages were studied ([Figure 3.33](#)). In particular, we found that the liver markers *Afp* and *Ttr* were not strongly up-regulated in comparison to the high increase of digestive enzymes. In addition, *Cdx2*, which marks the intestine, was not up-regulated showing that the protocol mostly promotes the development of acinar progenitors. Surprisingly, although the treatment with DM at Stage 2 strongly decreased the expression of hepatic markers ([Figure 3.10](#)), 12 days later the level of expression was similar to the cultures that were not treated at this stage. Those results suggest that DM treatment blocks the liver pathway at stage 2, but FCR mix is not sufficient to redirect hepatic endoderm to a pancreatic fate. Then after retrieval of the DM, the hepatic progenitors differentiate to hepatocyte lineage.

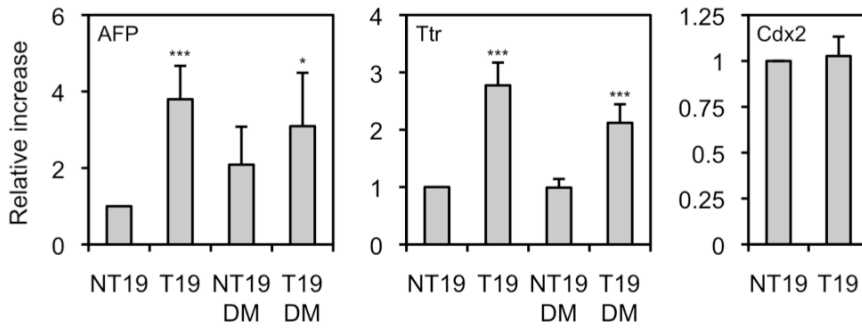


Figure 3.33. qRT-PCR analysis of liver (*Afp* and *Ttr*) and intestine (*cdx2*) marker expression at EB7P12. NT19, PEI-EBs not treated with pro-exocrine signals; T19, PEI-EBs treated with the Mix1 of soluble factors, in the presence or not of DM at stage 2. N \geq 3.

Furthermore, some of these findings were also studied at the protein level by IF. As shown in [Figure 3.36](#), in cells cultured along the whole protocol (T19), large cell clusters expressing both Amyl and Chymo (*b,c*) were observed as compared to control non-treated cells (N19) (*a*). Similarly, a large proportion of Chymo⁺ cells co-expressed Cpa1 with a cytoplasmic staining pattern (*e*). Counting immunofluorescent-positive cells demonstrated that 26.5% \pm 6.03% of total cells expressed digestive enzymes in T19 condition while only 4.9% \pm 1.05% were found in the NT19 condition, (P<0.05). Of note that in cultures treated only with SR we did not find any positive cell expressing digestive enzymes. Cells producing secretory enzymes expressed also nuclearly the transcription factor Rbpjl, showing their pancreatic identity (*f*). In some cases, these cells appear organized in small glands around a lumen-like structure (*l*). Moreover, in NT19 condition many AFP⁺ cells were found, with very few co-expressing Amyl while in the T19 condition we could not detect any Amyl or Chymo expressing AFP or

Glyc2 respectively. These results further support the notion that the exocrine cells arise from true pancreatic progenitors [220]. On the other hand, very few glucagon⁺ and none insulin⁺ cells were detected in the T19 condition (*k*) while they were present in large cell clusters from cultures progressing through stages 1 and 2 but not treated with the pro-exocrine signals (NT19) (*j*).

