Université de Montréal

Role of Ptf1a in the development of endocrine and exocrine pancreas of *Xenopus* laevis embryos

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Ce mémoire intitulé:
Role of Ptf1a in the development of endocrine and exocrine pancreas of *Xenopus* laevis embryos

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Résumé

Les injections d'insuline restent le seul traitement pratique pour le diabète insulinodépendant, mais causent beaucoup de problèmes parce qu'elle ne maintient pas la glycémie tel que le fait le pancréas. Les greffes de pancréas et d'îlots sont aussi faisables mais limitées par le petit nombre de donneurs et le traitement immunosuppresseur à vie. Une solution ultime serait la production de nouvelles cellules sécrétrices d'insuline à partir de cellules souches ou différentiées provenant du receveur diabétique lui-même. Il est alors crucial d'élucider les facteurs de transcription clés qui régulent le développement endocrine du pancréas. Ptf1a est un des facteurs de transcription exprimés au cours du développement précoce du pancréas et reconnu comme étant impliqué dans la détermination de la lignée pancréatique exocrine. Des résultats plus récents ont suggéré que Ptf1a peut aussi être impliqué dans le développement des cellules pancréatiques endocrines. Pour vérifier cette hypothèse nous avons étudié le rôle du Ptf1a au cours du développement du pancréas chez le *Xenope*. Nous avons montré que Ptf1a est essentiel pour le développement des deux lignées pancréatiques endocrine et exocrine. En outre, nous avons testé la suffisance du Ptf1a à promouvoir un sort pancréatique de façon ectopique et ceci en utilisant les techniques de surexpression par transfert de gènes et par injection d'ARN messager. Nous avons montré que Ptf1a est capable de promouvoir les deux sorts pancréatiques endocrine et exocrine dans une région bien définie de l'endoderme. En conclusion, nos résultats démontrent que Ptf1a joue un rôle potentiel au cours du développement du pancréas et éclaircissent la voie de génération de cellules insulinoproductrices *in vitro* en vue d'une application thérapeutique.

Mots clés : *Xenopus*, Pancréas, Ptf1a, spécification, transdifferentiation, insuline, îlots de Langerhans, endocrine, exocrine, organogenèse

Summary

Insulin injections remain the single practical treatment for type 1 diabetes, but still cause many problems even under the best conditions because it cannot reproduce the exact maintenance of euglycemia as the normal pancreas. Transplantation of whole pancreata or islets is a feasible alternative but still faces problems in the lack of donors and lifelong immunosuppressive treatment. Generating new insulin producing cells from one's body would be the ultimate solution. Whether the sources to be used are stem cells or differentiated cells the crucial need is to elucidate the key transcriptional regulators controlling endocrine pancreatic development. Ptf1a is one of the earliest genes expressed in the pancreas and is known to be one of the key transcriptional regulators of exocrine pancreas development. Recent results have suggested that the Ptf1a may also be involved in endocrine cell fate specification. To address this question we studied the function of Ptf1a in *Xenopus* pancreas development. We show that Ptf1a is essential for proper development of both endocrine and exocrine cells. Furthermore, we also tested its sufficiency to ectopically promote a pancreatic cell fate using transgenic and mRNA overexpression assays, and found that it is able to promote both endocrine and exocrine cell fates within a defined region of the endoderm. In conclusion, our results demonstrate that Ptf1a is a master regulator of pancreatic fate in *Xenopus* and give insight into the generation of endocrine cells *in vitro* for therapeutic application.

Key words: *Xenopus*, Pancreas, Diabetes, Ptf1a, specification, transdifferentiation, insulin, islet of Langerhans, endocrine, exocrine, organogenesis

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List of abbreviations

#	Number
5' UTR	5' untranslated region
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
CMV	Cytomegalovirus
d	Duodenum
Dll1	Delta like 1
dp	Dorsal pancreas
dpc	Days post-coitum
DSL	Delta, Serrate, Lag2
E	Embryonic day
EF1α	Elongation factor 1-alpha
EGF	Epidermal growth factor
Elas	Elastase
ep	Ectopic pancreas
FGF	Fibroblast growth factor
Fig.	Figure
Frp5	Frizzled related protein 5
GFP	Green fluorescent protein
HepG2	Human hepatocellular liver carcinoma cell line
Hes	Hairy / enhancer-of-split
Hex	Hematopoietically expressed homeobox
Hlxb9	Homeobox 9
IDDM	Insulin-dependent diabetes mellitus
IFABP	Intestinal fatty acid binding protein
int	Intestine
Ipf1	Insulin promoter factor 1
Isl1	Islet 1
l	Liver
Mist1	Muscle, intestine and stomach expression 1
MO	Morpholino

MO1	Ptf1a morpholino targeting the initiation codon
MO2	Ptf1a morpholino targeting the exon-intron boundary
mPtf1a	Mouse Ptf1a
mRNA	Messenger RNA
NeuroD/beta2	Neurogenic differentiation
Ngn3	Neurogenin 3
NIDDM	Non-insulin-dependent diabetes mellitus
Nkx	Homeobox Nk transcription factor
Notch ICD	Notch intracellular domain
p	Pancreas
Pax	Paired box gene
PCR	Polymerase chain reaction
Pdx1	Pancreas duodenum homeobox 1
PP	Pancreatic polypeptide
PTF	Pancreatic transcription factor
Ptf1a	Alpha subunit of the pancreatic transcription factor 1
RACE	Rapid amplification of cDNA ends
RBP-Jκ	Recombining binding protein suppressor of hairless-J Kappa
s.t.m	Septum transversum mesenchyme
Shh	Sonic Hedgehog
st	Stomach
St.	Stage
STZ	Streptozotocin
TD	Transdifferentiation
TGF	Transforming growth factor
TTR	Transthyretin
U	Unit
vp	Ventral pancreas
VP16	Herpes simplex virus regulatory protein
X.laevis	Xenopus laevis
X.tropicalis	Xenopus tropicalis
Xlhbox8	*Xenopus* laevis homeobox 8
XPtf1a	Xenopus Ptf1a

To my Parents

To my Sister and Brother

To my Husband

To my Sons

Acknowledgment

I am grateful for Dr.Marko Horb for the opportunity he gave me to do my Masters in his laboratory. I am proud of being Marko's first student, seeing the lab growing year after year, welcoming some people and saying bye to others. I thank Dr. Marko for the scientific formation he gave me, he taught me how to plan, perform and discuss my experiments. His enthusiasm and interest for this project are very encouraging and motivating. I really appreciate him being so close to us, understanding and sharing our bad and good times.

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1. Introduction

1.1. *Xenopus* laevis

Xenopus laevis is an allotetraploid African clawed frog that has been used for many years to study early periods of embryonic development. Many reasons make *Xenopus* a great experimental model for studying organogenesis: 1) organ development in frogs is much faster than in mammals; 2) the same genetic pathways operate during early stages of cell fate specification in both species; 3) a single female can lay between 1000 and 2000 eggs per day; 4) fertilization is easily done *in vitro* by mixing the eggs with sperm; 5) embryos develop externally which facilitate manipulation and observation; 6) cut-and-paste embryology is possible; 7) transgenesis is readily available for overexpression studies; 8) morpholino oligonucleotides permit loss of function studies; 9) microarray analysis is available to compare both normal and mutant (gain or loss of function) tissues for changes in gene expression; 10) the diploid genome *X. tropicalis* a close relative to *X. laevis,* is completely sequenced permitting genomic analysis (Horb 2005; Heasman 2002; Amaya & Kroll 1999).

1.2. The pancreas

1.2.1. Anatomy and Function

The mammalian pancreas is an elongated organ located across the back of the abdomen behind the stomach. It has three main sections: head, body and tail. The head, located at the right extremity is the widest part of the organ and lies in the curve of the duodenum. The body is the main portion of the pancreas located in the middle. The tail forms the left part of the organ and extends toward the spleen (Figure 1.1 A). The pancreas is a mixed exocrine and endocrine gland. The exocrine pancreas is a lobulated branched tissue that forms the bulk of the pancreas. Pancreatic exocrine cells are arranged in grape-like clusters called acini and are packed with numerous secretary granules containing several digestive enzymes such as lipases, amylases, proteases and nucleases (Figure 1.1 B). Most of these enzymes are exocytosed as inactive precursors into the lumen of the acinus to prevent auto degradation and auto digestion of the pancreas. From there, the enzymes are transported via a network of larger and larger ducts which coalesce into the main pancreatic duct that drains into the duodenum. In the duodenum, the pancreatic enzymes are activated by gastrointestinal enteropeptidases. In the small intestine, the digestive enzymes help break down carbohydrates, proteins, fats and acids as part of the pancreatic juice. On the other hand, the ductal cells secrete bicarbonate and water which form the other components of the pancreatic juice to

buffer the acidic chyme from the stomach (Slack 1995). Endocrine cells are grouped into spheroid structures called islets of Langerhans which are scattered amongst the exocrine tissue. The islets are composed of five principal cell types, α, β, δ, ε and PP that secrete respectively, glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide hormones into the bloodstream (Figure 1.1 C) (Slack 1995). Hormonal secretion of the pancreas especially insulin and glucagon is very important to regulate the level of glucose in the blood, and insulin deficiency will lead to one of the most important diseases, diabetes mellitus. Somatostatin acts by both endocrine and paracrine pathways to inhibit the secretion of other hormonal and exocrine pancreatic products (Strowski et al. 2000). Pancreatic polypeptide has a function similar to somatostatin; it suppresses the pancreatic secretion and stimulates gastric secretion. Thus, the pancreas by its exocrine and endocrine activities achieves two functions in the body that are crucial for a person's life: food digestion and glucose homeostasis.

