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IDENTIFICATION OF A NOVEL GENE EXPRESSED DURING MOUSE PANCREAS DEVELOPMENT

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Introduction

1. Pancreas: Histology

The pancreas is a compound gland of the digestive system that consists of several functionally and morphologically distinct cell populations derived from the endoderm.

The exocrine part represents 95-99% of the total pancreatic mass. It consists of:

- acinar cells, arranged into clusters at the end of the ducts, that produce digestive enzymes (amylase, lipase, phospholipase) as well as pro-enzymes (elastase, procarboxypeptidase, trypsinogen, pepsinogen, deoxyribonuclease, ribonuclease), which are stored in zymogen granules located in their apical pole;
- dutal cells, which secrete mucus, chloride and bicarbonate, and compose the complex tubular system that drains acinar secretions to the to the duodenum, for the intestinal digestion of nutrients.

The endocrine compartment is organized in islets of Langerhans scattered within the exocrine tissue, representing 1-5% of the pancreatic mass. Adult islets are composed of different cell types characterized by the production of specific hormones: the glucagon-secreting α-cell, insulin-secreting β-cell, somatostatin-releasing δ-cell, ghrelin-producing ε -cell, and finally the pancreatic polypeptide-secreting PP-cells.

Insulin and glucagon control blood glucose levels, whereas PP and ghrelin are orexigenic hormones and somatostatin regulates the secretion of insulin, glucagon and PP (Fig.1).

The endocrine cells aggregate to form the islets of Langerhans, which are intermingled with blood vessels, neurons, and a mesodermally-derived stromal component. The intimate interaction between endocrine and vascular cells regulates hormone release, establishing a fine-tuned glucose homeostasis in the body [1].

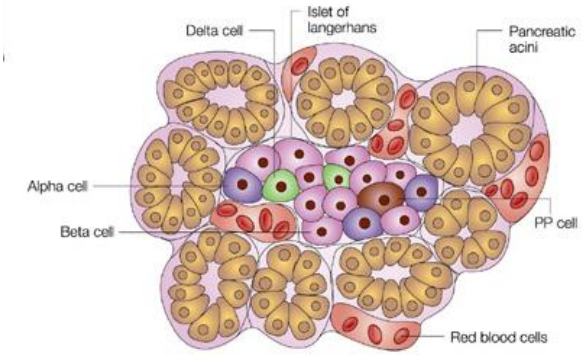


Fig.1 A pancreatic islet embedded in exocrine tissue.

2. Mouse pancreatic development

2.1 Pre-pancreatic endoderm patterning

During embryonic development, from the inner cell mass (ICM) of the blastocyst will develop the epiblast which undergoes gastrulation and forms the three principal germ layers: the ectoderm, which forms the skin and central nervous system; the mesoderm which forms the blood, heart and bones and the endoderm which forms the respiratory and digestive system [2]. The first sign of gastrulation is the formation of the primitive streak (PS) at day E5.5. It is characterized as a furrow in which epiblast cells will go on to form the mesoderm and endoderm through an epithelial-to-mesenchymal transition (EMT) [3]. *Nodal*, a member of the transforming growth factor-beta (*TGFβ*) family, promotes mesoderm and endoderm formation [4,5]. The Wnt signalling pathway is involved in the mesoderm versus endoderm cell fate choice; indeed, embryos lacking *β-catenin*, a key effector of the canonical *Wnt* pathway, have ectopic mesoderm cells in the endoderm germ layer [6].

In mouse, the transcription factors involved in endoderm formation, that act downstream of the Nodal and Wnt signals are: *Mixl1* [7], *Foxa2* [8], *Goosecoid* (Gsc) [9], *Gata4* [10] and *Sox17* [11].

In the mouse embryo at E7.5, the structure of the endoderm, consisting of 500-1000 cells, surrounds the mesoderm and ectoderm. At E8.5 the primordial epithelium of the primitive gut tube originates from the definitive endoderm, that is divided in three

parts along the anterior-posterior axis (A-P): the foregut, the midgut and the hindgut. The evaginations arising from these different domains of the foregut will give rise to organs, such as the thyroid, lung, liver, and pancreas [12,13]. Fibroblast growth factor (FGF) [14], Wnt [15], Hedgehog (Hh) [16] and Retinoic acid (RA) [17,18,19] signalling pathways have been implicated in formation and patterning of the foregut.

There are reports that FGF4 suppresses the expression of *Nkx2.1* and *Hhex*, which are required for foregut endoderm formation, and it promotes the expression of *Pdx1* and *Cdx1/Cdx2*.

In *Xenopus* embryos, Wnt/β-catenin activity must be suppressed in anterior endoderm to maintain foregut identity and to allow liver and pancreas development, whereas high Wnt/ β-catenin activity in the posterior endoderm promotes intestinal fate.

RA promotes pancreas organ allocation; indeed, blocking RA signaling inhibits pancreas specification. In *Raldh2* null mice, which lack a critical RA-synthesizing enzyme, the dorsal pancreas fails to bud and the early glucagon-positive cells do not develop. RA is also required to maintain the pancreatic progenitors and to promote the commitment of these progenitors to *Ngn3*-positive endocrine progenitors [20,21].

2.2 Dorsal pancreas induction

At E8.0, inductive signals (RA) from the paraxial mesoderm, together with suppression of *Shh* in the dorsal endoderm by FGF2 and Activin β2 from the notochord, are required to establish the dorsal pre-pancreatic domain. At first the inhibition of *Shh* is required for early pancreas specification, after its activity is needed to expand the pancreatic epithelium and to regulate the expression of insulin in the mature β-cells [22,23].

At 8.5, VEGF produced in the early dorsal aorta and vitelline veins induces the initial stages of pancreas development; it promotes the expression of *Pdx1* and *Ptf1a* in pre-pancreatic endoderm. In the absence of VEGF-A in the *Pdx1*-positive pancreatic epithelium, the endocrine cells signal back to the adjacent endothelium to induce a dense network of fenestrated capillaries within the islet [24,25].

By E9.5, condensation of the dorsal mesenchyme results in the evagination of the dorsal pancreatic rudiment. FGF10 stimulates bud outgrowth and proliferation of the pancreatic progenitor pool, inducing expression of the *Ptf1a* [26].

2.3 Ventral pancreas induction

The ventral endoderm forms ventral pancreatic endoderm receiving signals from cardiac mesoderm and the septum transversum mesenchyme (Fig. 2). Liver and ventral pancreas may diverge from an early common population of bipotential progenitors in the ventral foregut endoderm [27]. Probably, the ‘‘default fate’’ of ventral foregut endoderm is pancreas. In this way can be considered two aspects, the first is that the endodermal interaction with cardiac mesoderm is required for hepatic differentiation, and the second that the septum transversum mesenchyme produces BMPs that are necessary to induce a liver gene expression program and to allow some progenitor cells within a specific region of the ventral foregut endoderm to escape the pancreatic fate instruction.

A tissue-migration morphogenetic process allows part of the ventral endoderm to avoid hepatic-inducing signals; in fact, locking the relative movement of the ventral lateral foregut endoderm (the prospective ventral pancreas) prevents ventral pancreas specification from occurring.

Recent studies demostrate a strong link between the extrahepatobiliary (EHB) system (including gall bladder primordium, extrahepatic bile duct, cystic duct) and the ventral pancreas, more than with the liver. *Sox17*, a major regulator of endoderm formation, is required to establish and maintain distinct boundaries between the liver, EHB, and ventral pancreas domain.

Loss of *Sox17* function after gastrulation leads to biliary agenesis and ectopic pancreas formation, whereas *Sox17* misexpression suppresses pancreas development by promoting ectopic biliary-like tissue within the posterior foregut region that expresses *Pdx1*. *Sox17* upregulates *Hes1* levels in *Pdx1* expressing endoderm; *Hes1* then establishes a negative feedback loop to restrict $Sox17^{+}$ biliary

progenitors to the ventral foregut endoderm generating a boundary between EHB and ventral pancreas [28].

Later, as the gut tube forms, the prospective ventral pancreatic endoderm comes into contact with a pair of vitelline veins dorsal pancreatic mesenchyme is an important signalling tissue that allows proliferation and morphogenesis of pancreatic progenitors after the initial budding of the anlage.

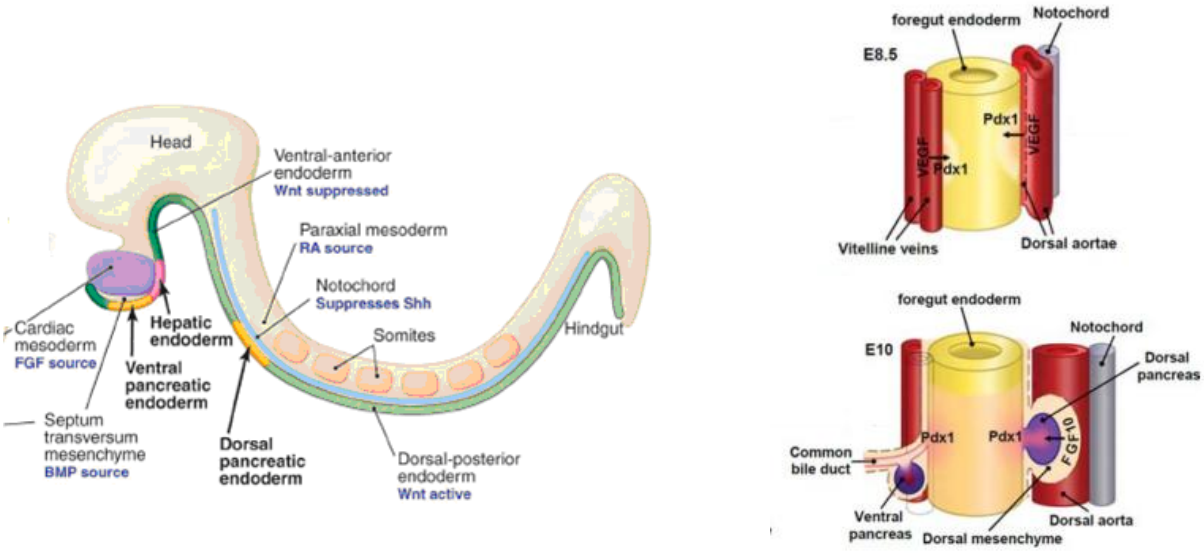


Fig.2 Schematic view of a mouse embryo showing the positions of the specified pancreas tissue domains.

2.4 The primary transition

In the mouse, pancreas development start at E9.0 when the dorsal foregut endoderm thickens and evaginates into the surrounding mesenchyme. This is followed at E9.5 by the emergence of the ventral pancreas bud from the ventral foregut endoderm. This early pancreatic epithelium comprises mainly multipotent pancreatic progenitor cells (MPC) together with a few early-differentiated ‘‘first wave’’ endocrine cells, which are mainly glucagon$^+$, although short-lived insulin$^+$ and glucagon$^+$/insulin$^+$ cells were reported [29].

In the first developmental transition (E9.5–E12.5), the epithelium of the two buds undergoes branching morphogenesis, and the compartmentalization of the

pancreatic epithelium commences, defining the epithelium into distinct ‘‘tip’’ and ‘‘trunk’’ domains. While the tip domains contain multipotential pancreatic cells (MPC), which are then believed to change into acinar-fated progenitors, the trunk domain consists of an endocrine-duct bipotential progenitor pool [30]. The comprtmentalization starting from a simple bud, the epithelium moves through a transient stratification. Tube formation starts at E10.75 when apical polarization of single cells occurs stochastically in the bud. These polarized epithelial cells undergo dramatic cell shape changes and rearrangements, forming “epithelial rosettes” that rather rapidly produce proper lumens at their common apical surface. The microlumens expand and eventually fuse, converting the epithelium through a multi-lumen intermediate into a complex epithelial network or plexus. Tubulogenesis sets up the initial tip (future acinar location) versus trunk compartmentalization. The progenitors that are in close proximity to the mesenchyme and receive a longer integrated exposure to extracellular matrix (ECM) acquire acinar fate, whereas progenitors receiving less mesenchyme exposure have a higher bias towards forming endocrine cells. At E12.0-E15.0, the tubular epithelial network expands and undergoes remodeling, generating the mature acinar and ductal cells. About the branching morphogenesis, it was proposed that ~85% of branching in these early stages was via lateral branching mechanism, and only about 15% by terminal bifurcation [31]. The lateral branching seemed frequent near the extending tip epithelium, which could include cells that have substantial “retained MPC-like”, in fact, these cells retain expression of the “primary multipotency markers” *Pdx1* and *Ptf1a*. The trunk epithelial domain, contains a pool of cells with duct/endocrine bipotency, scattered cells within the trunk epithelium upregulate *Ngn3* expression and commit to becoming endocrine precursors, that subsequently delaminate from the epithelial cord during the secondary transition. The remaining non-delaminating *HNF1β*$^{+}$ *Sox9*$^{+}$ *Nkx6.1*$^{+}$ trunk cells are fated to become duct cells. The resolution of *Nkx6.1* and *Ptf1a* expression into exclusive trunk and tip compartments, respectively, appears to correlate with the progressive restriction of trunk and tip cells towards the future duct/endocrine (trunk), or acinar (tip) lineages.

The progenitor cells allocated to the pancreas primordium in the first wave of development determines the final size of the mature pancreas [32], this type of organ size control is distinct from the ‘‘regulative programs’’ seen in other organs (e.g., liver, blood, central nervous system), in which reduction of progenitor numbers can be compensated by extensive proliferation, reduced apoptosis, with substantial effects from extrinsic factors [33].

2.5 Transcriptional program

As the buds grow, MPC progressively commit to the pancreas fate and move into a differentiated state and a high-proliferation, that reflects the expression of several TFs (transcription factors, Fig.3).

***Pdx*1** is expressed at E8.5 in the dorsal and ventral endoderm regions that give rise to pancreatic buds, as well as in the common bile duct, distal stomach, Brunner’s glands, and duodenal epithelium. *Pdx1*-expressing progenitors produce acini, ducts, and endocrine cells of the mature pancreas [34]. Pancreatic expression continues in the pancreatic epithelium and high level expression progressively becomes restricted to pancreatic *β* cells and a small subpopulation of *δ* and PP cells. Low levels are detected in acinar cells, it is required for their formation and differentiation [35,36]. In acinar cells, the Pdx1-Pbx1-Meis2 trimeric complex cooperates with PTF1 to activate the acinar-specific elastase1 promoter [37].

Furthermore, correct levels of *Pdx1* expression are important for proper pancreas formation. The precise regulation of Pdx1 levels within the various pancreatic cell types are affected largely by the conserved 5′ *cis*-regulatory elements. There are four highly conserved regions, termed Area I–II–III (the “proximal enhancer region”) and a more upstream Area IV (the “distal enhancer”) Areas I–II impart islet-specific *Pdx1* expression, while Area III working alone can confer β-cell-specific expression. Areas I–II–III harbor binding sites for many TFs such as HNF1α, Pdx1, Foxa2, Pax6, HNF6, and MafA.

Area IV and is required for expansion and differentiation of pancreatic progenitors while Area III binding is linked more to the differentiation and function of mature β-cells [38].

Dorsal pancreas development arrests after initial budding in *Pdx1* null mutants, resulting in a highly abrogated, developmentally retarded tissue, and a “ventral pancreas rudiment” cannot be found [39].

Hlxb9 is expressed in the dorsal bud prior to the dorsal induction of *Pdx1*. Between E10.5 and E12.5, its expression declines in both buds, but later is expressed in mature *β* cells. *Hb9* is an essential intrinsic signal for dorsal pancreas evagination as well as for initiation of the pancreatic program.

In mice lacking *Hlxb9*, the dorsal pancreas is entirely absent, *Pdx1* is not expressed, and the epithelium does not bud. The ventral pancreas, remarkably, develops normally until later stages. This may reflect an intrinsic difference between the dorsal and ventral buds, but because *Hlxb9* is also expressed in the notochord, which is known to promote dorsal pancreas development [40], its role in pancreas development may be indirect.

Ptf1a. The trimeric Pancreas Transcription Factor 1 complex (PTF1) comprises a tissue-specific bHLH protein, Ptf1a, the distinct mammalian Suppressor of Hairless (RBP-J) protein, or the RBP-JL paralog. *Ptf1a* is expressed as early as E9.5 in most cells of the nascent pancreatic buds, and appears to play an important role in early specification of pancreatic progenitor cells and later, in regulating exocrine differentiation. Mice lacking the *Ptf1a/p48* bHLH transcription factor exhibit normal dorsal bud formation, whereas the ventral pancreas not only fails to bud but becomes integrated into the surrounding duodenum. In addition to the complete lack of acinar cells, endocrine cell numbers are greatly reduced [41].