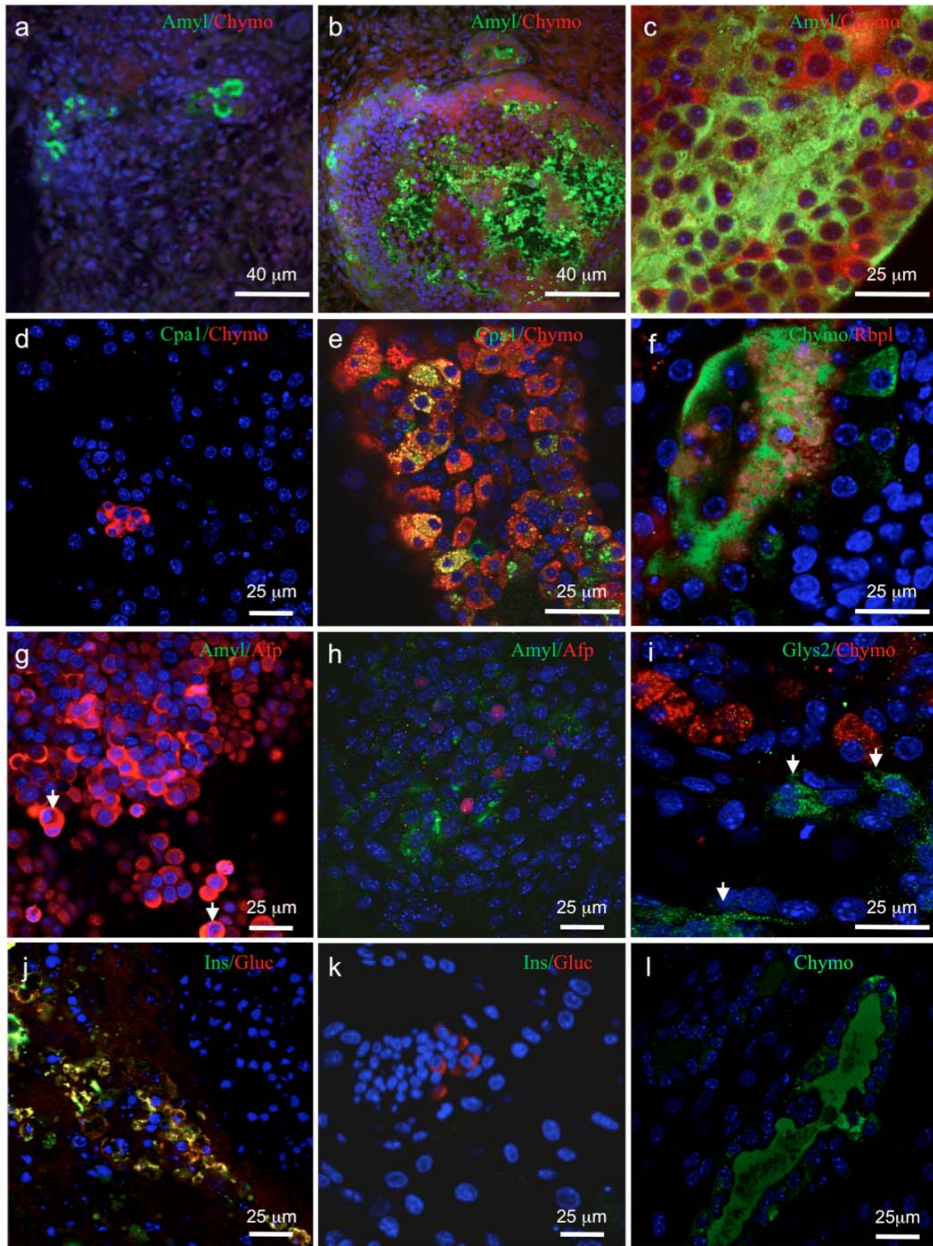


Figure 3.36. Immunofluorescence analysis of pancreatic and hepatic markers. Cells progressing through stages 1 and 2 and incubated without (a, d, g, j) or with (b, c, e, f, h, i, k, l) the pro-exocrine signals were double stained for the indicated markers. Nuclei were stained with DAPI. Arrows in g show double labelled cells whereas in i they show the Glyc2⁺ cells.

In order to measure the overall efficiency of the whole protocol we additionally analyzed acinar marker expression in cultures spontaneously differentiated in the presence of SR by qRT-PCR. As shown in (Figure 3.37), the huge increase in the expression of these markers further demonstrates the importance of the benefit brought by the endoderm formation, pancreatic endoderm induction and pancreatic acinar differentiation.

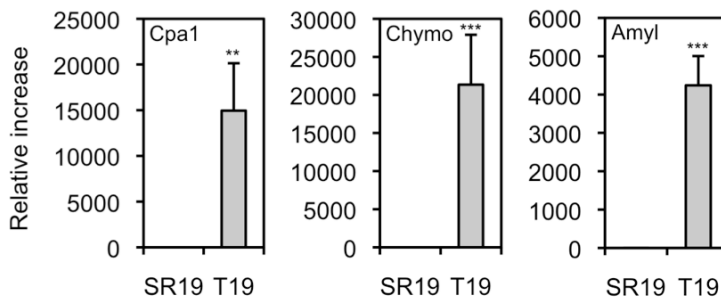


Figure 3.37. qRT-PCR analysis of digestive enzymes expression at EB7P12. SR19, spontaneously differentiated EB7-EBs plated on 1%SR gelatin and not treated with pro-exocrine signals; T19, PEI-EBs treated with the Mix1 of soluble factors. $N \geq 3$.