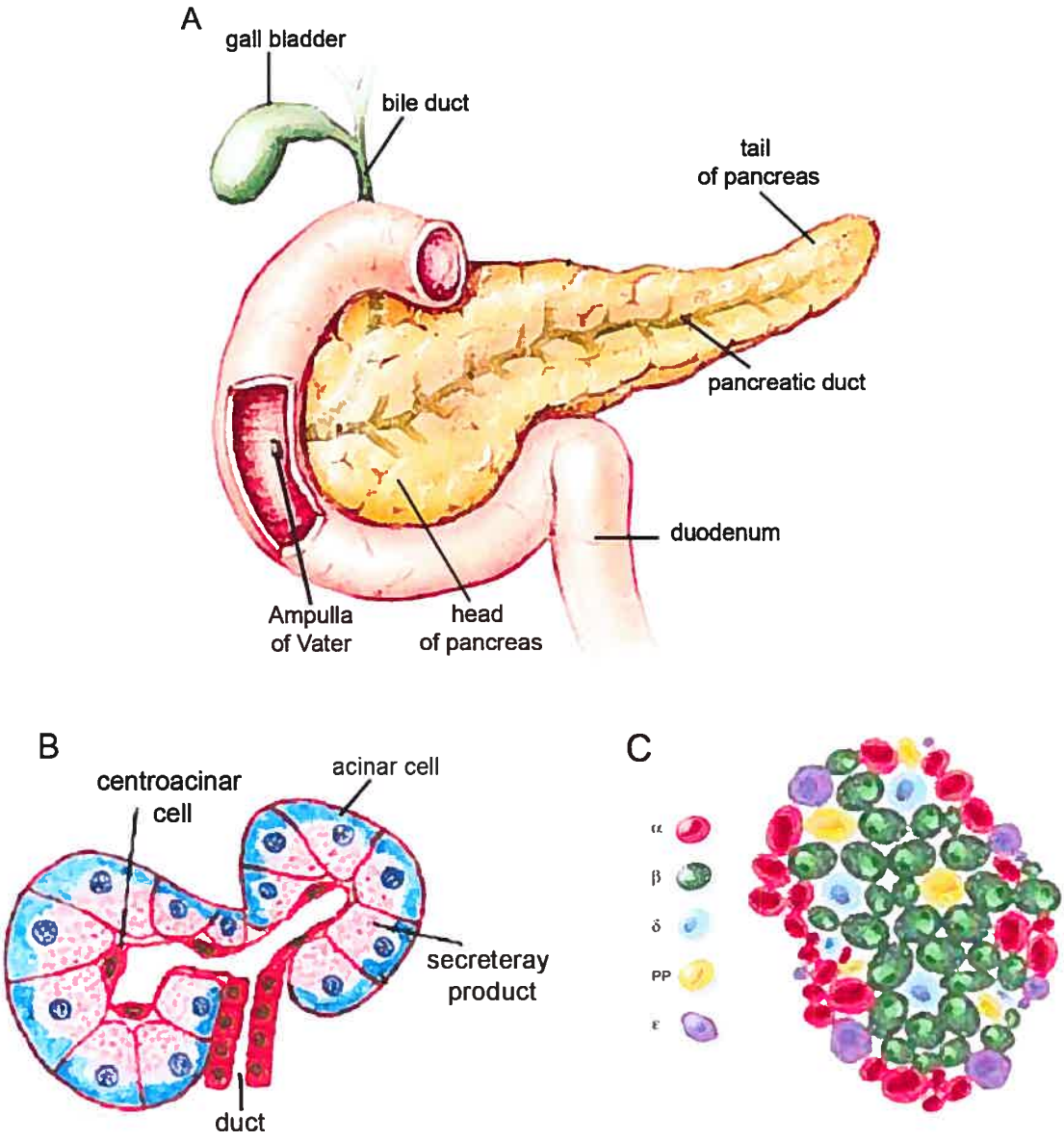

Figure 1.1 Anatomy of the human pancreas. (A) The adult pancreas contacts the duodenum. **(B)** Histology of pancreatic acinar. **(C)** Histology of rodent pancreatic islet.

1.2.2. Diseases of the pancreas

As the pancreas is a mixed gland containing a wide variety of cell types that accomplish different physiological functions, malfunction of the pancreas can give rise to many health complications. Pancreatic diseases include pancreatitis, cystic fibrosis, pancreatic cancer and diabetes. I will now discuss briefly each of these diseases highlighting in particular diabetes mellitus.

1.2.2.1. Pancreatitis

Pancreatitis is an inflammation of the pancreas and can be acute or chronic. Gallstones and alcohol are the major causes of acute pancreatitis which will lead to back up of the exocrine secretions in the pancreas or the adjacent organs. Acute pancreatitis will improve on its own after treatment if no kidney or lung complications occur (Mergener & Baillie 1998). Chronic pancreatitis is usually associated with alcoholism and can also occur following an acute pancreatitis. The digestive enzymes attack and destroy the pancreas and the nearby tissues causing scaring and pain. Chronic pancreatitis can lead to different complications (Steer et al. 1995) such as pancreatic insufficiency, bacterial infection and type 2 diabetes and can be fatal in some cases .

1.2.2.2. Cystic Fibrosis

Cystic Fibrosis is an inherited genetic disorder that disrupts the normal function of the epithelial cells which line passageways inside the lungs, liver, pancreas, digestive and reproductive systems. The epithelial cells are unable to regulate chloride transport through the cell membrane, consequently the balance of water and salt is disrupted and the mucus in the pancreas becomes thick, sticky and hard to move (Quintom 1999). The thick mucus leads to obstruction of the pancreatic ducts and impaired digestive problems.

1.2.2.3. Pancreatic Cancer

Pancreatic cancer is the fifth leading cause of cancer deaths worldwide (NCIC 2004). Most pancreatic cancers are ductal adenocarcinomas developing in the exocrine tissue (Yeo et al. 2002). Pancreatic adenocarcinoma is usually diagnosed at a late stage because symptoms are either absent or nonspecific and only about 10% of cancers are still within the pancreas at the time of diagnosis. Survival rate is less than six months (Warshaw & Fernandez-del Castillo 1992). Pancreatic adenocarcinomas are characterized by an overproliferation of pancreatic ductal cells, yet it is still unclear whether cells responsible for the disease originate from pancreatic ducts or from transdifferentiation of other pancreatic cell type such as acinar cells (Pour et al. 2003; Hall & Lemoine 1992). The weak knowledge on

pancreatic cancer is in first place due to the fact that very little is known about ductal cell development. However, in recent years several studies have attempted to reproduce the disease by overexpressing several signaling molecules within the pancreas or creating pancreatic-specific mutations related to human mutations identified in the disease. For example, several studies showed that pancreatic adenocarcinoma can be induced by overexpressing, in the developing pancreas, signaling molecules that are normally present during pancreas organogenesis such as TGFα (Wagner et al. 1998; Greten et al. 2001). Recently, another model of adenocarcinoma was generated by specifically deleting in the pancreas the either type 2 receptor of TGFβ (TGFβ-R2) or Smad4, another downstream mediator of TGFβ (Ijichi et al. 2006; Bardeesy et al. 2006). Expression of an active form of Kras(G12D) in TGFβ-R2 knockout mice produce pancreatic ductal adenocarcinoma in 100% of animals with a survival rate of 59 days (Ijichi et al. 2006). Similarly, deletion of Smad4 promotes activation of neoplasia by Kras and accelerates development of the tumor (Bardeesy et al. 2006). Thus, understanding how different pancreatic lineages develop and differentiate will help us identify the molecular mechanisms underlying pancreatic cancer development.