During early stages of organogenesis, the RBP-J form is required for pancreas specification and progenitor maintenance. PTF1-RBP-J complexes activate the expression of *RBP-JL*, and increased RBP-JL then begins to displace RBP-J, switching PTF1-RBP-J to PTF1-RBP-JL and initiating the acinar differentiation program [42]. 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.

Sox9 is a member of the SRY/HMG box (Sox) family, is involved in the proliferation, survival, and maintenance of pancreatic progenitors. *Sox9* expression is first detected at ~E10.5 in the early pancreatic MPC, and lineage tracing revealed that the "first-wave" *Sox9*$^+$ progenitors, like *Pdx1*$^+$ and *Ptf1a*$^+$ progenitors, produce cells of all three pancreatic lineages. In the adult pancreas, *Sox9* expression is maintained in a duct epithelium subpopulation. The pancreas-specific *Sox9* inactivation caused hypoplasia of both buds, through the decreased proliferation and increased cell death of progenitors, so it is essential in MPC proliferation and survival. Sox9 regulates the expression of several TF genes in the primary pancreatic MPC such as *Onecut1/Hnf6, Tcf2/Hnf1*β, *Foxa2* and *Ngn3* expression suggesting a role in initiation of endocrine differentiation [43].

Hnf1β is expressed broadly through the foregut-midgut region at E8.0, and in the liver and both pancreas anlagens at E9.5. *Hnf1*β-expressing pool is multipotent and can seed all three compartments: acinar, duct, and endocrine. During the secondary transition, *Hnf1*β expression becomes confined to the duct/endocrine bipotent progenitor domain of the central epithelial cord. In the absence of *Hnf1*β, there is the transient formation of a dorsal pancreas bud that expresses *Pdx1* and *Mnx1*, but these progenitors fail to expand; the ventral bud is absent [44]. In addition to *Ptf1a*, *Hnf1*β seems to form a transcription regulatory cascade with *Hnf6* in controlling pancreatic progenitor generation and endocrine progenitor induction [45].

Gata4 and ***Gata6*** are initially expressed broadly over the early foregut endoderm, and later in the pancreatic anlagen. During the secondary transition, *Gata4* and *Gata6* expression becomes uncoupled and restricted to distinct pancreatic domains. Gata4 expression is restricted to acini between E13.5-E18.5. In the adult pancreas, *Gata4* is expressed in a subset of α and β-cells and is no longer detectable in acinar cells. *Gata6* mRNA is detected in the bipotential central epithelial cord of the secondary transition.

In *Gata4* nulls, the ventral pancreas is not specified while the dorsal pancreas bud forms essentially normally. *Gata6* nulls exhibit a similar phenotype with a few detectable $Pdx1^+$ ventral foregut cells compared to none in the *Gata4* null [46].

Mnx1 is expressed at E8.0 within the notochord and the entire dorsal and ventral endoderm at the prospective pancreatic level, the expression remaining in both pancreatic buds and the stomach until E12.5, becoming confined to $Pax6^+$ endocrine precursors during the secondary transition. In adult pancreas, *Mnx1* is expressed in mature β-cells.

Mnx1 expression in the dorsal pancreatic rudiment precedes *Pdx1. Mnx1* null mice show dorsal bud agenesis and the normal Pdx1 upregulation during early bud formation does not occur.

Mnx1 expression in the ventral pancreatic rudiment occours after *Pdx1*. In *Mnx1* null mice, ventral pancreas development proceeds normally, producing all pancreatic cell types, but with increased δ-cells and reduced β-cell numbers that are are immature in that they lack *Pdx1*, *Nkx6.1*, and *Glut2* [47].

Ngn3 is expressed exclusively in endocrine precursor cells, and subsequently down-regulated during differentiation. *Ngn3* promotes endocrine fate in cells descended from *Pdx1* progenitor cells.

Notch signaling has two major functions during the development of the pancreas: a role in the maintenance of early progenitor cell in an undifferentiated state and a role in lateral inhibition, allowing selection of endocrine cells between non-endocrine cells. Regarding the first function, Notch signaling appears to control directly and indirectly the expression of Ngn3.

In the mechanism of lateral inhibition, Notch signaling inhibits the expression of Ngn3, leading to maintenance of the population of precursors, because preventing differentiation. This mechanism is triggered by the expression of Ngn3 in commissioned cells to differentiate, with the production of an extracellular ligand of Notch, Delta-like ligand 1 (Dll-1), which activates the NOTCH1 receptor in surrounding cells by cutting an intracellular domain of the receptor. This domain

interacts with RBP-Jκ , activating the expression of *Hes1*, which inhibits the promoter of Ngn3 [48].

Mice deficient for Notch signaling pathway components such as RBP-Jk, Dll1, or Hes1, display increased numbers of *Ngn3*-positive cells, premature endocrine differentiation and a hypoplastic pancreas that is likely the result of premature depletion of pancreatic precursor cells [49,50,51].

Hes1 encodes a bHLH transcription factor, present in the pancreas from the day E9.5. It has been observed that the number of pancreatic endocrine cells in *Hes1* null mouse increases***.*** So that Hes1 have a role in suppressing the development of endocrine cells through a pathway involving *Notch*. In *Hes1* null mouse *Ngn3* expression is high both in stomach and in intestine, confirming the conflicting effect of *Hes1* and *Ngn3* on endocrine cells development. The reduced expression of *Hes1* could, therefore, facilitate the differentiation of pancreatic endocrine cells and this hypothesis was confirmed by conversion of the developing biliary system in the pancreatic tissue in *Hes1* deficient mice [52].

Hnf6 is expressed in the epithelial cells at the stage E9.5, and subsequently its expression becomes specific of the ductal and exocrine cells.

Hnf6 mutant mice lack most *Ngn3*-expressing cells during development and instead accumulate primitive, undifferentiated epithelium; the block to endocrine differentiation in *Hnf6* mutants is only temporary, and islets begin to bud out of the abnormal epithelium shortly after birth.

Nkx2.2 is expressed early in developing pancreatic buds and is later restricted to *α*-, *β*-, and PP cells of islets. *Nkx2.2* mutants completely lack insulin expression; in place of normal β-cells, islets contain a large population of cells apparently arrested of β-cell fate.

Nkx6.1 is expressed primarily in *β*-cells of adult islets. *Nkx6.1* mutants have a phenotype with a small number of insulin-producing cells during early pancreatic development, the normally exponential increase in *β*-cell generation that initiates during the secondary transition is completely absent, and no immature β-like cells are formed.

Beta2*/*NeuroD1 is a key regulator of insulin gene transcription in pancreatic *β*-cells. *Beta2/NeuroD* expression occurs in a subset of pancreatic epithelial cells as early as E9.5, co-localizing with glucagon expression. On E14.5, expression appears within and in proximity to ductal epithelium, and by E17.5, expression is restricted to islets. Mice homozygous for a targeted disruption of the *Beta2/NeuroD* gene survive to birth, but die within 3–5 days post partum of severe hyperglycemia. The islets of these mice are dysmorphic and have markedly diminished numbers of endocrine cells arranged in streaks and irregular.

Pax4 is expressed at E9.5 in both ventral and dorsal buds of the developing pancreas, and by the time of birth, expression is restricted to *β*-cells. The *Pax4*-null mice survive birth, but die within 3 days of hyperglycemia and dehydration. There is a virtual absence of *β*- and *δ*-cells, but *α*-cells are increased. *Pdx1* expression in *Pax4*-null pancreas is absent, a finding consistent with the absence of differentiated *β*-cells. Thus, *Pax4* functions early in the development of islet cells to promote the differentiation of *β*- and *δ*-cells.

Pax6 is expressed early in the epithelium of the developing pancreas on E9.0 in both dorsal and ventral pancreatic buds and is expressed in differentiated *α*-, *β*-, *δ*-, and PP cells. Knockout *Pax6* mouse dies within minutes of birth. These animals fail to form islets. The disorganization of islets is proposed to relate to a possible function of *Pax6* in the regulation of cell adhesion molecules.

Brn4 (*Pou3f4*) regulates the promoter of the glucagon. Its expression begins in the pancreas at *E10.0*, prior to that of *Pax6*, which is believed to be the first gene that is expressed specifically in glucagon-positive cells of the pancreas. However, the defective mice to *Brn4* develop normally the endocrine portion, so that the expression of this gene is considered not necessary to determine the fate of glucagon-positive cells [53].

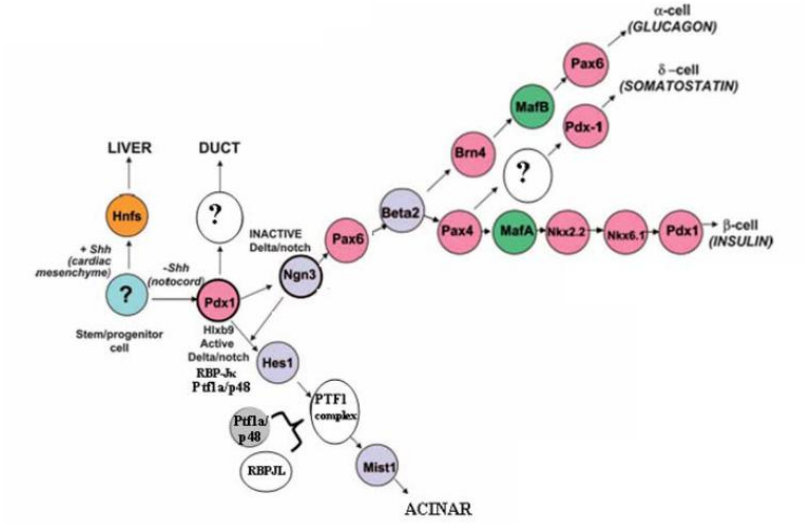


Fig.3 Flow of transcription factors during pancreatic development.

2.6 Signalling pathways involved in pancreatic development

The default fate of embryonic pancreatic epithelium is endocrine, and that this fate is redirected by mesenchymal signals to allow production of exocrine fates.

During early stages (E9.5), the fibroblast growth factor receptor 2b (*Fgfr2b*) expression is detected in the epithelium, and its high affinity ligand *Fgf10* is expressed in the pancreatic mesenchyme. Transgenic mice expressing dominant-negative *Fgfr2b* under the inducible metallothionein promoter, or mutants that lack either *Fgf10* or *Fgfr2b*, display pancreatic hypoplasia [54,55], while, excess FGF10 signaling leads to sustained Notch activation and promotes proliferation at the progenitor state [56,57].

Mesenchyme-derived BMP4/7, which signals through the epithelial ALK3 receptor, is also critical for pancreatic epithelium expansion and branching morphogenesis in mouse and chick. Inhibiting BMP signaling, either by over-expression of *Noggin* or a dominant-negative BMP receptor (*dnALK3*), causes severe pancreas hypoplasia, reduced epithelial branching, and excessive endocrine differentiation [58].

Isl1 plays a pancreas development-promoting role, both as an extrinsic factor produced by the dorsal mesenchyme, and intrinsically expressed in pancreatic progenitors. Targeted disruption of *Isl1* renders the mesenchyme unable to condense

around the dorsal pancreatic bud. In absence of *Isl1*, no endocrine cells differentiate. In addition, exocrine cells appear only in the ventral bud.

The activin-follistatin 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 [59]. Inversely, Follistatin, an activin-binding protein that acts as an antagonist, is expressed by early pancreatic mesenchyme [60]. Exogenous activin profoundly alters epithelial branching morphogenesis; it causes severe disruption of normal lobulation patterns *in vitro* whereas Follistatin counteracts its effect [61].

2.7 The secondary and third transition

Between E13 and E14, the dorsal and ventral pancreata rotate and fuse into a single organ (Fig.4). During E14.5 and E15.5, the exocrine pancreas differentiates from the ductal epithelium; at E15.5, acinar cells arise from the extending tip epithelium and continue to undergo active proliferation to increase the number of acinar tips by duplication processes acini are clearly discernible from ducts. Endocrine cells are present from the very beginning of development (E9.5), but up until E14.0 they are arrayed as single cells within the ductal epithelium, after which they undergo extensive proliferation (second developmental transition).

In general, the first endocrine cells to form mature cells are *α* cells, *Gcg*-positive cells, followed by *β* cells (E13.5), the *δ* cells (E15.5) and finally PP cells (E17.0). Once differentiated, endocrine cells leave the epithelium and migrate to the extracellular matrix, thought an epithelial-to-mesenchymal transition (EMT) where they recognize and are aggregated to form the islet of Langerhans [62]. This cluster of endocrine cells and the subsequent formation of the islet is realized (E18.5) just before the birth (third developmental transition).

In the mouse, predominantly β-cells form the islet core, and they are surrounded by mantle of α, δ, and PP-cells, whereas the very minor ε-cell population is dispersed throughout the islet. About 80% of the islet cell mass present at birth is generated by

the proliferation and differentiation of endocrine progenitors, with the other 20% coming from islet cell proliferation [63]. After birth, the predominant mechanism for islet cell mass maintenance is self-duplication. There is a shorter refractory period for β-cell proliferation observed in the first four weeks postnatally and a longer refractory period after weaning.

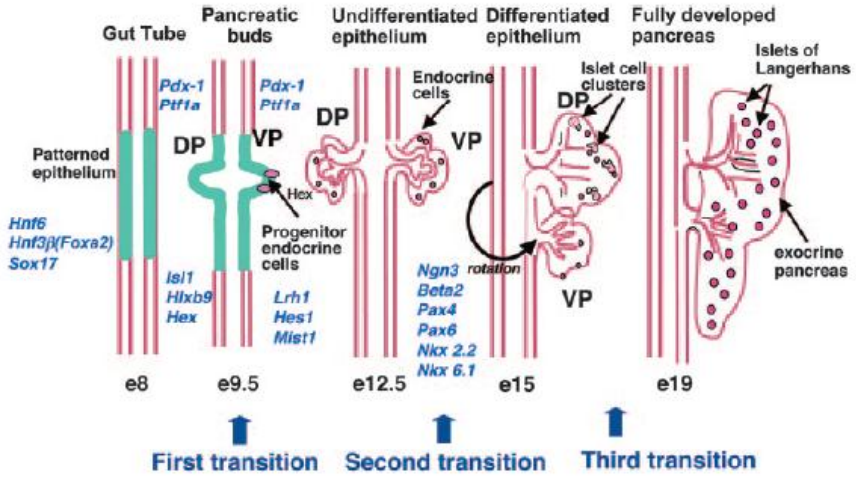


Fig.4 Schematic representation of pancreas development in mice with an indication of the main transcription factors involved.

2.8 Endocrine specification

The current model of ductal and endocrine cell fate segregation considers Notch-regulated lateral inhibition, so the endocrine progenitors expressing *Ngn3* autonomously induce the production of Notch ligands, which signal to neighboring cells and activate *Hes1* expression. *Hes1* in turn suppresses *Ngn3* expression, and, thus, inhibits endocrine fate acquisition in the neighbors. *Ngn3* initiates the endocrine developmental program by inducing pro-endocrine TFs and factors involved in delamination of the pro-endocrine cells from the epithelium. The direct targets of *Ngn3* include *NeuroD1/Beta2*, *Pax4*, *Arx*, *Insm1*, *Rfx6*, *Nkx2.2*, *Myt1*, and*Snail2/Slug*. *Ngn3* activation in a limited number of cells within the trunk epithelium to take input from TFs such as *Sox9*, *Hnf1β*, *Hnf6*, *Hnf3β*, and *Pdx1*. Mice lacking *Ngn3* function fail to develop endocrine cells [64], whereas ectopic *Ngn3* expression under the *Pdx1* promoter induces premature differentiation of the entire pancreatic bud progenitor

pool into endocrine cells with predominantly α-cells. Lineage tracing studies showed that all endocrine cell types seem to be born from Ngn3-expressing endocrine progenitors. The inducible *Ngn3* expression under the control of the *Pdx1* promoter in the *Ngn3* null background, to demonstrate that early *Ngn3*$^{+}$ progenitors (E8.75) produce α-cells, whereas endocrine progenitors acquire the competence to make β-cells and PP cells at ~E11.5 and E12.5, respectively, with much increased competence towards the β-cell fate at E14.5. Endocrine progenitors become competent to produce δ-cells at E14.5.