Overall, these results show the development of a protocol for pancreatic differentiation, which directs selectively the generation of exocrine acinar progenitors with respect to both pancreatic endocrine and hepatic lineages.

Functionality of the differentiated cells

A hallmark of the functionality of acinar cells is their ability to secrete digestive enzymes in response to specific secretagogues. Therefore, we analyzed the ability of stepwise differentiated and spontaneously differentiated-cells to secrete Amyl upon stimulation with carbachol and CCK *in vitro*. As shown in [Figure 3.38](#), we found that in T19 cultures, Amyl activity was already detectable in SN whereas it was not in spontaneously differentiated cells, which is indicative of a constitutive secretion. Treatment with physiological concentrations of secretagogues induced a very slight increase in both the intracellular and extracellular Amyl activity only in the T19 condition, which supports the notion of an increased synthesis and secretion in response to secretagogues. Nevertheless, the fact that Amyl is already released in non-stimulated cells suggests that the cells display an immature functional phenotype.

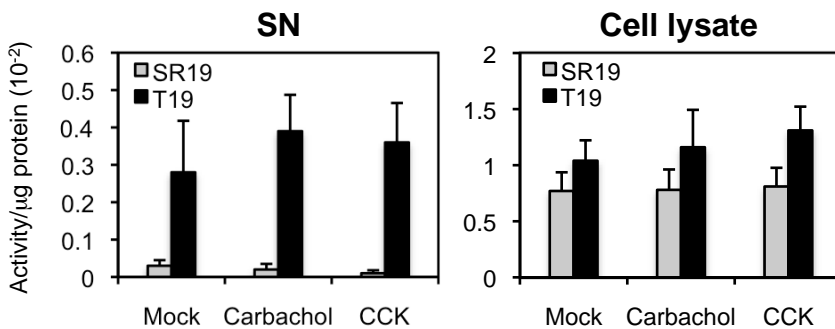


Figure 3.38. Exocytosis in differentiated cultures in response to secretagogues. T19-EB and SR19-EB cultures were stimulated with 5 mM Carbachol and 1 pM CCK for 45 min. Amyl activity was measured both in cell culture supernatants (SN) and in cell lysates. Controls are non-stimulated cells. N=1.

DISCUSSION

While there are many reports of differentiation of pancreatic endocrine cells from ESCs, only few reports studied their ability to generate acinar cells [72, 201, 203, 218, 225, 227, 247]. Both endocrine and exocrine pancreatic cells are derived from common progenitor cells that express Pdx1 [51, 248], suggesting that the know-how developed for the first stages of endocrine protocols can be useful for the improvement of exocrine differentiation procedures.

Definitive endoderm formation

Therefore, we took advantage of several studies that direct endocrine cells through DE formation and PE specification [196, 213] based on the recapitulation of early developmental stages (*Figure 3.7*). To that end, mESCs were cultured in suspension to form EBs containing endoderm, ectoderm, and mesoderm. After one day, high concentration of Activin A (100 ng/ml) were supplemented to the EB culture in order to enforce DE formation in a low knockout serum replacement medium as FBS were shown to impair Activin A effect. As a result, increased expression of many endodermal genes at stage 1 including *Gsc* and *Foxa2* (*Figure 3.3*) was observed, in line with similar studies [27, 193, 197, 200, 201]. In addition, genes that mark the neuroectoderm were down-regulated only in the presence of Activin A as SR alone promoted mostly the expression of these genes. In addition, mesodermal markers displayed a modest increase in comparison to endodermal ones, further supporting that our conditions promote preferentially the endoderm lineage. The fact that for reducing the cost of the experiments we did not daily supplemented the cultures with Activin A may account for these findings.

Moreover, several recent reports of Activin A-induced DE formation in mESCs performed a previous EB aggregation step before supplementation of the Activin A [27, 193, 197, 200, 201], supporting our choice and suggesting that the presence of the three germ layers could play an important role in the DE differentiation of mESCs. Nonetheless, in our hands, we observed a certain level of variability of the level of endodermal induction that could be explained by the fact that we used distinct lots of SR. Although SR has a defined composition it contains BSA, which has been shown to interfere with the Activin A induction ability [249] depending on its concentration but also of the quality. In addition, recent data indicate that the timing of Activin A application in EBs is critical for an optimal response and suggest that a previous step of spontaneous differentiation (at least two days) is necessary for the induction. Although we aggregated mESC cells in suspension for one day leading to heterogeneity in the EB size before Activin A supplementation, these findings suggest that our results could be further improved by delaying the spontaneous differentiation step. On the other hand, based on Melton's group results we incubated the cells during 4 days with Activin A leading to the additional enhancement of genes expressed in the foregut/pancreatic endoderm [27]. Shorten of this step1 could therefore enhance the results obtained during stage 2 of the protocol.