1.2.2.4. Diabetes

Diabetes mellitus is a chronic, incurable disease that occurs when the body encounters some insulin insufficiencies, leading to an excess of sugar in the blood. There are two main forms of diabetes: type 1 and type 2. Type 1 diabetes also known as juvenile diabetes or insulin-dependent diabetes (IDDM) usually develops in childhood or adolescence due to the autoimmune destruction of the pancreatic β-cells. Thus, daily insulin injections are required for proper glucose homeostasis and survival of type 1 diabetics. Type 2 diabetes also known as non-insulin-dependent diabetes (NIDDM) can occur at any time during person's life due to relative insufficiency of insulin in the body caused by insulin resistance and β cell dysfunction. Type 2 diabetes may progress to the destruction of the insulin producing cells of the pancreas and insulin administration might be required. Type 2 diabetes is the most common form of diabetes and affects 90% of diabetic people. Hyperglycemia associated with diabetes will slowly damage the small and large blood vessels in the body, resulting in a variety of long term complications including nephropathy, retinopathy, neuropathy and cardiovascular disease. In Canada, diabetes is a leading cause of death by disease; two million Canadians suffer from diabetes nowadays and it is expected to rise to three million in 2010 (Health Canada 2002). The economic costs of diabetes are also very high, it is estimated that 13.2 billion was spent in 2002 to treat people with diabetes and its complications.

The key goal of diabetes treatment is to prevent the complications associated with the disease. Insulin injections still cause many of problems even under the best

conditions because it cannot reproduce the exact maintenance of euglycemia as the normal pancreas. Moreover, the lack of donor tissue for whole pancreas and islet transplantations has not been resolved yet. Replacement of the pancreatic β-cells by new insulin secreting cells represents an adequate solution (Scharfmann 2003). One possibility is the use of pancreatic or other stem cells to produce β-cells *in vitro* for transplantation (Lechner & Habener 2003). Another solution will be the use of other differentiated tissues present in the body to generate pancreatic tissue; this will overcome the two major transplantation problems which are the lack of donor tissues and the immunosuppressive treatment (Slack & Tosh 2001; Tosh & Slack 2002). To achieve such a therapeutic application, a detailed analysis of the molecular mechanisms underlying endocrine pancreatic development is necessary.

1.2.3. Embryological origin

In mammals, the pancreas originates as two endodermal buds developing on the dorsal and ventral side of the foregut endoderm that will become duodenum (Edlund 2002; Kim & MacDonald 2002). At E8.5 the dorsal bud arises just below the notochord, while the ventral bud develops at E9.5 adjacent to the hepatic diverticulum (Slack 1995). Although the cells in the pancreatic buds are committed to form a pancreas, yet they are not differentiated. Only few glucagon expressing cells are detected in the undifferentiated ductal epithelium. At E13.5 the two pancreatic buds rotate, fuse and start to differentiate into endocrine and exocrine lineages.

Subsequently, the differentiated pancreatic cells proliferate extensively such that by E16 the endocrine and exocrine cells start to aggregate in islets of Langerhans and acini respectively. At E18.5 organogenesis of the pancreas is completed and the dorsal and ventral buds form a single functional organ (Figure 1.2).

In the amphibian *Xenopus laevis*, development of the pancreas proceeds in an almost identical manner to that seen in mammals (Kelly & Melton 2000). The dorsal bud is the first to appear at stage 35/36 in the archenteron roof endoderm just below the notochord. The two ventral buds develop by stage 37/38 adjacent to the hepatic diverticulum. Subsequent morphogenesis of the endoderm and dynamic movements of the gastrointestinal tract result in the fusion of the two ventral buds at stage 39 followed by the fusion of the dorsal and ventral buds at stage 40 to form a single organ (Figure 1.2). Differentiation of exocrine and endocrine cells occurs in a spatially and temporally distinct manner (Kelly & Melton 2000; Horb & Slack 2002) such that exocrine cells are initially specified in the ventral pancreas, and endocrine cells in the dorsal. The exocrine markers amylase, elastase and trypsinogen first appear in the ventral pancreas at stage 41; expression then spreads to the dorsal pancreas at stage 42/44 and by stage 45 all three are detected throughout both the ventral and dorsal pancreas (Horb & Slack 2002). Insulin is the first endocrine marker to appear in the dorsal bud at stage 32 prior to overt morphogenesis but is not detected in the ventral pancreas until stage 47 (7 days) (Horb & Slack 2002; Kelly & Melton 2000). In contrast, the other endocrine markers glucagon and somatostatin are not detected in the pancreas until stage 45 and only in the dorsal side, though expression is detected in the stomach and the intestine between stages

41 and 44. At later stages 47/48 expression spreads to the ventral pancreas. Thus, in both mammals and amphibians, the pancreas originates from two separated and distinct endodermal buds.

Even though the two pancreatic buds derive from the same endodermal germ layer, the corresponding developmental programs leading to their specification are not the same and the type of tissue specified by each bud is different, at least initially. The dorsal bud gives rise to the body, tail and part of the head of the pancreas. The ventral bud forms the remainder of the pancreatic head and the uncinate process. Interestingly, the distribution of endocrine cells within the islets in each region of the pancreas differs: islets in the tail are rich in beta and alpha cells, but contain few PP cells, while islets in the head are rich in PP cells with few beta or alpha cells. Those differences are due to the fact that the endodermal domains that specify the pancreatic buds are independent and receive different signals from their surrounding tissues.

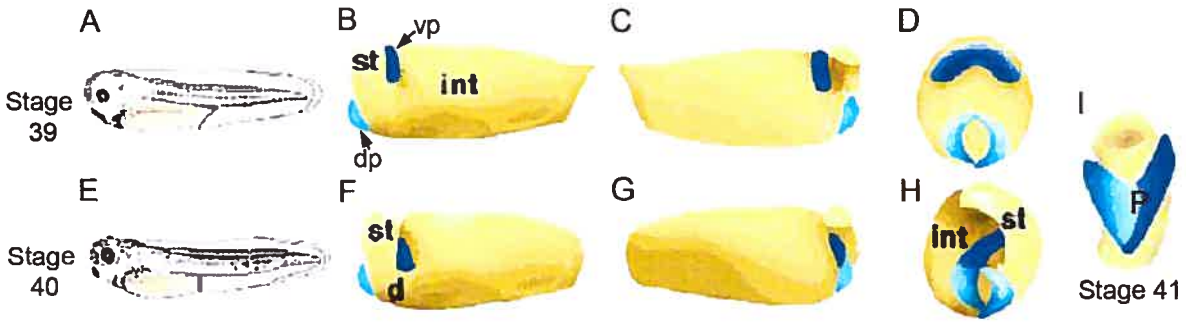

Figure 1.2 Pancreas development in *Xenopus* laevis. (A) Stage 39 tadpole. **(B,C)** Stage 39 dissected guts showing ventral pancreatic bud (dark blue) and dorsal pancreatic bud (light blue). **(B)** Left view. **(C)** Right view. **(D)** Transverse section of stage 39 gut showing the separated pancreatic buds, anterior view. **(E)** Stage 40 tadpole. **(F,G)** Stage 39 dissected guts. **(F)** Left view. **(G)** Right view. **(H)** Transverse section of stage 40 gut showing the pancreatic buds starting to fuse, anterior view. **(I)** Pancreas at stage 41. Stomach (st), intestine (int), duodenum (d), ventral pancreas (vp), dorsal pancreas (dp) and pancreas (p). (Adapted from Kelly and Melton 2000 with modifications)

1.2.4. Regional Specification of the pancreatic endoderm

During embryogenesis the prepancreatic endoderm contacts several mesodermal tissues that play a fundamental role in specifying the different pancreatic lineages, including the notochord and the heart (Figure 1.3). Those mesodermal tissues has been shown to secrete several different growth factors that have a direct influence on the prepancreatic endoderm and play a fundamental role in pancreas development including the Notch, Hedgehog, EGF, FGF, and TGF-β pathways. Early during embryogenesis, the dorsal prepancreatic endoderm in the posterior foregut contacts the notochord while the ventral prepancreatic endoderm is adjacent to the splanchnic mesoderm and to the aortic endothelial cells (Figure 1.3 A). (Kim & Hebrok 2001; Slack 1995). Later on between 9 and 9.5 dpc the fusion of the paired dorsal aorta separates the notochord from the endoderm (Figure 1.3 B), and by 10 dpc mesenchyme surrounds the whole gut (Figure 1.3 C). Thus signals between the endoderm and the mesoderm direct all stages of pancreatic development. Early during development the notochord is the signaling centre that permits dorsal budding and pancreatic gene expression. Later on signals from the mesenchyme are necessary for dorsal and ventral bud development (Kim et al. 1997a; Kim et al. 1997b). During pancreas organogenesis the notochord emits the first signals that initiate pancreatic budding in the foregut endoderm.