Three classes of endocrine regulators may be divided, based upon our current understanding of the phenotype:

- general endocrine differentiation (*NeuroD1, Islet1, Insm-1, Rfx6, Nkx6.1*).

NeuroD mutant mice have arrested endocrine cell development during the secondary transition, with reduced numbers of all endocrine cell types. The remaining endocrine cells fail to form islets [65]. Such deficiencies are also observed in Insulinoma-associated antigen 1 (*Insm1*), Regulatory X-box binding 6 (*Rfx6*), and Islet1 (*Isl1*) mutants.

Nkx6 family TF genes (*Nkx6.1* and *Nkx6.2*) could be the only non-*Ngn3* direct target that fall into this class. The *Nkx6.1* null has substantially reduced β-cell numbers while both β and α-cells are reduced in Nkx6.1/Nkx6.2 double null mice, demonstrating an intriguing overlapping yet distinct requirement for the different *Nkx6* TFs. The number of *Ngn3* endocrine progenitors and islet precursors are reduced dramatically in these mutants, suggesting that *Nkx6* might act upstream of *Ngn3*. These studies also proposed that *Nkx6* factors promote β-cell fate specification from *Pdx1* progenitors and that *Ngn3* subsequently promotes cell differentiation by initiating cell cycle exit.

- lineage-allocation (*Pdx1, Pax4, Arx, Nkx2.2*).

Lineage-specific factors control the flux of endocrine progenitors towards a specific endocrine type. As a consequence of removing factors such as *Nkx2.2, Pax4, Arx,* and *Pdx1*, endocrine cell numbers are largely unaffected, but fractional islet allocations are often severely altered. In *Pax4* null mice, β and δ-cells are lost

concomitant with a remarkable increase in α-cell and ghrelin^{+} ε-cell numbers. The increased ε-cell number is similar to the *Nkx2.2* null pancreas, suggesting possible genetic interactions between *Pax4* and *Nkx2.2*.

Pax4 and *Arx* induce, respectively, either an α-to-β or β-to-α conversion upon misexpression in terminally differentiated mature endocrine cell types. Thus, *Pax4* and Arx are required for β and α-cell fate allocation, respectively. Although Pax4 null mice also lack somatostatin+ δ-cells, a positive selective role for *Pax4* in δ-cell development remains unclear. In addition, *Pax4/Arx* double null mice show a massive increase in δ-cells, which suggests that δ-cell terminal differentiation requires the absence of *Pax4* and *Arx* [66].

Pdx1, apart from its early roles in MPC outgrowth and expansion, is also required for β-cell proliferation and survival. Deleting *Pdx1* function specifically in β-cells leads to a dramatic reduction in β-cell number concomitant with α/δ-cell overgrowth.

- maturation factors (*MafA, MafB, Foxa1, Foxa2, NeuroD1*).

Loss of these factors causes little or no defect during endocrine cell lineage diversification, but the cells begin to show aberrant function postnatally or in adulthood. During development, β-cells move from a *MafB*$^{+}$ immature state to *MafA*$^{+}$/*MafB*$^{+}$, and finally, to a *MafA*$^{+}$ mature state. Thus, *MafB* is essentially absent from mature adult β-cells and its expression is restricted to α-cells in the adult pancreas. *MafA* mutants have no effect on endocrine cell development but the mice become glucose-intolerant postnatally [67]. *MafB*$^{-/-}$ mice have reduced α and β-cell numbers, as well as some form of apparently delayed β-cell development [68].

Conditional inactivation of *Foxa2* (Foxa2-cKO) in early pancreatic epithelial precursors does not affect MPC development but leads to late-arising defects in α-cell terminal differentiation. Initial specification of α-cells in the *Foxa2*-cKO proceeds normally but differentiation is blocked with 90% reduction of glucagon expression and complete lack of prohormone convertase 2 (PC2) production in these mutant mice. The resultant neonatal *Foxa2* mutants die shortly after birth from severe hyperinsulinemic hypoglycemia.

Deleting N-cadherin in the *Pdx1* expression territory results in reduced insulin granules and impaired insulin secretion in β-cells. This suggests that cell adhesion or perhaps coordinated β-cell communication amongst the population is critical for proper regulation of insulin granule turnover.

The *NeuroD1* null islets responded poorly to glucose and displayed a glucose metabolism profile similar to immature β-cells. Therefore, *NeuroD1* is required for the complete transition to β-cell maturity, and maintenance of full glucose-responsiveness [69].

Some of the TF mutants show more than one phenotype, suggesting multiple functions during endocrine development.

2.9 Exocrine development

The mature ductal network has four subcompartments: a main duct as the common conduit to the duodenum; the interlobular ducts connected to the main duct; intralobular ducts draining exocrine enzymes into the interlobular ducts; intercalated ducts, also called terminal ducts or centroacinar cells (CAC), which connect the acini with intralobular ducts. Extensively abnormal duct development was reported in both global and pancreas-specific *Hnf6* gene inactivation [70], although these duct defects are likely relatively directly connected to an earlier problem in the development and maturation of the secondary transition trunk epithelium, and not in the differentiation program of duct cells. Indeed, the cells in the “duct epithelial” tissue of the secondary transition trunk epithelium transiently represents a bipotent pool of endocrine and duct-competent progenitors. Deleting *Pdx1* function at progressively later stages using an on-off doxycycline-regulated transgenic/knock-in system allows formation of interlobular ducts first, followed by intralobular ducts. It is possible that the large cystic ducts formed in the *Pdx1* and *Ptf1* null mutants adopted a common bile duct or intestinal character, and thereby lost the competence and microenvironment to produce the proper numbers of second-wave endocrine cells, but this hypothesis remains to be tested. Acinar differentiation starts at the distal tip epithelium at around E13.5. At this stage, the tip MPC undergo a switch to the pro-acinar fate by down-

regulating sets of progenitor-specific TFs that maintain MPC status, while upregulating expression of *Ptf1a* and various digestive enzymes [71]. In comparison to endocrine development, fewer transcription factors have been shown to be involved in acinar development. *Ptf1a* is the key player in acinar formation; PTF1RBP-J switching to PTF1RBP-JL initiates the pro-acinar differentiation program during the secondary transitionits. The loss of *Ptf1a* causes acinar agenesis. The PTF1RBP-JL complex can bind promoters of all the genes encoding secretory zymogens, including *Amylase*, *Elastases 1* and *2, CpaI*, *chymotrypsin B*, *trypsin*. *Mist1*, a bHLH factor, is required to establish proper apical-basal polarity and full acinar differentiation. Initiation of acinar development occurs in *Mist1*-deficient mice, suggesting action downstream of *Ptf1a*. However, acini in *Mist1*$^{-/-}$ mice do not acquire the typical rosette-like morphology, possibly as a consequence of loss of functional gap junctions that was attributed to reduced connexin-32 levels. To date, canonical Wnt signaling is the only reported extrinsic cue with direct implication on acinar development. Acinar differentiation fails early on in the absence of β-catenin [72].

3. Comparison between the human and mouse pancreas development

Human pancreas formation largely mimics the process in the mouse. Dorsal and ventral pancreas budding is first evident at 26–35 dpc (day post coitum), and fusion of the anlagen occurs at 6 weeks of gestation (G6w, gestation 6 weeks). The dorsal bud produces most of the head, body, and tail of the mature pancreas, whereas the ventral bud contributes to the inferior part of the head of the organ [73].

The spatio-temporal patterns of key TFs suggest that similar transcriptional regulatory mechanisms operate in both human and mice. Pdx1 is found in pancreatic progenitors broadly through the epithelium from G7w, and later in insulin-producing cells. Ngn3^{+} cells are found scattered in the epithelium at G9w, concomitant with the emergence of β-cells first at G8w, which are followed by α-cells and δ-cells at G9w. A

rare population (<5%) of insulin$^+$/glucagon$^+$ cells was also reported in the early human fetal pancreas.

Islet-like clusters appear from approximately G11w in human pancreas, the aggregated insulin and glucagon-expressing cells in the human fetal islets (around G14–16w) seem to be arranged similarly to the mouse adult islet, with a β-cell core and peripheral mantle of α and δ-cells. However, at G19w, it is proposed that there is a transient separation of the peripheral α-cells and δ-cells, away from the β-cell core, to form homogeneously mono-hormone-producing clusters. Presumably, the mono-hormone islets reintegrate amongst each other after G22w, and there is significant intermixing to generate the adult islet architecture [74].

4. Injury-Induced Reprogramming

Four main hypotheses were proposed for the origin of β-cells during the response to injury, including:

- replication of pre-existing β-cells. Under normal physiological conditions, β-cell turnover is slow and decreases progressively with age. Upon injury, it appears that at least some β-cells can re-enter the cell cycle. Under partial pancreatectomy conditions, have demonstrated that duplication of pre-existing β-cells represents a predominant mechanism of generating new β-cells in normal pancreas and under injury response [75].

- neogenesis from duct epithelium. The adult pancreatic ductal cells could give rise to insulin-producing cells upon injury, as a neogenetic birth process. Pancreatic duct ligation (PDL) is an injury model that generates tissue damage and cellular responses similar to those in acute pancreatitis. *Ngn3*$^+$ cells were induced in the duct epithelium after PDL, and thus identified a potential source of new endocrine cells *in vivo*. These *Ngn3*$^+$ duct cells, upon isolation, could produce all endocrine cell types when cultured in *Ngn3* null pancreatic bud explants *in vitro* [76].

- acini-to-endocrine or acini-to-duct-to-endocrine transdifferentiation. Several *in vitro* acinar cell culture studies demonstrated a potential acinar to endocrine/duct conversion when treated with EGF, nicotinamide, or TGF-α [77]. Upon caerulein

treatment *in vivo*, acinar cells transiently dedifferentiate to a progenitor-like state, based on the observation of *Pdx1*, *Foxa2*, *Sox9*, and *Hes1* positivity [78]. In this case, transient dedifferentiation of acinar cells is followed by acinar regeneration with no contribution of acinar lineage to ductal and endocrine lineages.

- endocrine cell interconversion, for example, α-to-β cells. With the expression of the diphtheria toxin (DT) receptor and the administration of the DT to achieve ~99% β-cell destruction, the α-cells move through a bihormonal glucagon+/insulin+ state on their way to generate authentic insulin^{+} β-cells [79].

5. *In vitro* differentiation of embryonic stem cells

The most important characteristics of Embryonic stem cells (ESCs) include the capacity to self-renew and the potential to differentiate into all embryonic cell types, a potential termed pluripotency, under *in vivo* and *in vitro* conditions. 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 murine ESCs (mESCs)in culture.

However, directed differentiation of ESCs toward specific cell lineages has proven to be a challenging task. This is in part because directed induction of a desired cell type has to be balanced with the inhibition of the formation of unwanted cell fates. These protocols have been based on the findings obtained from decades of developmental biology research that has outlined a cascade of signaling molecules and transcription factors that guide undifferentiated progenitor cells toward fully mature, functional cells.

During *in vitro* differentiation, both murine and human ESCs recapitulate early stages of embryonic development [80].

5.1 Endoderm induction

The first crucial step in the differentiation of ESCs toward the pancreatic lineage is the specification of DE.

In vivo, BMP4, Wnt and Nodal signallings 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. Then, incubation with Activin A a member of the TGFβ 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.

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. Serum at high level negatively influences endoderm induction by Activin A. 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 [81]. It was established that concentrations higher than 30 ng/ml and up to 100 ng/ml are sufficient to induce DE in mESCs.

In the differentiation studies the time and duration of Activin A application vary substantially and depend on the culture system used.

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 [82].

The formation of DE can be confirmed by testing for the up-regulation of endodermal markers, including *Sox17, Foxa2, Gata4*.

5.2 Pancreatic specification

The next desired step after the formation of primitive gut endoderm is the induction of pancreatic epithelium. Suppression of the activity of *Shh*, a member of the Hedgehog signaling pathway, is required for the initial specification of pancreatic buds *in vivo*. *Hedgehog* inhibition can be mimicked in cell culture via treatment with Cyclopamine (CYC), a cholesterol analog known to block the function of Smoothened, an essential element of the Hedgehog signaling cascade. Addition of CYC to the differentiation

media at this stage promotes the formation of pancreatic endoderm marked by the expression of *Pdx1*, the earliest marker of pancreatic epithelium, among a collection of other transcription factors important for pancreas organogenesis. All trans retinoic acid (RA) is added to promote the commitment of the *Pdx1*-positive pancreas progenitors toward the endocrine lineage over pancreatic exocrine cells. Later on, FGF signalling is implicated in the proliferation of the pancreatic progenitors at the expenses of differentiation [83].

Accordingly, d'Amour *et al.* applied successfully in ESCs 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*.

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 µM and RA, between 0.1 and 2 µM. In addition, the timing for the pancreatic specification step varies between 2, 3, 4 or 6 days.

Aim of Thesis

Cell therapy offers good prospects for the treatment of pancreatic diseases, for this reason more attention has been given to the pancreatic progenitor cells. Several studies show the improvement of differentiation of embryonic stem cells towards the definitive endoderm and pancreatic progenitors, but the identification of new molecular markers could further facilitate differentiation.

The aim of this study is to find new molecular markers involved in pancreatic specification. Therefore, our efforts will be directed towards *in vitro* and *in vivo* assessment of signature genes that would target the specification formation of pancreatic progenitor cells .

Our approach to identify *in vivo* pancreas progenitor cell markers will be based on the analysis of global gene expression profile of dorsal pancreatic buds isolated from E10.5 mouse embryos.

There is a significant gap in our knowledge on the endodermal players that act in the time window between the early stages of endoderm formation and the regionalized expression of the posterior-foregut/pancreas marker gene *Pdx1*. Development of more precise lineage-tracing tools allow a deeper focus on the functional delineation between stages and mechanisms of specification, commitment, lineage allocation, and differentiation.

In particular, conditional gene knockout strategy will be used to characterize spatio-temporal function of the putative markers during pancreatic specification.

The study will be followed by *in vitro* characterization of a candidate during differentiation of embryonic stem cells into the pancreatic lineage. We propose to develop a protocol based on the sequential activation of signaling pathways that recapitulate pancreatic development *in vitro*, through the definitive endoderm formation, the pancreatic specification.

We aim to generate genetically modified ES cell line expressing the beta-galactosidase under the promoter of the gene candidate to enable the stain and the

collection of the cell population that expresses the novel putative marker of pancreatic cell specification.

In this way, we would like to characterize both the global molecular signature of those cells, and eventually evaluate their ability to repopulate a diseased and/or damaged pancreas after current transplantation procedures.

Materials and Methods

1. Embryo dissection and embedding

Embryos were collected at E10.5 from C57/Bl6 mice; midday on the day of vaginal plug appearance was considered E0.5. All animals employed for the experiments were used in accordance with regulations and guidelines of Italy and the European Union and were approved by the local ethical committee. After dissection in cold phosphate-buffered saline (PBS), embryos were treated with 30% sucrose overnight at 4°C. They were rinsed in PBS, placed in sterile cryomolds biopsy and embedded in OCT (Sakura).

2. Immunofluorescence

8 µm thick frozen sections were collected on polylysine glass slides (Menzel-Gläser) and fixed with 4% paraformaldehyde (PFA) for 15 minutes. After, the sections were permeabilizated in 0.1% Triton X-100 in PBS (PBT) twice for 10 minutes and then fixed with 4% PFA for 5 minutes. The sections were pre-incubated in Protein block serum-free (DakoCytomation) at room temperature for 15 minutes. This product inhibits non-specific staining during the immunofluorescence detection of antigens. It may be especially useful when staining connective tissue, epithelial tissue, adipocytes, or other types of tissues susceptible to high background. The sections were incubated with primary antibodies monoclonal Rat anti-E-Cadherin 1:2500 (ECCD-2, Calbiochem), Rabbit Polyclonal anti-*Pdx1* 1:1000 (abcam) at room temperature for 2 hours; followed by the incubation with secondary antibodies Fluorescein-5-isothiocyanate (FITC) conjugated AffiniPure Donkey Anti-Rat IgG 1:200 (Jackson ImmunoResearch), Texas red dye-conjugated AffiniPure Goat Anti-Rabbit IgG 1:200 (Jackson ImmunoResearch) at room temperature for 30 minutes. Then the sections were treated with 4',6-Diamidino-2-Phenylindole, DAPI (Sigma Aldrich), diluted 1:10000 in PBS at room temperature for 5 minutes. After each incubation the sections were washed in PBS.

Slides were mounted with Fluorescent mounting medium (Dako Cytomation).

The images were Acquired with Zeiss Axioplan2 microscope at different magnifications and processed by AxioVision Rel.4.8 program.