Pancreatic endoderm specification

During stage 2, the supplementation of FCR mix increased the expression of pancreatic endoderm markers as *Pdx1*, *Nkx6.1*, *Ptf1a*, *Foxa2* and *GATA4* suggesting the specification of pancreatic progenitors. Further co-expression analysis of these markers is needed to quantify the

extent of the step2-efficiency. As suggested by other studies [184, 214] incubation with RA was critical for the induction of pancreatic genes and the efficiency was dependent of the concentration, being 10 μ M the optimal one (*Figure 3.8*). In addition, we showed that the ability to respond to the pro-exocrine signals is largely dependent on the cell competence acquired during this step (*Figure 3.29*). On the other hand, we showed an up-regulation of the hepatic markers upon incubation with the FCR mix, likely due to the presence of RA as recent data demonstrating that this pathway is involved in hepatic specification [250]. In addition, it is well known that embryonic liver initiation is regulated by BMPs signaling [57]. Consequently, qRT-PCR analysis showed a strong reduction of hepatic markers upon incubation with DM, an inhibitor of this signaling, while not affecting the level of expression of pancreatic ones, even if BMPs have been also shown to promote pancreatic specification few hours later than for the hepatic one. [251]. It is possible that the concomitant presence of FGF10 in the mix further supports the maintenance of the Pdx1 cell population as described in vivo [76, 77]. In any case, our results are also in line with other studies showing that blocking BMPs using Noggin is beneficial for the blockade of the hepatic lineage in ESC [220]. Since Noggin is limited to antagonize specific BMP ligands the use of DM which inhibits multiple BMP type I receptors [234] could be useful for pancreatic differentiation protocols. Nonetheless, this group showed that once Noggin acts the inhibition of the hepatic fate is fixed whereas we found that at later stages upon retrieval of DM at stages 3 and 4 the expression of hepatic markers was the same than in non-treated cultures (see NT19 versus NT19DM in *Figure 3.33*).

Pancreatic acinar differentiation

In the second part of our protocol (*Figure 3.28*) we investigated the role of defined molecules in inducing acinar ESC differentiation based on the knowledge about their role on pancreatic exocrine development. Thus, we investigated in a first attempt, which were the best conditions permissive for acinar differentiation and studied appropriate combinations of these signals in enforcing acinar gene expression. Thus, we demonstrated that culture in 3D-Matrigel embedded system and exposure to KGF, follistatin, and Dex in 1% SR supplemented medium enhanced efficiently the acinar differentiation of pancreatic endoderm-induced cells as compared to spontaneously differentiated cells “SR19” or “NT19” differentiated cells (*Figure 3.30-3.35*).

The rationale of the use of low concentration of SR is based on recent data showing that in 1% SR there was an increase in the number of cells expressing Amyl in long culture period (>25 days). In our hands, we did not observe at shorter times (7 days) an increase in the expression of transcripts encoding this enzyme and only did observe an increase in Cpa1 expression in cultures seeded on gelatin. This result suggests that 1% SR promotes the emergence of pancreatic progenitors or early acinar progenitors and that for inducing acinar differentiation additional signals are required. Indeed, seeding the cells in 3D Matrigel, which by itself promotes the expression of acinar genes, was sufficient to further increase the expression of Chymo and Cpa1 in 1% SR as compared to the same conditions in 10% FBS. As the assays were not performed at longer period time it is difficult to conclude.

The role of Laminin and Matrigel in pancreatic differentiation/morphogenesis has been documented on *ex-vivo* culture of embryonic pancreas and notably the induction of branching exocrine structures [90]. As shown in Fig, (reporter activity, matrigel concentration) the pro-exocrine differentiation ability of these molecules was dependent on their concentration. Moreover, culturing of the cells in 3D gels favored the emergence of cells expressing digestive enzymes that tend to organize in glandular structures (*Figure 3.34 and data not shown*), further supporting that 3D organization is one important factor to consider for future studies.