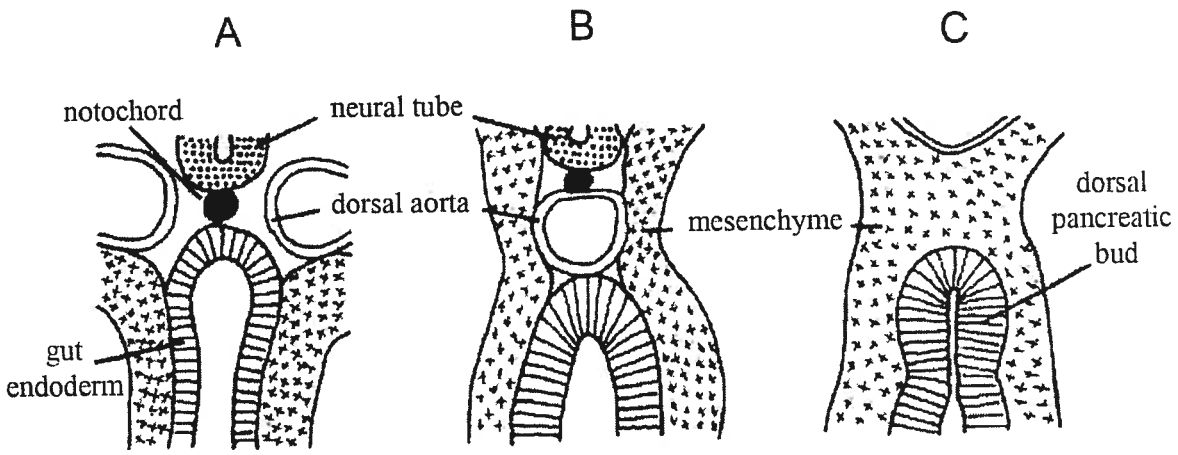

Figure 1.3 Early development of the dorsal pancreatic bud in mice. (A) At 15 somite stage the notochord contacts the gut endoderm. **(B)** At 20 somite stage the dorsal aorta separates the notochord from the gut endoderm. **(C)** By the 28 somite stage the mesenchyme surrounds the gut and the dorsal pancreatic bud has formed.

1.2.4.1. Role of the notochord

Signals from the notochord are required for early induction of pancreatic endoderm. Experiments in chick embryos showed that no pancreatic markers are detected when the notochord is separated from the endoderm. Yet this expression is restored in the anterior endoderm when endodermal epithelium is co-cultured with isolated notochord (Kim et al. 1997b). Early removal of the notochord results in ectopic expression of Sonic Hedgehog *Shh*, in the pancreatic endoderm disrupting both pancreatic morphogenesis and gene expression (Hebrok et al. 1998). The notochord secretes components of the TGF-β signaling pathways, *activin-β* and fibroblast growth factor *FGF2* which repress *Shh* expression in the pancreatic endoderm region. Purified *FGF2* and *activin-β* mimic the activity of isolated notochord in the pancreatic endoderm assay and induce *Pdx1* and *insulin* expression (Hebrok et al. 1998). *Activin-β* and *TGF-β2*, another member of the TGF-β family, have been shown to disrupt branching and acinar morphogenesis when overexpressed in embryonic mouse pancreas (Ritvos et al. 1995). Yet the notochord is not the only signaling centre that represses *Shh*; it is also repressed in the lateral endodermal region which does not contact the notochord that gives rise to the ventral pancreas (Kim et al. 1997a).

1.2.4.2. Role of the heart mesenchyme

It is unclear whether repression of *Shh* in the ventral prepancreatic endoderm is similar to its repression from the dorsal region. But it is clear that ventral pancreatic development differs from dorsal pancreatic development because the notochord does not contact the endoderm and because several gene knockouts have been shown to differentially affect the ventral versus the dorsal buds. The ventral pancreatic bud arises adjacent to the liver and organogenesis of both organs is closely related. The region of the foregut endoderm receiving high levels of *FGFs*, *FGF2* from the surrounding cardiac mesoderm initiates a hepatic program. While the other region, which is not in direct contact with the cardiac mesenchyme maintains the default pancreatic fate (Figure 1.4) (Deutsch et al. 2001; Duncan 2001). In addition, it appears that *BMPs* signaling from the septum transversum mesenchyme are required with *FGFs* to initiate hepatic development within the ventral endoderm (Rossi et al. 2001). In fact, *BMP4* and *FGF2* converge to change the pancreatic fate of ventral endodermal cells, with the latter forming liver instead. On the other hand it was shown that the homeobox gene *Hex* controls the specification of the ventral pancreatic bud (Bort et al. 2004). In the mouse embryo *Hex* is expressed at E7.0 in the ventral-lateral foregut that gives rise to the ventral pancreas and the liver (Bogue et al. 2000). In *Hex* null embryos, the ventral definitive endodermal cells do not proliferate and therefore cannot be positioned beyond the influence of the cardiac mesoderm, resulting in a complete failure of ventral pancreatic bud specification. Moreover, *Hex -/-* endoderm explants cultured in the absence of cardiac mesoderm

are able to activate early pancreatic genes (Bort et al. 2004). Thus, *Hex* controls the growth of the ventral endodermal cells and their positioning beyond the cardiac mesoderm, allowing them to escape the hepatic induction and form the ventral pancreatic bud.

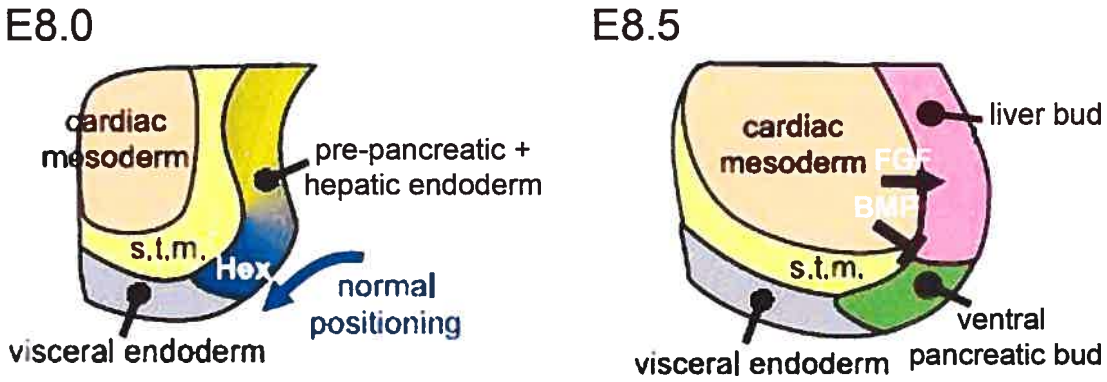

Figure 1.4 Early development of the ventral pancreatic bud in mice. At E8.0, Hex maintains the definitive endodermal cells in a proliferation state which lead to positioning them beyond the cardiac mesoderm. At E8.5, FGF and BMP signaling from the cardiac mesoderm induce hepatic fate (arrow), and prevent pancreatic fate (bar) in the adjacent endoderm. The region of the endoderm that does not contact the heart forms the ventral pancreatic bud. s.t.m: septum transversum mesenchyme (Adapted from Bort et al. 2004 with modifications)

1.2.4.3. Role of the pancreatic mesenchyme

Epithelial-mesenchymal signaling represents another interaction that governs later proliferation and differentiation of the pancreatic cells. The first classical studies showed that when the pancreatic mesenchyme is separated from the epithelium the latter fails to grow and to develop a mature pancreas, and concluded that the mesenchyme is indispensable for the pancreatic epithelium to proliferate and differentiate producing all pancreatic cell types (Wessels 1967). Recent studies show that the mesenchyme controls proliferation of the pancreatic epithelium via members of the fibroblast growth factor family (*FGFs*). It was shown that in *Fgf10-/-* mouse embryos the two pancreatic buds form normally but the cells fail to proliferate and subsequent differentiation is arrested (Bhushan et al. 2001). Implication of the mesenchyme in pancreatic epithelium differentiation is more understood and *in vitro* experiments demonstrated that the epithelium has different responses when exposed to different environments and that the default fate of the pancreatic epithelium is to form islets (Debas 1997). However, the mesenchyme has specific signals that permit the pancreatic epithelium to form acinar pancreatic tissue. The mesenchyme promotes exocrine pancreas development by repressing development of the endocrine lineage. Growth factors from the mesenchyme have been shown to induce cell division in the developing pancreas promoting exocrine differentiation and inhibiting endocrine differentiation (Horb & Slack 2000). The mesenchyme secretes Follistatin a potential inhibitor of Activin is expressed in the pancreatic mesenchyme at E12.5 and is able to mimic the effect of the mesenchyme

(Miralles 1998). Similarly in *Xenopus*, specification of the endoderm is controlled by the adjacent mesoderm (Horb & Slack 2001). Cultured explants from neurula and tailbud stages containing both endoderm and mesoderm, form specific morphological structures that express regional markers. Endodermal explants, lacking mesoderm, do not develop any recognizable morphological structures, while recombination between mesoderm and endoderm lead to the expression of endodermal markers with mesodermal characteristics (Horb & Slack 2001). Results from mouse, chick and fly have also shown that the gut mesoderm can specify the underlying endoderm (Kedinger et al. 1986; Yasugi 1993; Roberts 1998). Those experiments demonstrate that during gut development mesodermal signals are key players for proper patterning of the endoderm into distinct differentiated tissues.