Rat anti E-cadherin has a periplasmatic localization, it is expressed in the endodermic tissues such us pancreas, thyroid, lung, intestine.

Rabbit Polyclonal anti-*Pdx1* is a nuclear protein, it has a pancreas specific expression.

FITC is fluorescein isothiocyanate, detected as green signal (Absorption max: 492 nm; Emission max=520 nm).

Texas Red, derives from sulforodhamine and is detected as a red signal (Absorption max=596 nm; Emission max= 620 nm).

DAPI (4',6-diamidino-2-phenylindole) label DNA with blue emission.

3. Laser capture microdissection and RNA isolation

The embryos were equilibrated in the cryostat (-25°C) for 5 minutes and mounted on the specific support. They were dissected from right to left (pancreas is localized in left side) at 8 µm in thickness. When the central part of lung bud was visualized, indicating that the plane of sectioning was close to the midline, sections were collected on slides. The first section is collected on a slide destined to immunofluorescence analysis, the next three are collected on a slide for the LCM technique, and so on. The slides were immediately put on dry-ice and stored at -80°C overnight. During sectioning was minimized the room temperature section exposure time.

Before sections dehydration, all surfaces, and instruments were treated with RNase Zap (Ambion) for 5 minutes followed by 70% EtOH and 100% EtOH, to eliminate the extrinsic RNasees. The other utensils were treated, with RNase Zap for 5 minutes followed by two washings with H_2O-DEPC (water treated with 0.1% of diethylpyrocarbonate, a potent RNase inhibitor) and one washing with 100% EtOH. The utensils were keep at 60°C up to use, instead the surfaces and the instruments were allow to air dry.

In the step of dehydration, the slides were incubated sequentially in 75% EtOH (100% EtOH in ddH2O) for 30 seconds, ddH_2O for 30 seconds, 75% EtOH for 30 seconds, 95% EtOH for 30 seconds, 100% EtOH for 30 seconds and Xylene for 5 minutes. Allow slides dry for 5 minutes under a hood. Dehydration is crucial to stabilize the tissues and to allow the capture.

Laser capture microdissection (LCM) of dorsal pancreatic buds was performed in a maximum time of 60 minutes after dehydration and with a 36% of relative humidity in the room [84]. These parameters are important because a high ambient humidity could rehydrate the sections and reactivate endogenous RNases and the absorption of moisture of the atmosphere seems to cause the degradation of RNA and an LCM inefficient. LCM was performed using the PixCell II system (Arcturus) under 20x magnification with a laser spot size of 10 µm, laser output power of 100 mW and pulse duration of 1.5 ms. The LCM technique is performed by placing a thermoplastic film 12 µm above the dehydrated tissue section, this position serves to maintain the capture area above the surface of the section, avoiding direct contact. The cells of interest are localized with an inverted microscope, and on cells are directed pulses generated by an infrared laser at low energy to avoid damaging the tissue and therefore cells from which to extract RNA. Dorsal pancreas buds dissected from three embryos were captured with one cap. After that, the whole sections without pancreatic buds were scraped with a scalpel and collected in a 0,5 ml tube. Then, a cell extraction device ExtractSure (Arcturus) is placed on the film, creating a room for the extraction buffer. The extraction buffer, pre-heated at 42°C, should be applied to the cells immediately after the LCM to stabilize the RNA.

According to manufacturer's protocol of Pico-Pure RNA isolation kit:

Dispense 10 µl of Extraction Buffer (XB) on each sample and place a new 0.5 ml microcentrifuge tube onto the CapSure-ExtracSure assembly and incubate at 42°C in the thermoblock for 30 minutes. Centrifuge the microcentrifuge tube with the CapSure-ExtracSure assembly at 800 g for 2 minutes to collect cell extract into the same. During the incubation of the samples, the RNA Purification Column needs to be pre-conditioned. Pipette 250 µl of Conditioning Buffer (CB) onto the purification

column filter membrane. Incubate the RNA Purification Column with Conditioning Buffer for 5 minutes at room temperature. Centrifuge the purification column in the provided collection tube at 16000 g for 1 minute. After that is possible to proceed with the RNA isolation protocol. Pipette 10 µl of 70% EthOH into the cell extract, mix well by pipetting up and down and do not centrifuge. Pipette the cell extract and EtOH mixture into the preconditioned purification column.

To bind RNA, centrifuge for 2 minutes at 100 g, immediately followed by a centrifugation at 16000 g for 30 seconds to remove flow through. Pipette 100 µL Wash Buffer 1 (W1) into the purification column and centrifuge for 1 minute at 8000 g. At this step DNA may be removed. The DNase treatment utilizing the RNase-Free DNase Set (Qiagen). Pipette 5 µL DNase I Stock Solution to 35 µl Buffer RDD (provided with RNase-Free DNase Set). Mix by gently inverting. Pipette the 40 µl DNase incubation mix directly into the purification column membrane. Incubate at room temperature for 15 minutes. Finally pipette 40 µl PicoPure RNA Kit Wash Buffer 1 (W1) into the purification column membrane. Centrifuge at 8000g for 15 seconds and proceed with the RNA isolation protocol. Pipette 100 µl Wash Buffer 2 (W2) into the purification column and centrifuge for one minute at 8000 g. Pipette another 100 µl Wash Buffer 2 (W2) into the purification column and centrifuge for 3 minutes at 16000 g. Transfer the purification column to a new 0.5 ml microcentrifuge tube provided in the kit. Pipette 20 µl of Elution Buffer (EB) directly onto the membrane of the purification column (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of EB into the membrane) and incubate the column for 1 minute at room temperature. Centrifuge the column for 1 minute at 1000 g to distribute EB in the column, and then spin for 1 minute at 16000 g to elute RNA. The entire sample may be used immediately or stored at –80°C until use.

RNA yields were about 5 ng from 3 dorsal buds. RNA quality and integrity was determined by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) on RNA Pico Chips (Agilent Technologies).

According to manufacturer's protocol:

Cleaning the electrodes

Before running assay decontaminate the electrodes through the chip washing for 5 minutes with 350 µl of RNase Zap. Wash the pins with another chip washing for 2 minutes with 350 µl of RNase free water. To dry the pins leave the lid open for 2 minutes.

Preparing gel matrix

For the preparation of gel matrix allow all reagents to equilibrate to room temperature for 30 minutes. Place 600 µl of RNA 6000 Pico gel matrix (red) into the top receptacle of a spin filter and centrifuge for 10 minutes at 4000 rpm (rate is stable for one month).

Preparing the Gel-Dye Mix

Vortex and spin the tube RNA 6000 Pico dye concentrate (blue). Aliquot 65 µl filtered gel (gel matrix) in a tube 0.5 ml. Add 1 µl of RNA 6000 Pico dye concentrate to an aliquot of filtered gel (gel matrix), centrifuge for 10 minutes at 13000 rpm and keep in the dark.

Loading the Gel-Dye Mix

Denature 1.5 µl of each sample for 2 minutes at 70°C. Take an RNA Pico chip and place it on the chamber pressure. Pipette 9 µl of the gel-dye mix at the bottom of the well marked G and dispense the gel-dye mix. Set the timer to 30 seconds, make sure that the plunger is positioned at 1 ml and then close the chip priming station. Press the plunger of the syringe down until it is held the clip. Wait 30 seconds and release the clip. Slowly pull back the plunger of the syringe to 1 ml position. Open the chamber and pipette another 9 µl gel-dye mix in the other 2 wells G. Pipette 9 µl of the RNA 6000 Pico conditioning solution (white) into the well marked CS. Pipette 5 µl of RNA 6000 Pico Marker (green) into the 11 sample wells and the well marked with a ladder symbols. Load 1 µl of the diluted RNA 6000 Pico ladder into the well marked with the ladder symbols and 1 µl of each sample into each of the 11 sample wells. Centrifuge 1 minute at 2400 rpm the chip on vortex, and then insert it into the machine.

Using the software

The 2100 expert software screen shows that you have inserted a chip and closed the lid by displaying the chip icon at the top left of the Instrument context. In the Instrument context, select the appropriate Assay about RNA and the type of chip (in this case, Pico).

Click the Start button and wait for the outcome of the run. Assess the integrity of RNA through the presence 18s and 28s bands. The Bioanalyzer uses software that implements an algorithm of analysis obtaining from each electrophoresis a number, the RIN (RNA integrity number), representative of the quality of the sample (1, low quality; 10, highest quality). The quality of RNA extracted from cells obtained by LCM showed bands 18s and 28s, indicating that there is not deterioration in the quality of RNA and a good value of the RIN.

Three biological replicates with RNA integrity numbers (RIN) higher than 7 were considered to obtain labeled sense-strand cDNA (ss cDNA).

4. RNA amplification, labeling and hybridization

RNA samples obtained by LCM were amplified with the RiboAmp HS PLUS RNA Amplification Kit (Arcturus), for the second cycle of cDNA amplification and hydrolysis was used the Ambion WT Expression Kit (Ambion) and finally, to labeling ss cDNA was used the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix). The RiboAmp HS PLUS Kit provides sensitivity in linear amplification of small samples and enables the production of microgram quantities of amplified antisense RNA (aRNA) from picogram or low nanogram amounts of total RNA. The total RNA input recommended for RiboAmp amplification is 500 pg to 5 ng that allow to obtain over than 30 µg of aRNA. The success of amplification using the RiboAmp HS PLUS RNA Amplification Kit depends on the quality of the source RNA. Integrity is affected by exposure to internal and external sources of RNases. The three kits listed above generate labeled ss cDNA ready for microarray experiments and other downstream analysis methods. RNA amplification and labeling can be illustrated as in figure (Fig.5).

The RiboAmp HS PLUS RNA Amplification Kit utilizes two rounds of a five-step process for linear amplification:

1. 1st strand synthesis reaction that yields cDNA incorporating a T7 promoter sequence;
2. 2nd strand synthesis reaction utilizing exogenous primers that yields double-stranded cDNA;
3. cDNA purification using specially designed MiraCol™ Purification Columns;
4. *In vitro* transcription (IVT) utilizing T7 RNA polymerase yields antisense RNA (aRNA);
5. aRNA isolation with the MiraCol Purification Columns.

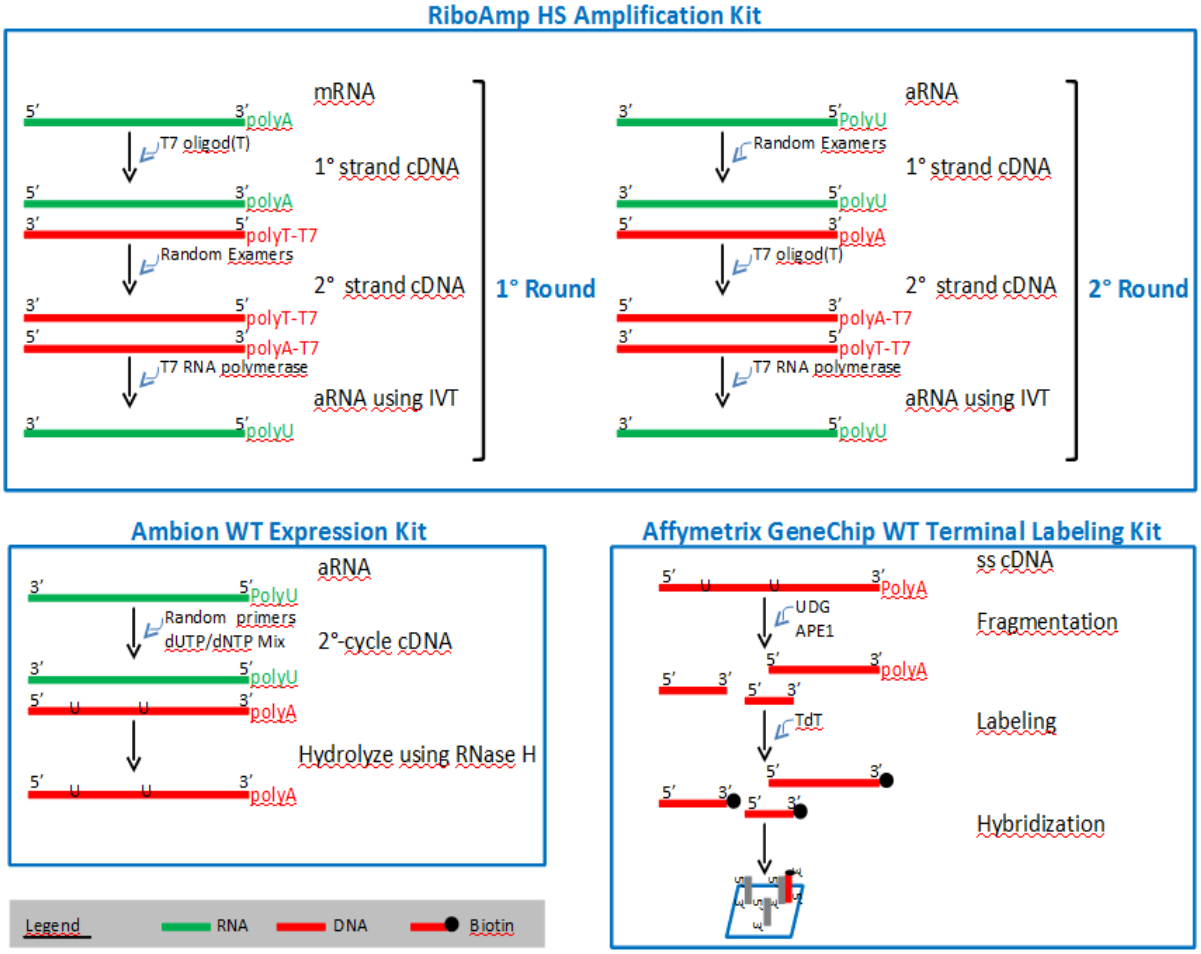


Fig. 5 Molecular events that occurs during RNA Amplification, Labeling and Hybridization.

According to manufacturer's protocol:

Round 1: first strand cDNA synthesis

RNA input amount was 4.5 ng. The input samples were prepared with H_2O DEPC in a final volume of 10 µl in 0.2 ml tubes and put at 4°C. Then add to each sample 1 µl of Primer 1 (Grey), titillate and spin. Incubate at 65°C for 5 minutes and then at 4°C for 1 minute. At the end spin and put in the cold block (4°C).

Prepare the first strand synthesis mix. The mix for a single sample is composed of 2 µl Enhancer (Yellow E), 5 µl 1st strand master mix (Red-1), 1 µl 1st strand enzyme mix (Red-2) and 1 µl SuperScript III Enzyme Invitrogen (200 U/µl). Titillate and spin the mix and add 9 µl of the mix to each sample. Incubate the samples at 42°C for 1 hour followed by 4°C for 1 minute. Then spin the samples and put them in the cold block.

Add 2 µl of 1st Strand Nuclease Mix (Gold) to the samples and incubate at 37°C for 30 minutes, 95°C for 5 minutes and 4°C for 1 minute. Spin the samples and put them in the cold block.

Round 1: second strand cDNA synthesis

Add to the samples 1 µl of Primer 2 (Grey-2), titillate, spin and incubate at 95°C for 2 minutes followed by 4°C for 2 minutes. Spin the samples and put them in the cold block. Prepare the second strand synthesis mix. The mix for a single sample is composed of: 29 µl of 2nd strand Master Mix (White-1) and 1 µl of 2nd Strand Enzyme Mix (White-2). Titillate and spin the mix and add 30 µl of the mix to each sample. Incubate the sample at 25°C for 10 minutes, 37°C for 30 minutes, 70°C for 5 minutes and 4°C for 2 minutes.

Round 1: cDNA purification

Incubate the purification column with 250 µl of DNA Binding Buffer (DB) for 5 minutes and then centrifuge at 16000 g for 1 minute.

Add 200 µl of DB to each sample, mix up and down and pipette the whole sample on

a column. Centrifuge at 100 g for 2 minutes followed by 10000 g for 1 minute. Eliminate the eluted DB.

Add 250 µl of DNA Wash Buffer to each sample and centrifuge at 16000 x g for 3 minutes. Put the column on a 0,5 ml tube and add 11 µl of Elution Buffer (DE). Incubate at room temperature for 1 minute and then centrifuge at 1000 g for 1 minute and 1 minute at 16000 g.