Regarding acinar specification and differentiation *in vivo* very little information is currently available. A part of Wnt/ β -catenin signaling which role is complex and largely dependent on the time and place of Wnt signalling, glucocorticoids and FGF have been suggested to play a role in the acinar versus endocrine fate choice, although the molecular mechanisms underlying this decision remain largely unknown. On the other hand, KGF is mostly recognized as stimulating both the proliferation of embryonic pancreatic epithelial cells and by increasing the presence of Amyl positive cells, but again it is not clear at the moment whether this is due to the recruitment of progenitor cells or the expansion of existing acinar progenitors. In addition, its role seems to be dependent on the developmental time as later on (E13) it is able to suppress endocrine differentiation [88].

In the ESC system, we found in preliminary assays that after 14 days all these soluble factors applied individually tend to increase the expression of acinar markers such as Chymo and Amyl in cells not primed by pro-endoderm and pancreatic signals, further supporting their

role in vitro in promoting exocrine differentiation. Indeed, culturing the cells with the three factors resulted in a synergic induction in the expression of these markers. Interestingly, in these assays, only Fol was able to decrease the expression of insulin, suggesting that this factor could mediate the anti-endocrine effect of the combination.

Taking into account this information we developed a stepwise differentiation strategy in a single Exo2 protocol (*Figure 3.28*). Therefore, cells progressing through stages 1 and 2 were incubated first with a combination of Fol, Dex and KGF to promote acinar specification and the proliferation of exocrine progenitors. Subsequently, KGF was retrieved to further enforce acinar differentiation.

In these conditions, qRT-PCR analysis demonstrated an important increase in the expression of a set of digestive enzymes including those expressed at early stages such as Chymo and Cpa1 and others such as Amyl which induction occurs slightly later in embryonic development [72]. Previous data in our group have demonstrated that in the Exo1 protocol, the occurrence of pancreatic foetal signals from E16.5 and the overexpression of *Ptf1a* results in a high enhancement of Cpa1 and Chymo B but not of Amyl. Indeed, in this study the detection of Amyl by IF required an amplification step. In the Exo2 protocol, the expression of Amyl was sufficiently elevated to be detected by standard techniques and was found in large cell clusters that co-expressed Chymo, suggesting an improvement not only in the pattern of digestive enzyme expression but also in the level of their expression. Interestingly, the increase of digestive enzymes was associated to an increase in the levels of mRNAs encoding for the transcription factors *Ptf1a* and *Pdx1*, involved in their expression. However, *Rbpjl*, which maximizes the expression of digestive enzymes,

was not significantly increased; coincident with the low enhancement of other genes such as *Prss3*, *Cel*, and *Ela* genes (figure 3.31), proposed to be regulated later in embryonic development with the accumulation of *Rbpjl* [73]. In addition, only a subset of the differentiated cells displayed a nuclear staining for *Rbpjl* (Figure 3.34).

Other groups have reported an enhancement of *Amyl* expression in response to Dex treatment *in vivo* and in the ESC model [98, 99, 225] (Figure 4.1). In this latter, cells differentiated into the endoderm by activin A and sodium butyrate treatment enhanced the expression of *Amyl* and *Ela1* but not of *Chymo*, but the extent of this induction was much more lower than the data presented in here, probably due to the lack of a pancreatic specification step in their protocol. The fact that Dex was the only pro-exocrine signal provided could also account for the limited induction (Figure 3.25). Finally, they found by FACS that around 17% of the cells were *Amyl* positive, whereas in our hands we detected a 26% using counting assays. We estimate that this number may be underscored in comparison to this study due to the different techniques used for the quantification.

Similarly, **KGF** has been shown to induce exocrine marker in mESC in an acinar differentiation procedure [247] (Figure 4.1). But here again the induction level of exocrine markers was lower than the data presented in here but higher than the Ren's procedure. The improvement could reside in the RA treatment, which has been successfully used to induce *Pdx1*⁺ pancreatic progenitors. Then, the slight induction of exocrine markers is likely due to the use of KGF as the single pro-exocrine factor. Moreover, KGF was used in combination with a cocktail of pro-endocrine factors, and according to our *in vitro* results,

supplementation of KGF could enhance *Ins2* expression in mESCs (Figure 3.25), then it is likely that their protocol is not as selective for acinar differentiation as our and finally their efficiency is lower as shown by their counting of Amyl⁺ cells (10%).