1.2.4.4. Retinoic acid in pancreas development

It is well known that in vertebrate, retinoic acid (RA) signaling is essential for patterning ectoderm and mesoderm (Gavalas & Krumlauf 2000; Maden 1999). Studying the role of RA in patterning the endoderm has emerged recently. First studies done on zebrafish embryos demonstrated that RA is essential for regionalization of the endoderm (Stafford & Prince 2002). No pancreatic and liver markers are detected in RA null zebrafish embryos. In *Xenopus* embryos, inhibition of RA at gastrula stage blocks development of both exocrine and endocrine pancreas (Stafford et al. 2004; Chen et al. 2004). On the other hand, a high

concentration of RA disrupts the antero-posterior patterning of zebrafish endoderm and ectopic pancreatic and hepatic tissue are detected in the anterior endoderm (Stafford & Prince 2002). In *Xenopus* embryos, overexpression of RA leads to an expansion of endocrine development and blocks exocrine development in the dorsal pancreatic endoderm. In the ventral endoderm, RA promotes exocrine pancreatic differentiation and inhibits hepatic development (Chen et al. 2004; Stafford & Prince 2002). Chen et al. showed that RA promotes pancreatic development by repressing *Shh* from the dorsal prepancreatic endoderm. They also demonstrated that expansion of endocrine cell population on the expense of the exocrine population after RA induction is due to inhibition of *Notch* signaling activity (Chen et al. 2004). In mice, retinoids have been shown to inhibit acinar differentiation and promote ductal differentiation through epithelial-mesenchymal interaction (Kobayashi et al. 2002). In conclusion we can say that RA signaling is essential at the gastrulation stage of pancreas, development to regulate exocrine pancreas lineage.

1.2.4.5. Notch signaling in pancreas development

Notch signaling in pancreas development is responsible for maintaining the undifferentiated cell population. The *Notch* signaling cascade was first described in the neural system, where lateral specification restricts neuronal fate; later, *Notch* has been shown to regulate cell fates and pattern formation in most tissues. *Notch* is a receptor that is able to receive extracellular signals and to regulate gene expression

in the nucleus. Upon activation via DSL (Delta, Serrate, Lag-2) ligand interaction, *Notch* receptor is activated and its intracellular domain (ICD) is released and translocated into the nucleus (Mumm & Kopan 2000). *Notch* ICD work in concert with another DNA binding protein *RBP-Jκ* to express hairy/enhancer-of-split (*Hes*) transcription factors which are known to repress key regulator genes such as neurogenin (Beatus & Lendahl 1998). In the pancreas, *Notch* signaling modulates the differentiation of progenitor cells. Loss of function experiments, affecting different components of the *Notch* pathway (*Hes1, Dll1, and RBP-Jκ*), showed that *Notch* signaling inhibits premature and excessive differentiation of pancreatic progenitors into endocrine cells (Apelqvist et al. 1999; Jensen et al. 2000). Directed misexpression of *Notch* ICD to mouse dorsal and ventral pancreatic buds using the *Pdx1* promoter, inhibits differentiation of both endocrine and exocrine cells and traps the pancreatic progenitors in an undifferentiated state (Murtaugh et al. 2003). Moreover, activation of *Notch* in normal mouse pancreas causes loss of acinar cell differentiation and expansion of the ductal cell population thus inducing acinar-to-ductal metaplasia - characteristic of pancreatic adenocarcinoma - (Miyamoto et al. 2003). More recently, it was shown that endogenous *Notch* signaling activates the initial commitment of the exocrine lineage but blocks terminal acinar differentiation (Esni et al. 2004). Thus, early during development, *Notch* signaling activates initial commitment to the exocrine fate and inhibits endocrine cell differentiation to maintain the undifferentiated pancreatic precursors. In the mature pancreas, *Notch* is silenced in the acinar cells and its reactivation results in an acinar to ductal dedifferentiation

while misexpression of *Notch* in mature β-cell does not alter their differentiation (Murtaugh et al. 2003; Lardon et al. 2004; Rooman et al. 2006).

1.2.5. Genetic network regulating pancreas development

1.2.5.1. Exocrine and endocrine pancreatic transcription factors

Pdx1

Several classes of transcription factors are involved in the specification and differentiation of endocrine and exocrine lineages from a common precursor population which is first established in dorsal and ventral pancreatic buds (Figure 1.5). Lineage tracing analysis show that pancreatic progenitors expressing the Para-hox transcription factor *Pdx1* give rise to all pancreatic cell types: endocrine, exocrine and ductal cells (Gu et al. 2002; Gu et al. 2003). Interestingly, the *Xenopus Xlhbox8* was the first *Pdx1* homologue to be isolated. *Xlhbox8* is first expressed in the dorsal and ventral pancreatic buds as well as in the duodenum (Wright et al. 1989). In mice, *Pdx1* has the same expression pattern. It is expressed in the pancreatic buds at E8.5. Later on at E9.5 and E11.5 expression is detected in the duodenum and stomach (Guz et al. 1995; Offield et al. 1996). In the adult pancreas

Pdx1 expression is restricted to the β-cells (Jonsson et al. 1995). Loss and gain of function experiments have shed light on the role of *Pdx1* in specifying the various pancreatic lineages and established *Pdx1* as the first master regulatory gene in pancreas development.

Mice lacking *Pdx1* do not form a pancreas, however a small dorsal pancreatic bud forms but without any expression of insulin or amylase (Jonsson et al. 1994; Offield et al. 1996). The primitive bud formed does contain few α and β-cells. These results suggest that initial budding of the endocrine cells can occur in the absence of *Pdx1* while the subsequent steps to reach a mature and functional stage are *Pdx1* dependent. Conditional inactivation of *Pdx1* in the differentiated β-cells shows that *Pdx1* is indeed required for maintaining the hormonal secretion characteristic of the β-cell (Ahlgren et al. 1998). Moreover, targeted depletion of *Pdx1* in the whole pancreas at late gestational stage leads to agenesis of the acinar tissue, instead immature duct-like cells are formed (Hale et al. 2005). Mutations in the human homologue, Ipf1, are also associated with pancreatic agenesis (Stoffers et al. 1997). In conclusion, these loss-of-function studies showed that Pdx1 is essential for initial pancreas development, but they have not addressed the sufficiency of Pdx1 in specifying pancreatic fates.

On the other hand, gain of function experiments prove that ectopic expression of *Pdx1* is able to generate functional pancreatic cells. The first misexpression experiment of *Pdx1* in the stomach and hindgut region did not show any ectopic pancreatic tissue even though agenesis of the cecum was promoted (Heller et al. 1998). Later, Ferber et al. showed that expression of *Pdx1* in mouse

livers activates endogenous expression of insulin and they also showed that mature hepatic insulin reduces hyperglycemia in diabetic mice (Ferber et al. 2000; Ber et al. 2003; Meivar-Levy & Ferber 2003). We have shown previously that ectopic expression of *Pdx1-VP16* in *Xenopus* liver is able to convert liver to pancreas containing both exocrine and endocrine markers (Horb et al. 2003). Similarly, *Pdx1-VP16* transdifferentiates rat hepatic cells into pancreatic endocrine precursor cells that rescue diabetes when exposed to high glucose levels (Cao et al. 2004). More recently, it was shown that overexpression of both *Pdx1* and *Ptf1a* was sufficient to promote a pancreatic fates in nonpancreatic endodermal cells (Afelik et al. 2006). These results suggest that transdifferentiation of hepatic and other endodermal cells into functional insulin producing cells will serve as a new therapy for insulin dependent diabetes.