Round one: in vitro transcription

Prepare the IVT Reaction Mix. The mix for a single sample is composed of: 2 µl of IVT Buffer (Blue-1), 6 µl of IVT Master Mix (Blue-2), 2 µl of IVT Enzyme Mix (Blue-3) and 2 µl of Enhancer (Yellow-E), titillate and spin the mix. Add 12 µl of the mix to each sample and incubate at 42°C for 6 hours and at 4°C for 2 minutes or overnight.

Add 1 µl of DNase Mix (Blue-4) to each sample, titillate, spin and incubate at 37°C for 15 minutes and then put the samples on a cold block. Proceed immediately with the aRNA purification.

Round one: aRNA purification

Incubate the purification column with 250 µl of RNA Binding Buffer (RB) for 5 minutes at room temperature and then centrifuge at 16000 g for 1 minute. Add 120 µl of RB to the samples, mix up and down. Pipette the whole sample on the membrane of the column and centrifuge at 100 g for 2 minutes followed by 1 minute at 10000 g. Eliminate the eluted RB.

Add 200 µl of RNA Wash Buffer (RW) to each sample and centrifuge at 16000 g for 3 minutes.

Put the column on a 0.5 ml tube and add 12 µl of RNA Elution Buffer (RE). Incubate at room temperature for 1 minute and then centrifuge at 1000 g for 1 minute and 1 minute at 16000 g.

Round 2: 1st strand cDNA synthesis

Add 1 µl of Primer 2 (Grey) to each sample, titillate, spin and incubate at 65°C for 5

minutes followed by 4°C for 1 minute. Spin the samples and put them in a cold block. Prepare the 1st Strand Synthesis Mix. The mix for a single sample is composed of: 2 µl of Enhancer (Yellow-E), 5 µl of 1st Strand Master Mix (Red-1), 1 µl of 1st Strand Enzyme Mix (Red-), 1 µl of SuperScript III Enzyme. Titillate and spin the mix. Add 9 µl of the mix to each sample, titillate, spin and incubate at 25°C for 10 minutes, 37°C for 1 hour and 4°C for 1 minute.

Round 2: 2nd strand cDNA synthesis

Add 1 µl of Primer 3 (Grey) to each sample, titillate, spin and incubate at 95°C for 5 minutes and at 4°C for 2 minutes.

Prepare the 2nd Strand Synthesis Mix. The mix for a single sample is composed of: 29 µl of 2nd Strand Master Mix and 1 µl of 2nd Strand Enzyme Mix. Titillate and spin the mix. Add 30 µl of the mix to each sample, titillate spin and incubate at 37°C for 30 minutes, followed by 70°C for 5 minutes and 4°C for 30 minutes.

Round 2: cDNA purification

Incubate the purification column with 250 µl of DNA Binding Buffer (DB) for 5 minutes and then centrifuge at 16000 g for 1 minute.

Add 200 µl of DB to each sample, mix up and down and pipette the whole sample on a column. Centrifuge at 100 g for 2 minutes followed by 10000 g for 1 minute. Eliminate the eluted DB.

Add 250 µl of DNA Wash Buffer to each sample and centrifuge at 16000 g for 3 minutes. Put the column on a 0.5 ml tube and add 11 µl of Elution Buffer (DE). Incubate at room temperature for 1 minute and then centrifuge at 1000 g for 1 minute and 1 minute at 16000 g.

Round 2: in vitro transcription

Prepare the IVT Reaction Mix. The mix for a single sample is composed of: 2 µl of IVT Buffer (Blue-1), 6 µl of IVT Master Mix (Blue-2), 2 µl of IVT Enzyme Mix (Blue-3) and 2 µl of Enhancer (Yellow-E), titillate and spin the mix. Add 12 µl of the mix to

each sample and incubate at 42°C for 6 hours followed by 4°C for 10 minutes.
Add 1 µl of DNase Mix (Blue-4) to each sample, titillate, spin and incubate at 37°C for 15 minutes and then put the samples on a cold block. Proceed immediately with the aRNA purification.

Round 2: aRNA purification
Incubate the purification column with 250 µl of RNA Binding Buffer (RB) for 5 minutes at room temperature and then centrifuge at 16000 g per 1 minute. Add 120 µl of RB to the samples, mix up and down. Pipette the whole sample on the membrane of the column and centrifuge at 100 g for 2 minutes followed by 1 minute at 10000 g. Eliminate the eluted RB.
Add 200 µl of RNA Wash Buffer (RW) to each sample and centrifuge at 16000 g for 3 minutes.
Put the column on a 0,5 ml tube and add 30 µl of RNA Elution Buffer (RE). Incubate at room temperature for 1 minute and then centrifuge at 1000 g for 1 minute and 1 minute at 16000 g. Store the samples at -80°C overnight.
At this step samples can be quantized with the Nanodrop instrument.
Using Ambion WT Expression Kit, sense-strand cDNA is synthesized by the reverse transcription of aRNA using random primers. The sense-strand cDNA contains dUTP at a fixed ratio relative to dTTP. 10 µg of aRNA is required for 2nd-cycle cDNA synthesis.

According to manufacturer's protocol:
Synthesize 2nd-cycle cDNA
Prepare aRNA samples in a final volume of 22 µl (in Elution Buffer-Arcturus) in which the final amount of aRNA is 10 µg. Add 2 µl of Random Primers and incubate at 70°C for 5 minutes, followed by 25°C for 5 minutes and 4°C for 2 minutes.
Prepare 2nd cycle Master Mix. A mix for a single sample is composed of: 8 µl of 2nd Cycle Buffer Mix and 8 µl of 2nd Cycle Enzyme Mix. Titillate and spin the mix. Add 16 µl of the mix to each sample, titillate, spin and incubate at 25°C for 10 minutes, 42°C

for 90 minutes, 70°C for 10 minutes and 4°C for 2 minutes.

Hydrolyze using RNase H

In this procedure, RNase H degrades the aRNA template leaving single-stranded cDNA.

Add 2 µl of RNase H to each sample and incubate at 37°C for 45 minutes followed by 95°C for 5 minutes and 4°C for 2 minutes.

Purify 2nd cycle cDNA

After synthesis, the second-strand cDNA is purified to remove enzymes, salts, and unincorporated dNTPs. This step prepares the cDNA for fragmentation and labeling. Before beginning the cDNA purification pre-heat the Elution Solution at 58°C for 10 minutes. Vortex the Nucleic Acid Binding Beads vigorously before use to ensure they are fully dispersed. Add to each sample 18 µl of H_2O Nuclease-Free, 10 µl of Nucleic Acid Binding Beads and 50 µl of Nucleic Acid Buffer Concentrate, for a final volume of 60 µl. Transfer each sample to a well of a U-bottom plate. Add 120 µl of 100% EtOH to each sample. Pipette up and down 3 times to mix. Gently shake for 2 minutes on the thermomixer. The cDNA in the sample binds to the Nucleic Acid Binding Beads during this incubation. Move the plate to a magnetic stand to capture the magnetic beads. When the capture is complete (~5 minutes), the mixture is transparent, and the Nucleic Acid Binding Beads form pellets against the magnets in the magnetic stand. Carefully aspirate and discard the supernatant without disturbing the magnetic beads.

Add 100 µl of Nucleic Acid Wash Solution to each sample, then shake the samples at moderate speed for 1 minute. Move the plate to a magnetic stand to capture the Nucleic Acid Binding Beads. Carefully aspirate and discard the supernatant without disturbing the Nucleic Acid Binding Beads. Move the plate to a shaker, then shake the plate vigorously for 1 minute to evaporate residual ethanol from the beads. Dry the solution until no liquid is visible. Elute the purified cDNA from the Nucleic Acid Binding Beads by adding 30 µl of pre-heated Elution Solution to each sample.

Incubate for 2 minutes at room temperature without shaking. Vigorously shake the plate for 3 minutes. Transfer the supernatant, which contains the eluted cDNA, to a nuclease-free multiwell plate.

Using the Affymetrix GeneChip WT Terminal Labeling Kit is possible to fragment, label and hybridize the ss cDNA samples on the GeneChip® Mouse Gene 1.0 ST Array. The latter is the product in the family of Affymetrix expression arrays offering whole-transcript coverage. Each of the 28.853 genes is represented on the array by approximately 27 probes spread across the full length of the gene, providing a more complete and more accurate picture of gene expression than 3'-based expression array designs.

According to manufacturer's protocol:

ss cDNA fragmentation

Prepare the ss cDNA in a final volume of 31.2 µl (in H_2O RNase Free) in which the final amount of ss cDNA is 5l5 µg. Add for each sample 10 µl of H_2O RNase free, 48 µl of 10x cDNA Fragmentation Buffer, 1 µl of UDG (10 U/µl) and 1 µl of APE (1000 U/µl). Incubate at 37°C for 60 minutes, 93°C for 2 minutes and 4°C for 2 minutes.

ss cDNA labeling

Add to each sample 12 µl of 5X TdT Buffer, 2 µl of TdT and 1 µl of DNA Labeling Reagent (5 mM). Incubate at 37°C for 60 minutes, 70°C for 10 minutes and 4°C for 2 minutes.

Hybridization

Each sample was hybridized on a GeneChip® Mouse Gene 1.0 ST Array at 42°C for 16 hours. Chips were then washed and scanned.

5. RNA extraction from embryos or cell cultures

In order to perform total RNA extraction, the samples were collected and preserved in TRIzol (Invitrogen, a monophasic solutions of phenol and guanidine isothiocyanate suitable for isolating total RNA, DNA and proteins). After homogenization in TRIzol, the samples were treated with 200 µl of chloroform, vortexed for 15 seconds and centrifuged at 12000 rpm for 10 minutes; this step is required to remove any trace of phenol. In order to precipitate RNA, 500 µl of isopropanol were added to the upper phase obtained from the previous step; the samples were incubated for 10 minutes at room temperature and centrifuged at 12000 rpm for 10 minutes. After the removal of the surnatant, pellets were washed with 1ml of EtOH 75% for 10 minutes at 14000 rpm and then resuspended in H_2O-DEPC.

RNA qualitative control by denaturing agarose gel electrophoresis

To check the integrity of RNA, each sample was analyzed by electrophoresis on agarose gel in denaturing conditions. Agarose was dissolved in H_2O-DEPC, then MOPS buffer 1x (MOPS buffer 10x: MOPS pH 7.0 200 mM, NaAc 200 mM, EDTA pH 8.0 10 mM) and of 37% formaldheide (J.T.Baker, the denaturing agent), were added. Samples (~800 ng) RNA were prepared adding 3 volumes of Loading dye 1x (500µl formamide, 100 µl MOPS 10x, 150 µl 37% formaldehyde, 100 µl DYE 10x, 1 µl ethidium bromide 10 mg/ml). Samples were denatured at 65°C for 5 minutes and loaded on the gel; the electrophoresis was performed at 100 V for about 30 minutes.

6. RT–PCR and quantitative PCR (qPCR) Analyses

One microgram of RNA was reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen) to obtain cDNA in 200 µl, 5 µl of which (25 ng per reaction) was used for qualitative PCR and quantitative PCR analysis.

According to manufacturer's protocol:

Elimination of genomic DNA

The purified RNA sample is briefly incubated in gDNA Wipeout Buffer at 42°C for 2 minutes to effectively remove contaminating genomic DNA. In contrast to other methods, the RNA sample is then used directly in reverse transcription.

Reverse transcription

After genomic DNA elimination, the RNA sample is ready for reverse transcription using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction takes place at 42°C for 30 minutes and is then inactivated at 95°C for 3 minutes.

Primers were designed with the Primer 3 program and their sequences are listed in Table 1.

PCR reactions were performed using a commercial Taq DNA polymerase and reaction conditions were as follows: initial denaturation at 95°C for 5 minutes, then for 35 cycles of amplification (30 seconds at denatured temperature of 94°C, followed by 30 seconds at annealing temperature of 60°C and 30 seconds at extension temperature of 72°C) and 10 minutes at 72°C for a final extension. The PCR products were analyzed using 2 % agarose gel electrophoresis and visualized by staining with ethidium bromide.

Quantitative PCR were performed in duplicate with the power SYBR Green PCR Master Mix (Applied Biosystems). The number of cycles of threshold (Ct) was measured with 7900 HT System (Applied Biosystems). The Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold as PCR amplification proceeds. The data were processed using SDS 2.3 software. The distinct dissociation curve of each product was used to determine the specificity of its amplification. All quantifications (ΔCt) were normalized with the *Gapdh* mRNA level, then the fold induction was calculated by the ΔΔCt method as described in the original paper [85].

Table 1

Oligo name	Sequence 5' to 3'	Oligo name	Sequence 5' to 3'
Bex1_Fw	ACCTGGTGGTGAGCATCTCT	Primer 3_Fw	CAATGCTACAGGCTCATGGA
Bex1_Rv	CCTCCTCTAGGTGCACAGTTTT	Primer 4_Rv	GTGCTGAAGGCGATTAAGT
Churc1_Fw	GAAAACTGCGTGACGATGTG	Pdx1_Fw	GCTCACCTCCACCACCACCTTC
Churc1_Rv	TTCTCCATCTTCCTCTTTCAGG	Pdx1_Rv	GGGTCCTCTTGTTTTCCTCGGG
Fat3_Fw	ACTGCCCATGATGAGGATTC	Prdxdd1_Fw	TGCGGGCTGAGTTAGAACA
Fat3_Rv	GGTGGAATAGAGAGGGACCAC	Prdxdd1_Rv_iso1	TTTTGTTTGTGGCACAAGCG
Foxa2_Fw	CTGGGAGCCGTGAAGATGGAAG	Prdxdd1_Rv_iso1_ iso2	CCCAACTCCTAACTGCTTGC
Foxa2_Rv	TCCAGCGCCCACATAGGATG	Pyy_Fw	GTCGCAATGCTGCTAATCCT
Gapdh_Fw	AATGGTGAAGGTCGGTGTG	Pyy_Rv	GTCGCTGTCGTCTGTGAAGA
Gapdh_Rv	GAAGATGGTGATGGGCTTCC	Rbm39_Fw	AACGGCCATGAAGAACGTAG
Gcg_Fw	GAAGACAAACGCCACTCACA	Rbm39_Rv	GGCCTCGGAATCTTCTATCC
Gcg_Rv	CTGGCCCTCCAAGTAAGAACT	Rnf160_Fw	CTGTGATGTGGTCCATAGCTTC
Ghrelin_Fw	TCATCTGTCCTCACCACCAA	Rnf160_Rv	TCCTTCAGCCACAGAACAGA
Ghrelin_Rv	GCTTGTCCTCTGTCCTCTGG	Secretin_Fw	AGGACCCCAAGACACTCAGA
Hnf6_Fw	CAAAGAGGTGGCGCAGCGTATC	Secretin_Rev	GGCAGAAGCCAGTCCTGTAG
Hnf6_Rv	GCTCTTTCCGTTTGCAGGCTG	Slc10a4_Fw	TGCTGTCCTGGTGGAAATCT
Nanog_Fw	AACCAGTGGTTGAAGACTAGCAATG GTC	Slc10a4_Rv	ACGCCTAGCCCAATAGGAAT
Nanog_Rv	TTCCAGATGCGTTCACCAGATAGC	Slc2a1_Fw	ACTGGGCAAGTCCTTTGAGA
Nepn_Fw	AACCTCTGTGTTGGACAATGC	Slc2a1_Rv	GGCTGGGATGAAGATGACAC
Nepn_Probe _Fw	TACATGAGCATGGAGAACAACC	Sox17_Fw	GCCGATGAACGCCTTTATGGTG
Nepn_Probe_Rv_SP6	GCGATTTAGGTGACACTATAGCTGG CTCGTAGGAGACTGCAA	Sox17_Rv	CATGCGCTTCACCTGCTTGC
Nepn_RV	TCAGAGTTTTGAAGGTGTCATTTT	Tff3_Fw	CCTGTGCAGTGGTCCTGAA
Nkx6.1_Fw	ACTTGGCAGGACCAGAGAGA	Tff3_Probe_Fw	GGATAGCTGCAGATTACGTTGGCC
Nkx6.1_Rv	AGAGTTCGGGTCCAGAGGTT	Tff3_Probe_Rv_SP6	CGATTTAGGTGACACTATAGAGCAATC AGATCAGCCTTGTGTTG

Oligo name	Sequence 5' to 3'	Oligo name	Sequence 5' to 3'
Oct4_Fw	CCGTGTGAGGTGGAGTCTGGAGAC	Tff3_Rv	CAGGGCACATTTGGGATACT
Oct4_Rv	CGCCGGTTACAGAACCATACTCG	Tmem50b_Fw	GGCCGGAGTGTGAGTGTATT
Pcbd1_Fw	AGGCCGAGATGCTATCTTCA	Tmem50b_Rv	ATAGCTGTCACCTCGCACCT
Pcbd1_Rv	CACATTCATGGGTGCTCAAG		
Primer 1_Fw	CACTATTGCCAGTCAATACAAGTCT ACGAG		
Primer 2_Rv	CACAACGGGTTCTTCTGTTAGTCC		

7. In situ Hybridization

Digoxigenin labeled RNA probes

Templates for riboprobes were generated by PCR from cDNA obtained by reverse transcription of mRNA from E10.5 mouse embryos using forward and reverse primers extended at their 5′ ends with either SP6 promoter sequences (CGATTTAGGTGACACTATAGA). The resulting PCR products were resolved on agarose gels and desired bands excised and purified using the QIAGEN gel extraction kit (QIAGEN). The purified products were used to obtain a new amplification, in this way the products can be purified using QIAquick PCR purification kit (QIAGEN), analyzed on an agarose gel.