In summary, we have set up the first selective protocol for acinar differentiation using a large cocktail of factors that allowed us to mimic the in vivo development of the acinar cells. Nevertheless the functionality of the differentiated cells has not been achieved beside a constitutively secretion of active amyl into the medium. As a consequence improvements of our procedure have to be investigated.

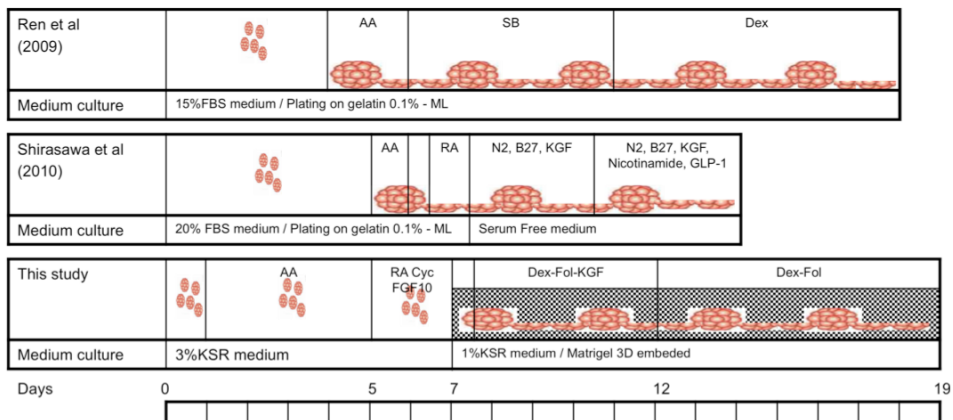


Figure 4.1. Schematic view of the three stepwise-differentiation protocols of acinar cells from mESCs. Ren et al. [225]; Shirasawa et al. [247] and exo2, this study. Activin A (AA); sodium butyrate (SB); glucagon-like peptide-1 (GLP-1).

Improvement of the stepwise differentiation protocol

The Activin A-induced DE formation stage could be improve by the combination with sodium butyrate, as it has been shown to enhance Activin A-induced DE formation [204].

The plating of cells progressing through stages 1 and 2 on AR42J-ECM with supplementation of soluble factors (Dex, Fol and KGF) could have been a way to improve the acinar differentiation of our procedure. Actually the data of differentiated cells on AR42J-ECM show that *amyl* mRNA level was not regulated (*Figure 3.14.C*) but the combination of Dex, Fol and KGF could have compensated it as it enhances the regulation of *Amyl*.

Moreover, scaffolds are biodegradable polymer that can act as signalling templates to forming tissue, elicit controlled cellular adhesion, or maintain differentiated phenotypic expression. Those polymers could be used as template for acinar differentiation. A large variety of scaffolds are available depending on the pore structure and subdivision of 3D space, the surface properties of the interfaces between cells and scaffolds, the concentration and release of soluble growth factors and the clustering of cells, among others figure. The previous data of Dr Shakesheff's lab (Nottingham University) on liver epithelial cells [252] and pancreatic insulinoma RINm cells [253] pushed us to contact him to collaborate for improving the use of 3D scaffold in our protocol. The expertise and the materials of his specialized laboratory were needed, then we try to get a fellowship to realize the experiments there but we didn't succeed. Actually, scaffold could be coated with Matrigel. The achievement of our acinar differentiation protocol inside a scaffold could have mimic the morphogenetic induction of the surrounded pancreatic mesenchyme and further enhance cytodifferentiation and/or maturation.

CONCLUSIONS

- 1) Activin A treatment of derived mESC EBs for 4 days in low serum replacement concentration medium induces endoderm differentiation, while inhibits the expression of markers characteristic of the neuroectoderm and mesoderm lineages.

- 2) Retinoic Acid, Cyclopamine and FGF10 promote pancreatic endoderm specification of DE cells. At this stage, hepatic markers are also induced.

- 3) Acinar differentiation from mouse ES cells can be selectively induced by a diverse range of signalling clues including:
 - Components of the extracellular matrix such as Matrigel and laminin in a concentration manner.
 - Combination of glucocorticoids, TGF β and FGF signalling.

- 4) The integration of these findings in a stepwise protocol results in
 - A high increase in the expression of a set of digestive enzymes, including some characteristics of a more mature phenotype such as *Prss3* and *Ela2*.
 - The co-expression of Cpa1, Chymo and Amyl proteins with a typical cytoplasmic pattern.
 - The release of active amyl into the medium.

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Et en parlant de rose... Moltes gracies nena... t'estimo molt !!!

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