Both loss and gain of function experiments demonstrate that *Pdx1* plays a fundamental role in the specification of all pancreatic lineages. In concert with specific protein partners, *Pdx1* has the ability first to switch the fate of endodermal progenitor cells into a pancreatic fate and second to convert differentiated cells into functional pancreatic cells.

Hlxb9

Hlxb9 is a homeodomain transcription factor with different expression patterns during pancreas development. *Hlxb9* expression is first detected around E8

in dorsal and ventral pancreatic buds even before initiation of *Pdx1* expression, yet *Hlxb9* expression is transient and by E10.5 very low levels are detected only in the ventral pancreas (Li et al. 1999). Later during development, Hlxb9 becomes restricted to the differentiated endocrine cells. Homozygous mutant embryos fail to develop a dorsal pancreatic bud. The ventral pancreatic bud develops with aberrant islet structure and a reduction in β-cell number (Harrison et al. 1999; Li et al. 1999). These loss of function studies demonstrate that *Hlxb9* is required in a specific period of time in the dorsal pancreas for pancreatic specification and in the ventral pancreas for endocrine differentiation. In agreement with this, overexpression of *Hlxb9* from the *Pdx1* promoter results in continuous expression of *Hlxb9* in early pancreas development resulting in aberrant development of the pancreas. It seems that the pancreatic epithelium and its neighboring mesenchyme adopt an intestinal fate (Li & Edlund 2001). This gain of function experiment confirm further that only transient expression of *Hlxb9* is required for pancreatic development, and that in extended expression *Hlxb9* may function as a repressor in the developing pancreas.

Ptf1a/P48

Ptf1a first identified as *P48* is the cell specific component of the pancreatic transcription factor one *Ptf1* (Krapp et al. 1996). PTF is a heterooligomer that contains three different subunits p75, p48 and p64. p75 is required to transport the complex into the nucleus and does not contact the DNA (Sommer et al. 1991). p48

and p64 are the DNA binding subunits, they contact the DNA as a heterodimer recognizing two different motifs. p48 binds to consensus sequence CANNTG, while p64 recognizes the TGGGA motif (Cockell et al. 1989). The tissue specific component of *Ptf1* is *p48* and will be referred to as Ptf1a throughout the thesis. *Ptf1a* is a basic helix loop helix (bHLH) transcription factor that was initially identified as a key transcriptional regulator of exocrine pancreas development that binds to the 5' promoter regions of all acinar digestive enzyme genes and activates them (Cockell et al. 1989; Krapp et al. 1996). Recent results have suggested that *Ptf1a* may also be involved in endocrine cell fate specification (Krapp et al. 1998; Kawaguchi et al. 2002).

In mammals, *Ptf1a* is expressed in early pancreatic progenitors (dorsal and ventral buds) (Kawaguchi et al. 2002), but in adults it is only expressed in acinar cells (Krapp et al. 1996). In *Xenopus* endoderm, *Ptf1a* is expressed in the dorsal and ventral pancreatic anlagen at stage 32 prior to overt morphogenesis and differentiation. Later *Ptf1a* expression is maintained in the entire pancreas ((Afelik et al. 2006). Aortal endothelial cells induce *Ptf1a* specifically in the dorsal pancreatic endoderm (Yoshitomi & Zaret 2004), while *Fgf10* is necessary to maintain this dorsal expression (Jacquemin et al. 2006). Ventral induction of *Ptf1a* does not require the vitellin veins (Yoshitomi & Zaret 2004).

Loss-of-function studies in mice have demonstrated that *Ptf1a* is essential for acinar cell development and plays an important role in endocrine cell development as well. Mice homozygous for a null mutation of *Ptf1a* lack exocrine pancreatic tissue, and almost no endocrine cells are detected prior to E18. From

E18 however, pancreatic endocrine cell markers are detected in the spleen, but they are not organized into islets (Krapp et al. 1998). In humans, PTF1A gene mutations are associated with pancreatic and cerebellar agenesis (Sellick et al. 2004). In *zebrafish, Ptf1a* is only expressed in a subset of pancreatic progenitors in the left ventrolateral endoderm, and not in the dorsal posterior endoderm (Lin et al. 2004; Zecchin et al. 2004). Morpholino knockdown studies in zebrafish have shown that *Ptf1a* is required for development of all acinar cells and a subset of endocrine cells (Lin et al. 2004). In *Ptf1a* morphants, acinar development is inhibited while the early endocrine cells are unaffected (Zecchin et al. 2004). Another study showed that late endocrine cells that normally develop from ventral anterior endoderm are absent in *Ptf1a* morphants (Lin et al. 2004). Several other studies have suggested that *Ptf1a* may function as a master regulator of pancreatic cell fate. Lineage tracing analysis based on Cre-mediated recombination showed that pancreatic cells lacking *Ptf1a* fail to initialize a pancreatic program and acquire a duodenal fate instead (Kawaguchi et al. 2002). Knockdown studies in Xenopus (Afelik et al. and current study) are concordant with what have been shown previously in mice and zebrafish; implicating *Ptf1a* only in exocrine pancreas development and establishing the early insulin cells as *Ptf1a* independent (Lin et al. 2004; Krapp et al. 1998). Thus, loss of function experiments demonstrate that *Ptf1a* is essential for exocrine pancreas development but they do not ask whether *Ptf1a* is sufficient to initiate a pancreatic program.

Gain of function experiments have revealed the importance of *Ptf1a* in specifying the pancreas in the endodermal progenitors. Loss of *Hes1*, a downstream target of the *Notch* signaling pathway, results in ectopic expression of *Ptf1a* in the

stomach, duodenum and bile duct regions of mice embryos. Endodermal cells ectopically expressing *Ptf1a* switch their fate into pancreatic progenitors that will later differentiate into endocrine, exocrine and ductal cells (Fukuda et al. 2006). In *Xenopus,* direct overexpression of *Ptf1a* in the anterior gut endoderm leads to the loss of stomach and duodenum at the expense of exocrine pancreatic cells (Afelik et al. 2006). In the same study, they showed that overexpression of *Ptf1a* and *Pdx1* promotes ectopic pancreas differentiation in the posterior endoderm. Altogether, work from Afelik et al. with our current study demonstrate that *Ptf1a* is implicated in specifying all pancreatic lineages and suggest that Ptf1a plays a central role in the decision to become stomach, duodenum, bile duct or pancreas.

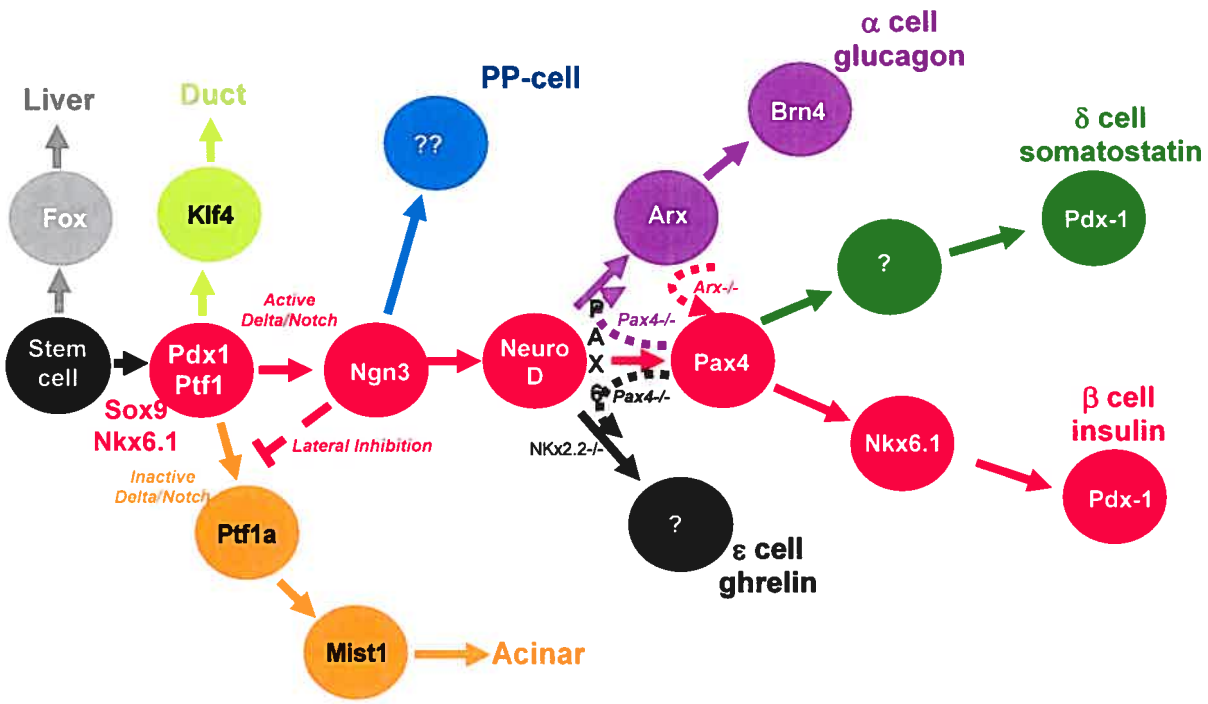

Figure 1.5 Transcription factors in pancreas development. Hierarchy of transcription factors in the developing pancreas showing the relationships among the various transcription factors. Lineage relationships are based on gene expression patterns and phenotypes from mouse knockout studies. Different colors represent different pancreatic lineages.