Digoxygenin labeled riboprobes were generated by *in vitro* transcription using DIG-labeling RNA kit (Roche); in each reaction the follow reagents were added:

Template DNA	0.5 µg
RNA labeling mixture (DIG)	2 µl
10 x transcription buffer	2 µl
RNase inhibitor (40 U/µl)	0.5 µl
RNA polymerase (SP6 20 U/µl)	2 µl
Adjust with DEPC-treated water to total	20 µl

The reactions were mixed gently, briefly centrifuged and incubated for 2 hours at 37°C. After the incubation with 2 µl of DNase I (2 U/µl) at 37°C for 15 minutes, the reaction was stopped with 50 µl of STE and purified in equilibrate Quick Spin Columns (Roche) at 1000 g for 4 minutes.
To check the integrity of RNA probes, each sample was analyzed by electrophoresis on agarose gel in denaturing conditions.

Hybridization
E10.5 embryos of wild-type C57/Bl6 mice were fixated in 4 % PFA, cryoprotected in 30% sucrose, embedded in OCT and stored at - 80°C. Frozen sections (10µm) were collected on Superfrost slides (Mentzel Gläser). *In situ* hybridization of frozen sections was performed following the protocol previously described [86]. Briefly, sections were fixed in 4% PFA, washed in PBS and treated with 1 µg/ml of Proteinase K (PK) in PBS for 10 minutes at room temperature. After pre-treatment with PK, sections were washed in PBS and subjected to acetylation step using 0.25% Acetic Anhydride in 1M Triethanolamine-HCl for 10 minutes.
Thereafter, slides were hybridized over night (50% Formamide; 5x SSC, pH 4.5; 50 µg/ml yeast tRNA; 1% SDS; 50 µg/ml Heparin) using a probe concentration of 0.5-1 µg/ml at 68°C.
For the detection of hybridization, sections were incubated with anti-digoxigenin alkaline phosphatase-conjugated Fab fragments (Roche) at 1:4000 dilution. Staining was development according to probe-signal, with NBT/BCIP (Roche). Finally, slides were fixed in 4% PFA - 0.2% gluteraldehyde and mounted in Dako Glycerol Mounting Medium (DakoCytomation) .
Images were obtained using an Axioplan2 microscope equipped with an Axiocam digital camera (Zeiss). Images were processed using the Axion Vision software and Image J software.

8. ES cell culture and differentiation

JM8A cell line are considered feeder independent. These cells are derived from C57BL/6N mice and the Agouti allele has been modified to correct the black

mutation. Mice derived from these cells will have Agouti coat color and are heterozygous for the Agouti allele (A/a). The Nepn mouse strain used for this research project was created from ES cell clone EPD0686_5_C01 obtained from Komp Repository and generated by the Wellcome Trust Sanger Institute (Fig.6). This gene has 2 wild type transcripts, of which 2 are protein-coding. Following removal of the floxed region, 2 transcripts are predicted to produce a truncated protein product of which 0 may be subject to non-sense mediated decay (NMD).

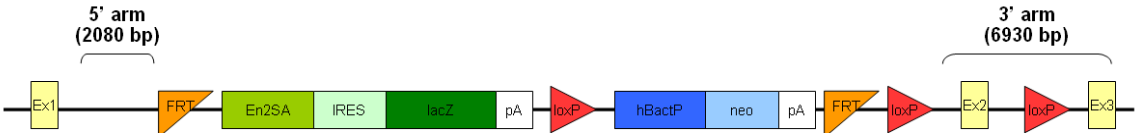


Fig.6 Strategy of homolog ricombination to obtain ES cell clones with conditional potential.

mESCs were cultured at 37°C in a 5% CO_2 atmosphere on 0,1% gelatine in ES medium composed of Knockout DMEM high glucose (Gibco) supplemented with 15% fetal bovine serum (Gibco), 0.1 mM 2(β)-Mercaptoethanol (Sigma), 1mM non-essential amino acids (Gibco), 2 mM glutamine (Gibco), and 1000 units/ml leukemia inhibitory factor (Millipore). Culture media was changed every day and cultures passaged every two days.

For differentiation, 1 million of mESCs were plated in 35 mm dishes, as a feeder free monolayer. The differentiation medium consists of the in DMEM Low Glucose (Gibco) supplemented with 5% fetal bovine serum, 0.1 mM 2(β)-Mercaptoethanol, 1mM non-essential amino acids, 2 mM glutamine, depleted of LIF, overlayed with 200 µg/ml Matrigel (BD Biosciences) and treated with various factors sequentially.
These factors included Activin A (50 ng/ml, R&D Systems), all trans retinoic acid (RA, 10 µM, Sigma), fibroblast growth factor 10 (Fgf10, 10 ng/ml, R&D Systems), Cyclopamine (CYC, 10 µM, Sigma), N-N-(3,5-difluorophenacetyl)-L-alanylsphenylglycinet-butylesterm (DAPT, 5 µM, Sigma). Medium was changed every two days.

9. DNA genomic extraction

The lysis buffer (50mM Tris Hcl pH 8.5, 25 mM EDTA, 1% SDS, 1M NaCl, 1mg/ml Proteinase K) was added in 60 mm dishe of cell culture. Digestion is complete after 4 hours at 60°C with agitation (1000 rpm). The sample was centifuged, at full speed at 4°C for 10 minutes, after adding 250 µl of NaCl 6M and 1 ml of Isopropanol. The pellet was washed with 1 ml of cold 70% EtOH, then dried at room temperature. Finally suspended in 100 µl of MilliQ water on a mixer and then store at 4°C.

10. β-galactosidase staining

Cells were fixed with a fixation solution (formaldehyde 37%, glutaraldehyde 25% in PBS) for 2 minutes, then washed with PBS and incubated overnight with a PBS solution containing Potassium ferro-cyanide (5 mM), Potassium ferri-cyanide (5mM); $MgCl_2$ (2 mM) and X-Gal (1 mg/ml) at 37°C.

11. Southern Blot

Restriction digestion of genomic DNA

40 µg genomic DNA was digested in 300 µl total volume with 5 units restriction enzyme (SacII and AccI, Biolabs) per µg DNA at 37°C overnight.

The digestion was purified adding the same volume of Phenol:Chloroform:Isoamylalcohol solution and then centrifuged. In the surnatant were added 0.1 volume NaAc 3M pH 5.2 and 2.5 volumes ice-cold 100% EtOH, precipitated at -20°C for 2 hours. After 20 minutes of centrifugation, pellet was washed with 1 ml of cold 70% EtOH, then dried at room temperature. Finally suspended in 30 µl of MilliQ water on a mixer.

Agarose gel electrophoresis and transfer to Nylon membrane

The digested samples were runned on a 0.8% agarose gel without Ethidium Bromide, and transferred to a nylon membrane as described previously [88]. Briefly,

taking picture of agarose gel and lined up with the phoshorescent ruler along side it, in this way can be later estimate the size of interest band on nylon membrane.

The next step of depurination is necessary for transfer of the DNA out of the gel, so it was placed in 0.2 N HCl for ~ 30 minutes.

To neutralize, the gel was placed in transfer buffer (0.4M NaOH, 1M NaCl) for 15 minutes two times. To transfer the DNA onto a nylon membrane overnight, was prepared a large dish filled with transfer buffer and glass plate on top of it to rest the gel. Two pieces of wick- blotting paper were cut to the width of the gel and length such that the wick is in contact with the bottom of the dish. Wet the wick with transfer buffer and smooth out the bubbles gently with a glass pipette. From the bottom to the top, the disposition is as follows:

Agarose gel, turned upside down, with a nick in the bottom right hand corner for orientation.

Wet Hybond N+ nylon membrane cut to the exact size of the gel, with a nick in the corner for orientation.

Two pieces of wet blotting paper cut to size of the gel, and other 30 on top.

Glass plate and additional weight to keep blot in place.

Hybridization

A 514-bp Neomicin probe was amplified on Neo^R with primers Neo-Probe-Fw (5'-GACCGACCTGTCCGGTGCCCTGAATGAACT-3') and Neo-Probe-Rev (5'-TATGTCCTGATAGCGGTCCGCCACACCCAG-3'). Hybridization with this probe revealed a 11.8-kb band from the targeted locus.

Taking off the gel and membrane together, can be marked the wells with a pencil.

After fixation of DNA on membrane with Stratalinker, the filter can be prehybridized in Hybridization Buffer (HB, Maleic Acid 1x and Blocking Reagent-Roche) at 55°C for at least 30 minutes, and then hybridize adding the probe in HB at 55°C overnight.

Detection

To detect the bands of interest, the membrane was washed with SSC 0.2x and 0.1 % SDS at 68°C two times for 15 minutes each. After pre-blocking, the membrane was

placed with Anti-Digoxigenin-AP-Fab (Roche). The signal was developed with CDP-Star (Roche) according to manufacturer's instructions.

Results

1. Capture of dorsal pancreas bud by LCM

In order to dissect the dorsal pancreatic bud at E10.5, and considering the small size of the organ at this stage, it was necessary to proceed with LCM.

To identify the structure of pancreas at E10.5, we performed immunofluorescence experiments using two genes as markers: *E-cadherin* and *Pdx1.* *E-cadherin* is expressed in endodermic tissues as pancreas, intestine, lung, thyroid, and *Pdx1* is specific of pancreas. The localization of Pdx1 protein is nuclear, while E-Cadherin localizes at periplasmatic level.

In particular, we progressively collected the 10 µm sections to perform both bud localization through immunofluorescence, and bud microdissection through LCM; while in the last case sections were stored in dry ice during the cutting, for immunofluorescence we optimized a protocol on the other sections (Fig.9). The immunofluorescence protocol has been developed considering that the embryos have been included after treatment with sucrose, without any fixtation in paraformaldehyde. Because of the restricted number of sections in which the pancreas dorsal bud is distinguishable, we referred to the progression of branchial arches, to define the starting and the ending point of pancreas containing sections (Fig.7). In particular, the ventral pancreas bud appears when the third branchial arch disappears; in the same sections the right lung and thyroid buds appear; while the dorsal pancreatic bud appears when the second branchial arch, thyroid bud and trachea disappear. In the following sections the dorsal pancreatic bud is present until the second branchial arches and left lung bud reappear. Using this methodology we tightly estimated the section coordinates and thickness of several endoderm buds.

The thickness in which is present the entire pancreatic structure is about 140 µm, so we can collect 14 sections of 10 µm. In fact, we find sections only with the dorsal pancreas bud or only the ventral pancreas bud. Then, there are sections in which both buds are present; near the ventral bud we identify also the gall bladder (Fig.8).

Moreover, it was possible to select the sections even before the dehydration allowing to reduce the time of exposure of the same to environmental temperature. Following the microdissection of dorsal pancreatic buds (DPB), the same sections without dorsal pancreatic buds (SwDPB) were collected in order to compare the gene expression profile *versus* the developing pancreatic bud.

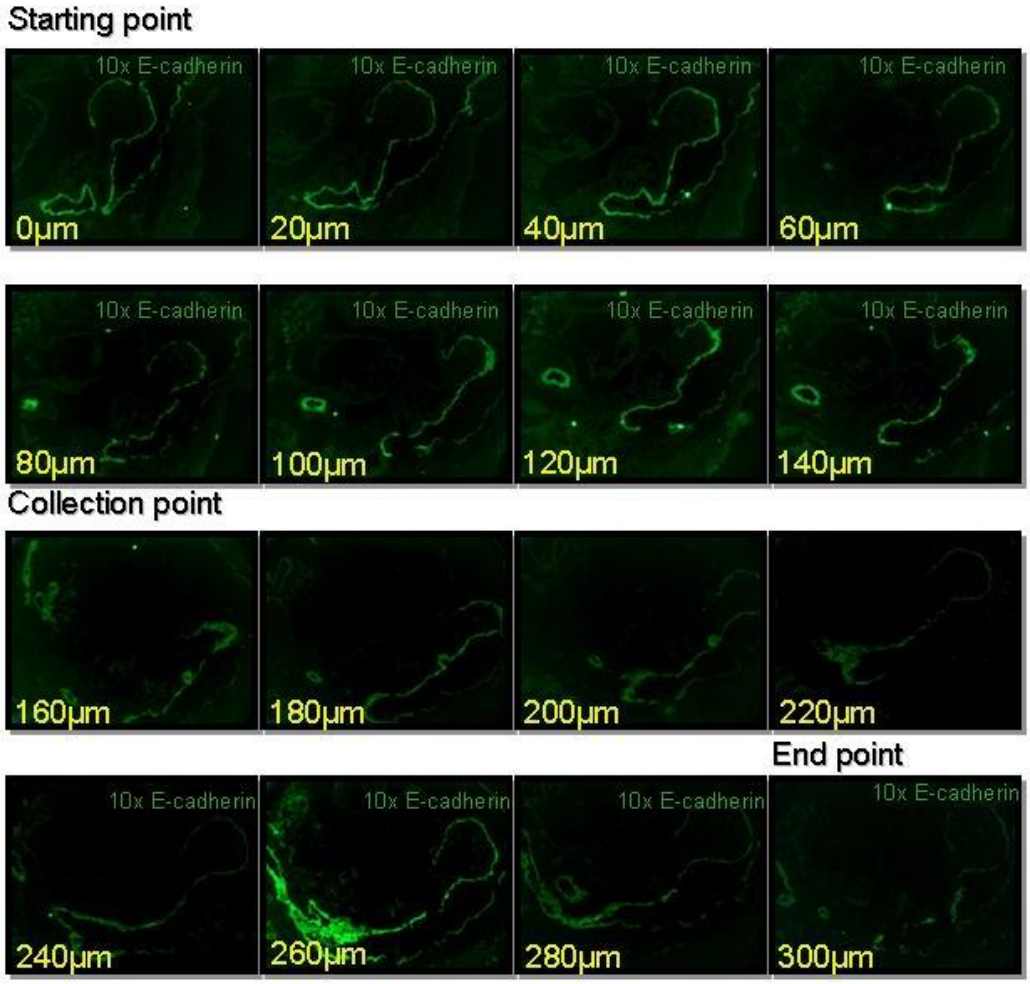


Fig. 7 Progression of branchial arches by E-cadherin staining.

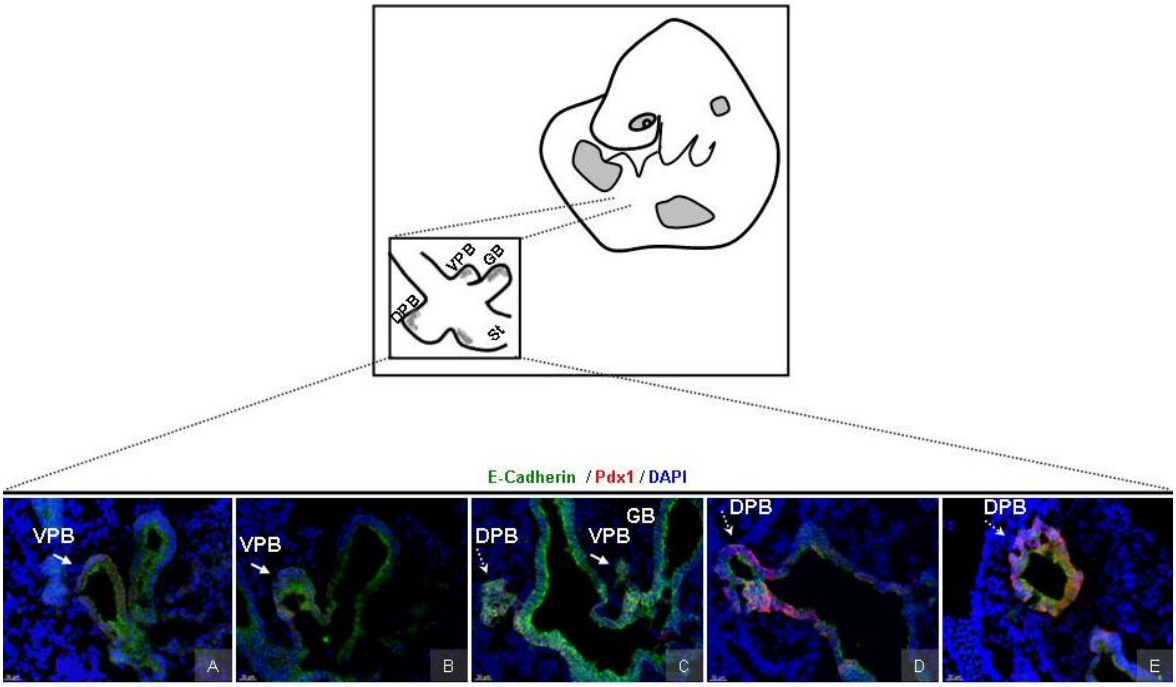


Fig. 8 Progression of pancreatic structure. DPB (dorsal pancreas bud), VPB (ventral pancreas bud), GB (gall bladder).