1.2.5.2. Exocrine specific transcription factors

Mist1

Most of the studies on pancreatic development focus on identifying regulatory genes involved in β-cell formation. Very little is known about exocrine pancreas development and function. Only few transcription factors specific to the exocrine tissue have been identified, including *Ptf1a* and *Mist1*. *Mist1* is the only pancreatic gene restricted to exocrine cells. *Mist1* is a bHLH transcription factor expressed in a variety of exocrine tissues including the salivary gland, pancreas, stomach and prostate (Pin et al. 2000). This type of expression pattern suggests that *Mist1* may be involved in regulating the process of exocytosis. Inhibition of *Mist1* disrupts first the organization of the acinar cells; later on exocrine tissue is dramatically injured with loss of acinar cells at the expense of ductal cells (Pin et al. 2000). These results indicate that *Mist1* is required to maintain exocrine function and identity.

1.2.5.3. Endocrine specific transcription factors

Understanding the transcriptional regulatory network underlying endocrine cell development is of great interest for generating β-cells either from stem cells or

from differentiated tissues. Some transcription factors are required to differentiate the endocrine lineage from the pancreatic progenitor cells, others permit to specify the different endocrine cell types.

Ngn3

The bHLH transcription factor *Ngn3* is the most important endocrine transcription factor. *Ngn3* is transiently expressed between E9.5 and E15.5 in the pancreatic epithelium prior to endocrine differentiation. Epithelial pancreatic cells expressing both *Pdx1* and *Ngn3* represent the endocrine precursor population (Schwitzgebel et al. 2000; Jensen 2004). Inactivation of *Ngn3* by homologous recombination results in diabetes. Islets of Langerhans are missing in the mutant pancreas and all four major hormones (insulin, glucagon, somatostatin and PP) are undetectable (Gradwohl et al. 2000). However, overexpression of *Ngn3* under the *Pdx1* promoter in pancreatic progenitor cells promotes an endocrine fate, characterized by an accelerated differentiation, of glucagon producing cells (Apelqvist et al. 1999). Although *Ngn3* is necessary for the development of the endocrine precursors before their differentiation it seems that other factors are required for their subsequent specification.

NeuroD/beta2

NeuroD is another member of the bHLH class of transcription factor which plays a fundamental role in pancreas development. *NeuroD* was first isolated from the pancreas and the brain. In the pancreas, the activity of the insulin and glucagon promoters depend on the presence of an E-box that binds to the cell specific bHLH *NeuroD/Beta2* (Naya et al. 1995; Dumonteil et al. 1998). *NeuroD* is expressed in the endocrine cells, the intestine and the brain (Naya et al. 1995; Lee et al. 1995). Mice lacking *NeuroD* die from severe diabetes. *NeuroD*-deficient pancreases form all types of endocrine cells, yet morphogenesis of the islets is disrupted and the number of β-cells is reduced due to prenatal apoptosis (Naya et al. 1997). In the endocrine transcriptional cascade, expression of *Ngn3* precedes and overlaps with expression of *NeuroD* (Naya et al. 1997), which precedes that of other endocrine differentiated markers such as *Pax* genes. Thus, *Ngn3* seems to be the upstream activator of *NeuroD*, which in turn activates other transcription factors such as *Pax6* (Huang et al. 2000; Marsich et al. 2003).

Isl1

Isl1 is a LIM homeodomain transcription factor expressed early during development in the dorsal pancreatic mesenchyme and in the adult pancreas *Isl1* expression is detected in the islet cells. Inhibition of *Isl1* leads to dorsal agenesis,

confirming the role of the mesenchyme in the development of the dorsal pancreatic bud (Ahlgren et al. 1997). In addition, *Isl1* embryos lack any differentiated endocrine islets, which reveals the requirement of endodermal *Isl1* for endocrine cell differentiation in the pancreas.

Pax4 and Pax6

Pax4 and *Pax6* are members of a subclass of the Pax gene family, containing both a paired domain and a homeodomain (Dahl et al. 1997). *Pax4* expression is detected as early as E9.5 in the dorsal pancreatic; one day after *Pax4* is present in dorsal and ventral pancreatic buds. Expression peaks at the secondary transition (E13.5-E15.5) and then diminishes to very low levels at birth (Sosa-Pineda et al. 1997). *Pax4* null embryos fail to develop any β- and δ- cells; instead an expansion of α- and ε- cell lines is observed (Sosa-Pineda 2004; Prado et al. 2004). *Pax4* colocalizes with other endocrine specific transcription factors such as *Ngn3*, *Isl1*, *Nkx2.2* and *Pax6*. Moreover, it has been shown that *Pax4* is a target of *Ngn3*. Thus, the role of *Pax4* in pancreas development is to control β- and α- cell specification after initiation by *Ngn3* (Sosa-Pineda et al. 1997; Sosa-Pineda 2004). Based on the knockout phenotype, one can tell that early during endocrine development *Pax4* promotes β- and α- cell differentiation by repressing α- and ε- cells. In fact, Peterson et al. (2002) have demonstrated *in vitro* that endogenous glucagon expressed in rat cells is inhibited by *Pax4* expression.

In the same study the authors showed that *Pax4* competes with *Pax6* to repress insulin transcription mediated by *Pax6*. Expression of *Pax6* is detected in all endocrine cells at early stage of development and in the mature pancreas. First gene inactivation analysis leads to a total loss of glucagon producing α-cells (St Onge et al. 1997), which indicates that *Pax6* is required for α-cells development but not necessary for that of β- and δ- cells. Interestingly, in *Pax4-Pax6* double mutant all endocrine islet cells are missing. Thus, *Pax4* and *Pax6* seem to have reciprocal and non-redundant functions during endocrine pancreatic development; *Pax4* is responsible for the differentiation of β- and δ- cells while *Pax6* is responsible for differentiating the α-cells.

Nkx2.2 and Nkx6.1

Nkx2.2 and *Nkx6.1* are members of the homeodomain class of transcription factors. *Nkx2.2* is detected very early in pancreatic precursor epithelium, and becomes restricted to endocrine β, α, and PP cells when differentiation is initiated (Sussel 1998). In *Nkx2.2* mutants, endocrine cells do not show any obvious phenotype and a large population of islet cells is still present. However, these cells do not express insulin and expression of glucagon and pancreatic polypeptide is dramatically reduced. Cells in the mutant islet clusters were thought to be immature β-cells that fail to complete their differentiation (Sussel 1998). Further analysis show

that β-cells in the *Nkx2.2* mutant mice are replaced by ghrelin producing ε-cells (as we have described before for *Pax4* mutants) (Prado et al. 2004).

Early expression of *Nkx6.1* is similar to that of *Nkx2.2* but later expression is limited to β-cells only (Jensen J et al. 1996; Oster et al. 1998). Deletion of *Nkx6.1* does not affect endocrine precursor cells, however after the secondary transition there is a great decrease in islet size due to a reduction in the number of β-cells. The other islet cells are present with normal hormonal expression. Unlike *Nkx2.2*, *Nkx6.1* mutant pancreases do not show any evidence of ghrelin cells expansion (Sander et al. 2000; Prado et al. 2004), and expression of *Nkx2.2* is normal. Interestingly *Nkx6.1* is not detected in *Nkx6.1* mutants and double deletion of both *Nkx2.2* and *Nkx6.1* is similar to *Nkx2.2* mutant phenotype. Taken together, all these data place *Nkx6.1* downstream of *Nkx2.2* in the endocrine transcriptional network and suggest that the phenotype observed in *Nkx2.2* mutant pancreas may be due to the loss of other transcription factors such as *Nkx6.1* (Sussel 1998; Sander et al. 2000). In conclusion, it is important to know that *Nkx2.2* is essential for the specification of the mature β-cell phenotype and *Nkx6.1* is essential to stabilize the β-cell phenotype rather than to induce the endocrine program.