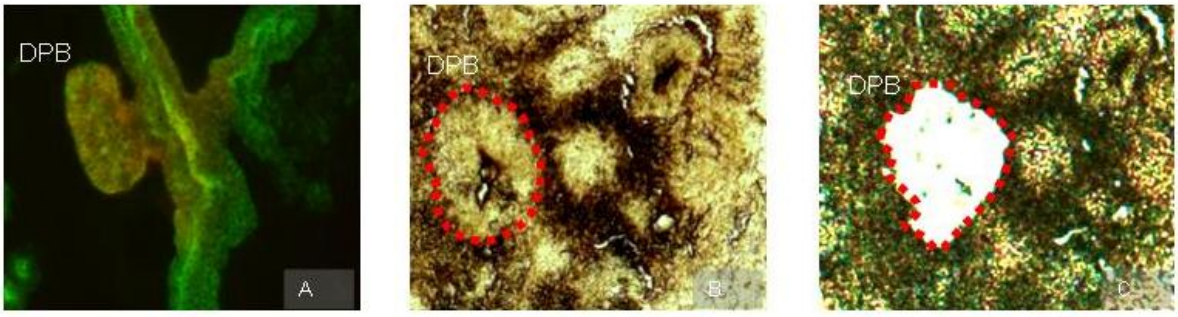


Fig. 9 The first panel shows the immunofluorescence of dorsal pancreas bud, the second panel shows the next section after dehydration and the third panel shows the sections after capture in which there isn't dorsal pancreas bud. DPB (dorsal pancreas bud).

2. Global gene expression profile of dorsal pancreatic bud

DPB cells of a single embryo are insufficient to obtain the amount of RNA necessary to perform the hybridization to microarrays. For this reason, we pooled pancreatic

buds of three embryos belonging to three different litters. In this way, we can increase the amount of RNA, but also reduce the biological variability.

Considering that the amount of RNA obtained from pancreatic buds is from 200 pg to 1.5 ng, we used to linearly amplify the amount of RNA necessary for the following assay.

The overall integrity of RNA isolated with the protocol provided by Arcturus was determined by capillary electrophoresis on Bioanalyzer 2100 (Agilent). After several experiments, we obtained a RIN between 6.0 and 8.4. The quality of RNA extracted from cells obtained by LCM showed 18s and 28s bands, indicating that there was no deterioration of RNA.

Therefore, the samples were selected considering the parameter of RIN and concentration, in order to have samples qualitatively and quantitatively similar (Fig.10).

Subsequently, we performed the linear amplification of the same samples following the instructions of the RiboAmp HS RNA Amplification Kits (Arcturus), so as to amplify the RNA up to 30 µg maintaining the original representation of the mRNA. The linear amplification allows us to appreciate the difference in gene expression.

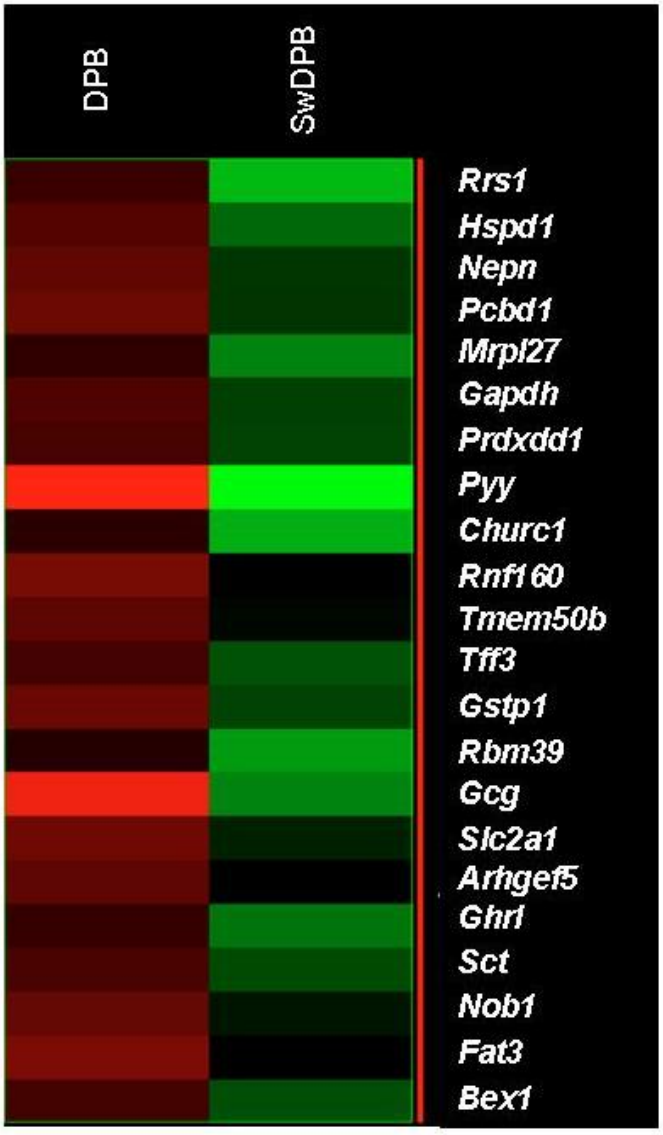
10 µg of aRNA (antisense RNA) were used to obtain ss cDNA, necessary for hybridization to GeneChip Mouse Gene 1.0 ST Array - Affymetrix. In order to have the biological and technical triplicate, three samples of DPB and three samples of SwDPB were amplified and hybridized to six different chips.

	Sample 1. Dorsal Pancreatic Bud	Sample 2. Dorsal Pancreatic Bud	Sample 3. Dorsal Pancreatic Bud	Sample 4. Sections without Dorsal Pancreatic Bud	Sample 5. Sections without Dorsal Pancreatic Bud	Sample 6. Sections without Dorsal Pancreatic Bud
Pico LabChip Agilent Gel						
[ng/µl]	1.4	1.4	1.5	4	8	2.5

Fig.10 RNA runs on Agilent Pico LabChip

To find specific genes during pancreas development, we compared the expression level of each transcript in DPB with that in the SwDPB and sorted out transcripts with an expression level, in DPB, at least 1.2-fold higher than that in the whole embryo. By this procedure we detected 162 genes enriched in the DPB.

Considering that the entire embryo is a mixture of all three germ layers, with this approach, we can find an enrichment of general epithelial determinants in addition to organ specific transcripts.



To validate the results of the analysis we considered genes with fold change (FC) greater than 1.6 as shown in the panel.

In particular, we confirmed by qPCR the expression of both specific pancreatic markers known at E10.5 as *Pyy, Gcg, Sct, Ghrl,* and the expression of markers unknown as *Bex1, Nepn, Pcbd1, Prdxdd1, Rnf160, Slc2a1, Tff3* (Fig. 12).

Fig.11 Expression heatmap showing representative genes of DPB.

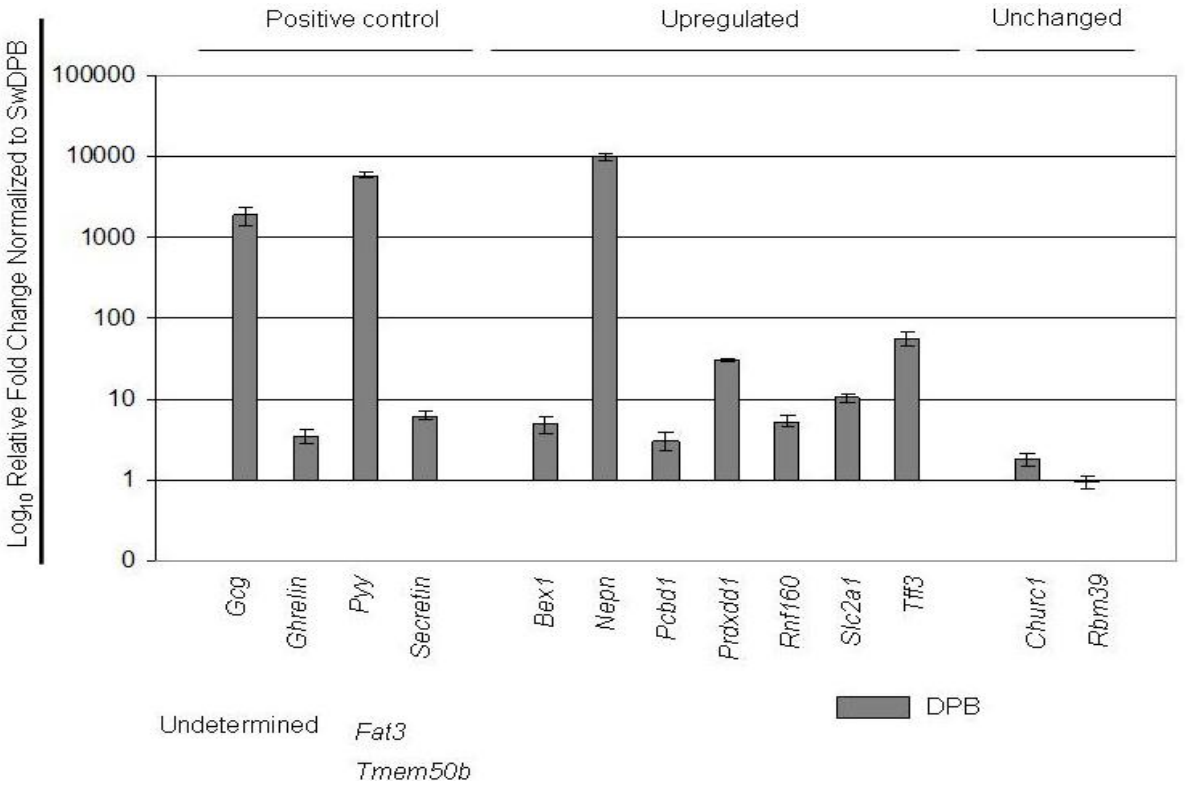


Fig.12 Validation of candidates by qPCR

3. Characterization of *Nepn* expression in the early stages of pancreas development

By RT-PCR, we selected *Nepn* (Nephrocan, Leucine-rich repeats), *Prdxdd1* (prolyl-tRNA synthetase associated domain-containing protein) and *Tff3* (Trefoil Factor 3, cysteine-rich domain) as specific and exclusive candidates of the pancreas. The result was confirmed by *in situ* hybridization for *Nepn* and *Tff3*, while for *Prdxdd1* was not possible to discriminate the isoform 1 (pancreas specific) from the isoform 2 for the high nucleotide identity percentage. The expression level during mouse embryogenesis was examined by *in situ* hybridization using specific riboprobe labeled with digoxigenin on sagittal sections from E9.5 to E12.5. We show in the panel of figure 14 that the expression of *Nepn* changed during embryonic development, in fact, at E9.5 and E10.5 we noted high expression levels that were reduced at E11.5, until E12.5 in which it was not expressed. *Tff3* developed a very

weak signal, this did not allow us to characterize very well this gene during early pancreas development (data not shown).

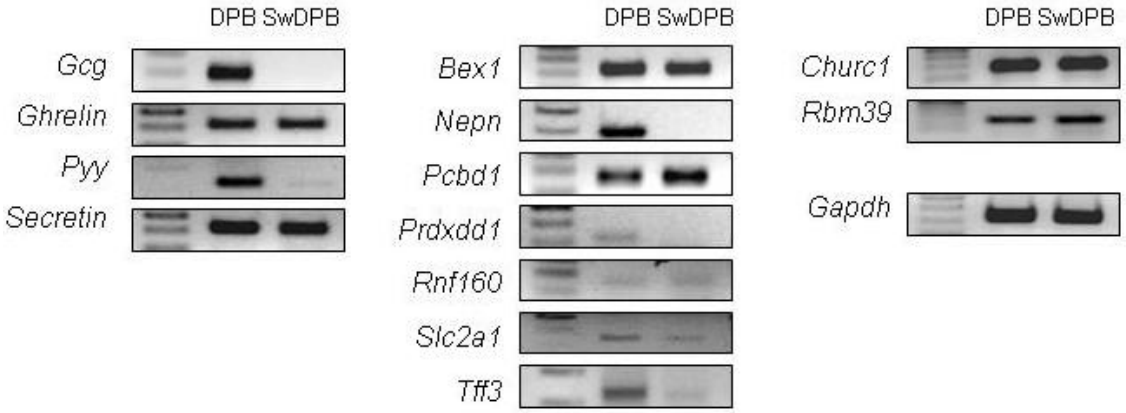


Fig.13 Validation of candidates by RT-PCR.

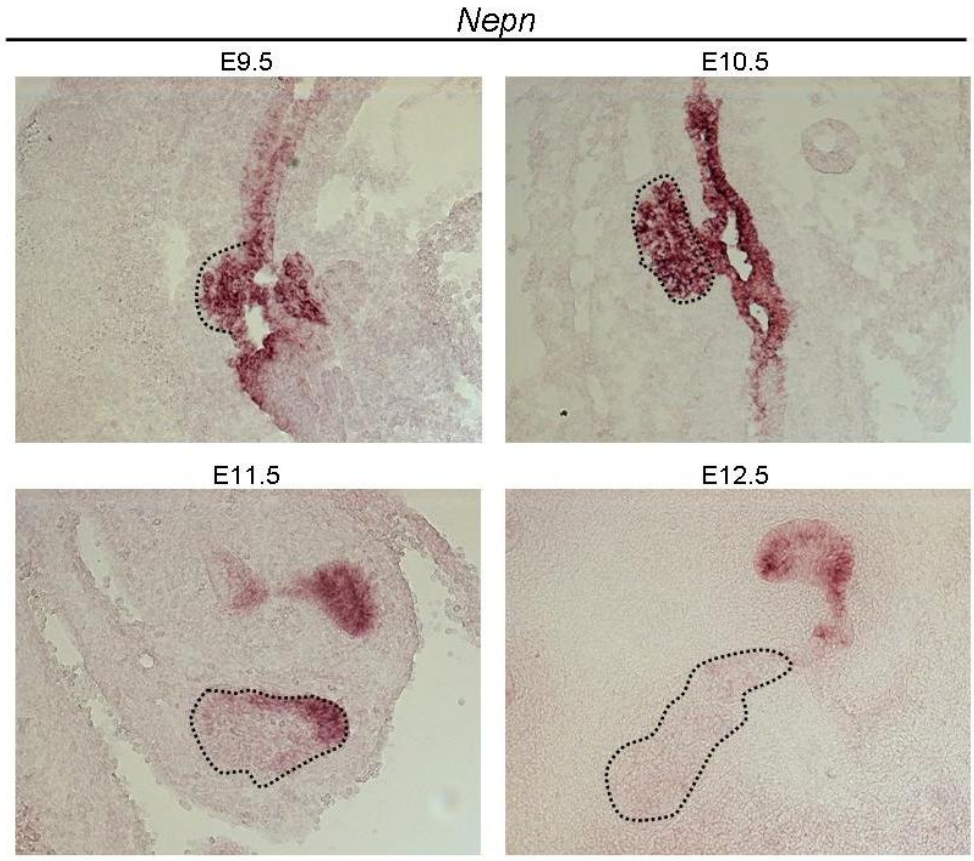


Fig.14 Validation of *Nepn* on developing pancreas by *in situ* hybridization, magnification 20x.

4. Differentiation of mESCs towards pancreatic lineage *in vitro*

In our study, we optimized the protocol to differentiate mESCs into the pancreatic progenitors, following the patterning of definitive endoderm and posterior foregut endoderm.

To achieve a high efficiency of directed differentiation, we induced ESCs in a monolayer format to minimize unwanted spontaneous differentiation [89]. In a low glucose environment a high concentration of Activin A promoted mESCs to give rise effectively to DE (definitive endoderm), marked by the expression of *Sox17* and *Foxa2*. *Sox17* plays a determinant role in the formation of DE cells while *Foxa2* marks the anterior primitive streak from which the DE emerges. Simultaneously, the expression of undifferentiated mESC pluripotent marker genes *Nanog* and *Oct4/Pou5f1* was down-regulated.

To induce the DE to give rise to PF (posterior foregut) cells, Activin A was removed from the culture medium and RA in combination with Fgf10 were added. At day 8, the expression of the gut tube marker gene *Hnf6* and *Pdx1* was detected.

In order to differentiate the PF into PP (pancreatic progenitor) cells, CYC, a known factor for pancreas specification and the γ secretase (a component of the Notch pathway) inhibitor DAPT were examined in combination with RA. Indeed at day 12, a group of PP marker genes including *Ptf1a* and *Nkx6.1* was expressed (Fig.15).

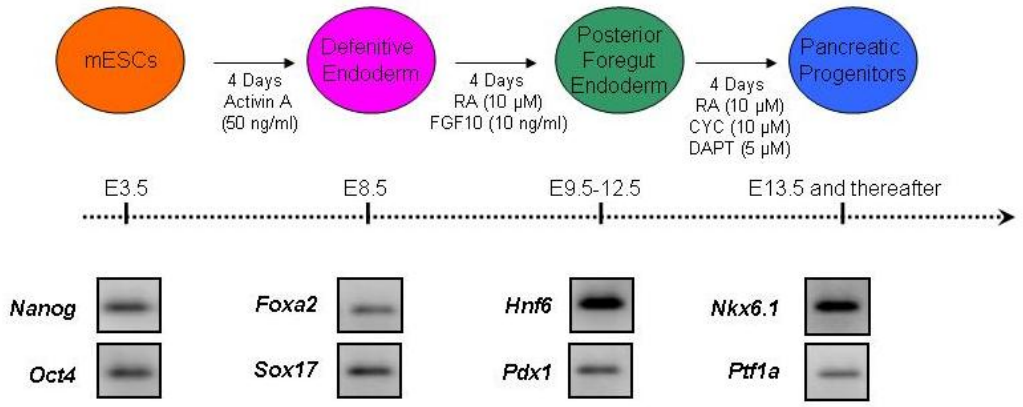


Fig.15 Validation of positive markers during differentiation by RT-PCR, rappresentative steps of differention at day 4, 8 and 12.

To verify if our candidate genes were expressed during differentiaiton, we performed qPCR for *Bex1, Nepn, Pcbd1, Prdxdd1, Rnf160, Slc2a1, Tff3.*
Four genes (Bex1, Nepn, *Pcbd1*, *Prdxdd1*) are up-regulated at day 8 compared to day 4 during differentiation of mESCs towards posterior foregut endoderm; the enrichment of our candidates is emphasized further at day 12.

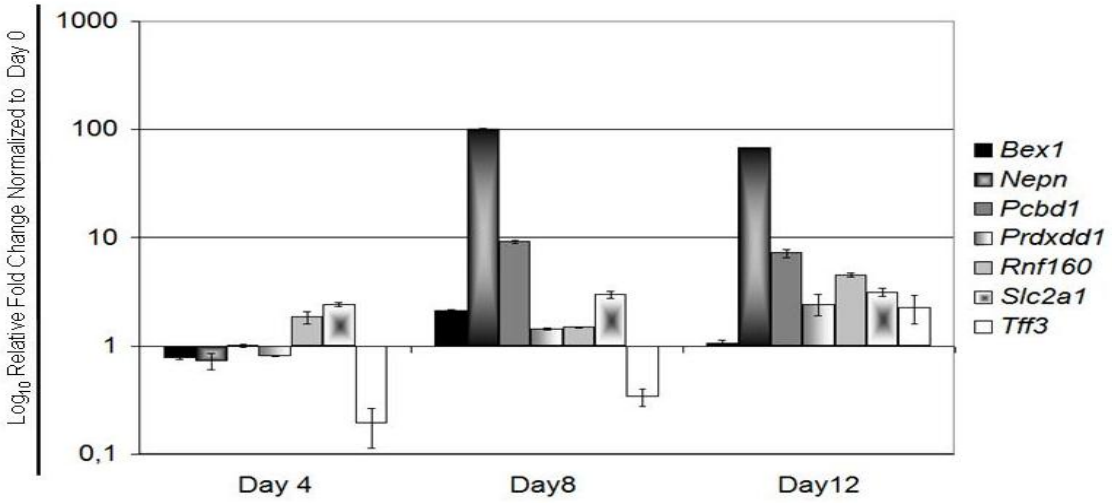


Fig.16 The histogram shows the expression of *Bex1, Nepn*, *Pcbd1, Prdxdd1, Rnf160, Slc2a1* and *Tff3* during differentiation of mESCs towards pancreatic progenitors at day 4, day 8 and day 12.

We have a cell line in which two loxP sites were introduced at the sides of *Nepn* Exon 2 by homologous recombination, in this way has been interrupted the transcription of *Nepn* on first exon to obtain a truncated protein. The knockout first allele is initially a non-expressive form, but can be converted to a conditional allele via Flp recombination. The correct DNA recombination was confirmed on 5' arm by PCR and on 3' arm by Southern blot. For PCR, we used the Primer 1 designed externally to the arm 5' and the Primer 2 designed internally to the region of recombination. For southern blot, we used the restriction enzymes *SacII* and *AccI* to digest genomic fragment between the neomycin resistance (Neo^R) and *Nepn* Exon 3. The fragment length of about 10 Kb was detected with Neo^R probe (Table 1 - Fig. 17).

As shown in the panel below, using *Nepn*$^{+/-}$ cell line the presence of the splicing acceptor site and the IRES sequence allows the transcription of Exon 1 and lacZ. Using a system that induces the expression of *Nepn* in ES cells, such as treatment with RA, we showed the expression on both the wild-type allele and the recombinant allele, so the expression of lacZ is correlated with the expression of *Nepn*. This was confirmed by PCR using the Primer 3 designed on exon 1 and the Primer 4 designed on the LacZ gene, and the translation of beta-galactosidase was confirmed by the assay of beta-galactosidase (Table 1 - Fig. 18).

In this way we could characterize *Nepn*-positive in the heterozygous cell line after induction of differentiation.

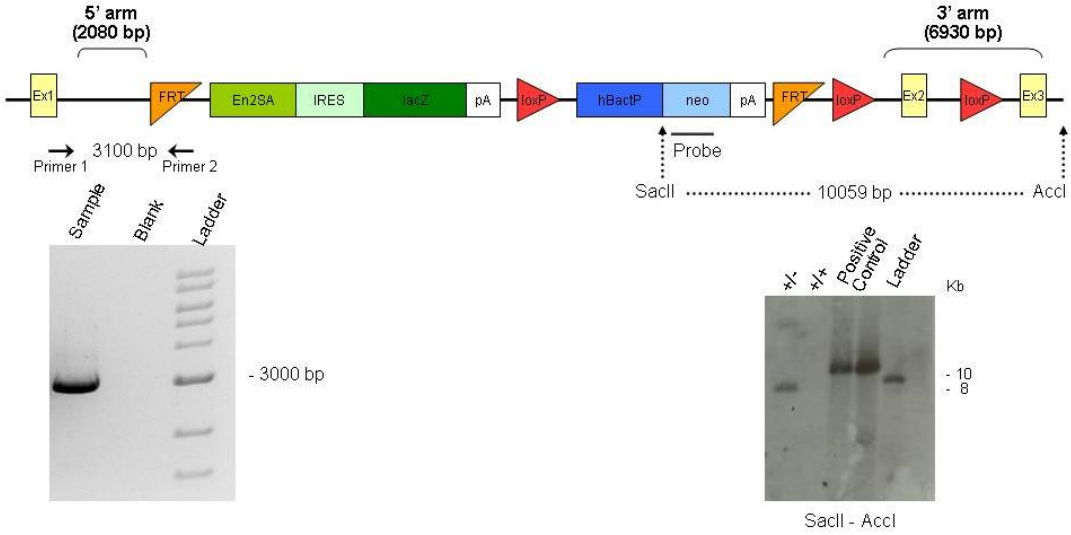


Fig.17 Assesment of the 5' arm by PCR in the left side, and assesment of the 3' arm by southern blot in right side.

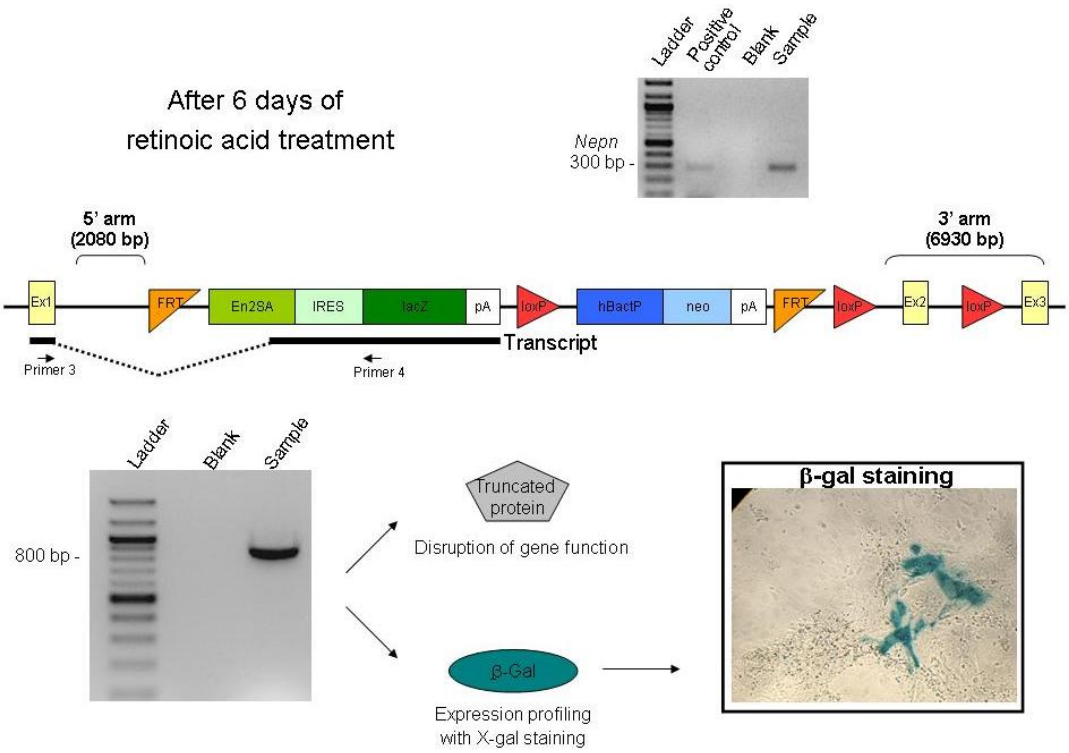


Fig.18 Expression of *Nepn* in the cell line *Nepn*$^{+/-}$.

Discussion

The pace of development of molecular analytical techniques has been rapid in recent years. High throughput methods such as DNA microarrays (“DNA chip technology”) and proteomics have enormous potential for revolutionizing the study of molecular events associated with physiological and pathological processes.

Although the technology has become increasingly sophisticated, the accuracy of the results obtained is still largely influenced by the selection of appropriate study material.

Tissues are composed of many different cell types, from which it is necessary to obtain a pure sample of the cells of interest to acquire meaningful data.

In this way, the cell specific expression is masked, especially if the cell type of interest represents only a small fraction of the entire sample, or if gene expression is elevated or suppressed.

Many techniques have been used in attempts to isolate cell populations, including cell culture studies, xenografts, flow cytometry, and various microdissection methods such as laser capture microdissection (LCM).

We considered the cell population that forms the dorsal pancreatic bud at E10.5 because the aim of our work is based on identification of signature genes involved in pancreas development.

To identify mouse dorsal pancreas bud at the stage E10.5, in which different transcription factors are expresses, some unknown and others known as *Pdx1* which is tissue-specific; Pdx1 was used as a marker of the pancreas in the different experiments such as immunofluorescence and LCM.

We used LCM technology to microdissect these cells from mouse embryos because the size of the pancreas, at this stage, is reduced; it’s impossible to dissect surgically.

To pick out cells of a specific type from the midst of others in the same microenviroment is a necessary step for subsequent molecular analysis. LCM provides a good way to collect a well defined cell population from a heterogeneous and complex tissue organization. The technique reported in this study provides a

simple and rapid method that combine excellent tissue morphology with good preservation of RNA.

Microarray require up to 10 µg of cellular RNA, and this is a barrier to the analysis of gene expression in low density cell population. To overcome this barrier, we performed the linear amplification of mRNA to increase the amount of product expression.

After hybridization to GeneChip Mouse Gene 1.0 ST Array, to find new genes expressed during pancreas development we compared the gene expression profile of DPB with the gene expression profile of SwDPB. Among the candidate genes we focused our attention on *Nepn* whose spatio-temporal expression was confirmed by both qPCR and *in situ* hybridization.

Nephrocan, a novel member of the small leucine-rich repeat protein family, is an inhibitor of TGF-beta/Smad signaling. *Nepn* is expressed in kidney in adult mice, and during mouse embryogenesis, the expression was markedly increased at E11.5 when early kidney development takes place. In the adult mouse kidney, Nepn protein was located in distal tubules and collecting ducts [90].

It was found that *Nepn* is highly enriched in endoderm by *in situ* hybridization at E8.5–E9.0, *Nepn* expression level reaches the highest in the midgut. At E9.5, the signal is retained but is down-regulated [91].

In situ hybridization for *Nepn* at E14.5 reveals epithelial expression at the pylorus. Expression is more robust toward the stomach and is primarily localized to epithelial cells closer to the basement membrane. Nephrocan expression at E16.5 is restricted to the base of the developing epithelial glands in stomach and developing villi in intestine [92].

Nepn expression increases in the *Nkx2.2*$^{-/-}$ pancreas at E12.5, E13.5, E14.5, and E15.5, its expression is detectable in exocrine compartment and overlaps with the elastase expression domain. In both wild type and *Nkx2.2*$^{-/-}$ pancreas the expression of gene was already considerably lower at E15.5 when compared to E13.5, although even at E15.5, *Nepn* levels were still significantly higher in the mutant pancreas

compared to wild type. This would suggest that *Nepn* is transiently up-regulated in the exocrine pancreas of the *Nkx2.2*$^{-/-}$ mice [93].

In future experiments we will propose the *in vivo* characterization of *Nepn* and its possible role in the pancreas development. The heterozygous ESCs clone for *Nepn* was microinjected into C57BL/6 blastocysts to generate chimeric mice, in particular we generate 3 mice with a high index of chimerism.

To generate *Nepn* null mutant mice, heterozygous mutant mice will intercross, and the genotypes of 30-day-old offspring will be determined by PCR.

At the same time, we propose the *in vitro* characterization of the cell population that expresses *Nepn* during differentiation of mESCs towards the pancreatic progenitors. In this case, we would like to sort *Nepn*-positive cells and define them by RNA-seq and qPCR.

We would like to evaluate the potential of *Nepn*$^{-/-}$ ES cells to differentiate in pancreatic progenitors compared to *Nepn*$^{+/-}$ cells.

Whereas preliminary data suggest that *Nepn* is not expressed in the adult pancreas, we will induce damage to the adult pancreas in order to observe whether there is a reactivation of the gene by RT-PCR and *in situ* hybridization. In this case, cells sorted will be injected in the damaged organ to verify a possible facilitated regeneration.

Among our candidates *Tff3* and *Pcbd1* are enriched during differentiation of mESCs towards the pancreatic progenitors. Mice homozygous for *Tff3* results in impaired intestinal mucosal healing and death from colitis after administration of dextran sulfate sodium. Mice show poor epithelial regeneration after injury.

Mice homozygous for *Pcbd1* display hyperphenylalaninemia, are mildly glucose intolerant, and are predisposed to cataract formation.

For this reason we propose *in vitro* characterization of the cell population that expresses them.

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