1.3. Aim of this work

The role of *Ptf1a* in pancreas development remains controversial since it appears to have different roles in different organisms. It is still unclear if Ptf1a is involved in endocrine pancreas development. We decided to investigate its role in embryonic pancreatic cell specification using the frog *Xenopus laevis* as our experimental model. *Xenopus laevis* has been widely used to study early embryogenesis. We have also shown in our previous work that *Xenopus laevis* is a good system to study pancreatic growth and differentiation since the development of the exocrine and endocrine cells occurs in a spatially and temporally distinct manner, such that the exocrine cells appear first in the ventral pancreas, while the endocrine cells come from the dorsal pancreas. In addition the fate map of early *Xenopus* embryos is well determined and by targeting specific blastomeres we can target specifically the ventral or the dorsal pancreas. We have cloned the full length *Xenopus laevis Ptf1a* cDNA and studied its expression pattern during embryos development. We carried out gain and loss of function experiments to study the functional role of *Ptf1a* in pancreas development. Overexpression of *Ptf1a* and *Ptf1a-VP16* has different ability to convert endoderm derived organ to pancreas. On the other hand, inhibition of *Ptf1a* using morpholino antisense oligonucleotides affects the initial specification of both endocrine and exocrine lineages. Taken together, our results demonstrate that *Ptf1a* is necessary and sufficient for endocrine and exocrine pancreatic cell fate in Xenopus.

2. Materials and Methods

2.1. Isolation of XPtf1a

Degenerate PCR primers used to amplify a partial fragment of the bHLH region of *Xptf1a* were based on the following peptides: PTLPYEKR, 5'-TCCCCACCCTGCCCtaygaraarmg-3' for the forward primer and ENEPPFEFV, 5'-CACGAACTCGAAAGGGggytcrttytc-3' for the reverse primer. The PCR product was cloned into the *pCR-Script* vector, and the sequence was used to identify a *Ptf1a* open reading frame from the *X. tropicalis* genome assembly. Based on the *X. tropicalis Ptf1a* sequence the following primers were designed to amplify the 5'end of *X. laevis Ptf1a* from stage 42 whole gut cDNA: forward 5'-CCGGCACCATGGAAACGGT-3' and reverse primer 5'-ATCCTCAGGAGTCCACACT-3'. The PCR product was cloned into the *pCR-Script* vector and ligated to the bHLH region of *Xptf1a* previously isolated by cloning into the *Not1-Bsu36I* sites. The 5' UTR of *Xptf1a* was cloned using 5' RACE (BD Biosciences). 5' ready cDNA was prepared from stage 42 *Xenopus laevis* whole tadpoles (gift of G. Andelfinger). Two different reverse primers were designed: the first primer was positioned 720 bp from the start site 5'-ATCCTCAGGAGTCCACACT-3' and the second 500 bp from the start site 5'-TGAGGAAGTTAATGTAGC-3'. The PCR product was cloned into the *pCRII* vector (Invitrogen).

For cloning the *X. laevis* intron, we designed the following primers 60 bp upstream and downstream of the predicted site of the intron: forward 5'-GTACAGTCCGATCTGCCGCT-3' and reverse 5'-CTCAGTTGCTTCTCATCAGT-3'. We expected the *X. laevis* intron to be approximately 500bp in size since the *X. tropicalis* and mouse introns are 477 bp and 330 bp in length, respectively. We amplified the *X. laevis* intron from stage 42 whole gut cDNA; this can be accomplished since *X. laevis* cDNA frequently contains intronic sequence. A single band of 832 bp was amplified and cloned into the *pCRII* vector and sequenced; the intron being 712 bp long. Interestingly, when compared to the *X. tropicalis* intron only one region of 43 bp was similar in sequence, showing 84% nucleotide identity (data not shown). The accession number for the complete cDNA sequence including 5'UTR is AY372268.

2.2. Embryological assays

2.2.1. *In vitro* fertilization

Pigmented *Xenopus laevis* females were injected in the dorsal lymph sacs with 500-600 U of human chorionic gonadotrophin (HCG) 10 to 12 hours before eggs collection. Freshly squeezed eggs were fertilized *in vitro* with minced testes in 0.1XMMR (0.1M NaCl, 2.0 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM HEPES

pH7.8, 0.1 mM EDTA). Fertilized eggs were then dejellied with 3 % cysteine hydrochloride (pH 7-8) and cultured in 0.1XMMR at different temperatures between 14 and 23°C. Embryos were staged according to Niewkoop and Faber (Nieuwkoop & Faber 1967) .

2.2.2. Microinjections

Embryonic injections were preformed in 2 % Ficoll, 1XMMR. Antisense morpholino oligonucleotides were designed by Gene Tools, LLC. MO1 5'-CAACTGCTCCAGGACCGTTTCCATG-3' targets the initiation codon of *Xptf1a*. MO2 5'-ACGTTGGACTTACTTGTGCCCCGG-3' targets the exon-intron boundary. Forty ng (which corresponds to 4.5 pM) of each morpholino were injected individually into the four vegetal blastomers of 8 cell stage *Xenopus* embryos. In case of double morpholino 20 ng of MO1 were injected along with 20 ng of MO2. As control for *Ptf1a* morpholinos we injected *Klf4* (*Klf4* is present but not specific to the pancreas) morpholinos in the vegetal blastomeres of *Xenopus* embryos and we did not get any phenotype. For *Ptf1a* mRNA, 800 pg were injected along with 400 pg CMV-GFP mRNA to track our injections. For Ptf1a-VP16 mRNA only 300 pg with 400 pg of the CMV-GFP were injected into the dorsal vegetal blastomers of 8 cell stage *Xenopus* laevis embryos. Synthetic mRNA transcripts were transcribed with the SP6 *in vitro* transcription kit mMessage mMachine (Ambion). Injected embryos were cultures in 2 % Ficoll, 1XMMR for 4 hours at 18°C, then changed to 2 % Ficoll, 0.1XMMR and

kept at 18°C overnight. The following day embryos were changed to 0.1XMMR at different temperatures.

2.2.3. Generation of Elas-GFP transgenics

F0 Elas-GFP transgenics were generated using the Elas-GFP transgene as described by Beck and Slack (Beck & Slack 1999). F1 offspring were generated by crossing F0 a female Elas-GFP adult with a wild type male, whereas F2 offspring were generated by fertilizing F1 female transgenic eggs with F1 transgenic sperm *in vitro*. Germline transmission of the Elas-GFP transgene from these F1 adults is found in 75% of the offspring when transgenic eggs are fertilized with F1 transgenic male sperm (n>300). When transgenic sperm is used to fertilize wild type female eggs, only 45% of the embryos are transgenic.

2.3. Embryos Fixation and In Situ Hybridization

2.3.1. Fixation

Embryos were collected at different stages of development as whole embryos or dissected guts. Samples were fixed in MEMFA for 1 hour at room

temperature, then washed with pure ethanol for 15 minutes and stored in fresh ethanol at -20°C until subsequent hybridization.

2.3.2. Antisense probe synthesis

Antisense digoxigen probes for amylase, elastase, and insulin were prepared as previously described (Horb & Slack 2002). Antisense digoxigenin probe for *XPtf1a* was synthesized from *XPtf1a* in pCR-Script linearized with SacII and transcribed with T7 RNA polymerase. Probe for *XHex* was prepared from *XHex* in Bluescript linearized with NotI and transcribed with T7 RNA polymerase. All the probes were purified using sephadex G50 columns. The Dig-labeled antisense RNA probes were then used for in situ hybridization.

2.3.3. In Situ Hybridization

Whole mount in situ hybridization were done as described using BM purple (Harland 1991). Embryos are subjected to three major steps: hybridization, anti-body incubation and staining each of which is completed in one day.

2.3.3.1. Hybridization

Fixed embryos must be rehydrated, treated with proteinase and prehybridized before adding the probe for hybridization.

Rehydration and Proteinase K treatment

5 min sequential washes at room temperature with:

- Methanol (MeOH)
- 75 % MeOH + 25 % H2O
- 50 % MeOH + 50 % H2O
- 25 % MeOH + 75 % PTw (1XPBS, 0.1% Tween20)
- 100 % PTw (4 times)
- 5 to 15 minutes with proteinase K
- 0.1 M Triethanolamine (TEA) (2 times)
- 0.1 M TEA + 12.5 µl acetic anhydride (2 times)
- 100 % PTw (2 times)
- 20 minutes with 4 % Paraformaldehyde
- 100 % PTw (5 times)

Prehybridization

- 10 minutes in hybridization buffer at 60°C

Hybridization

- 18 to 20 hours in hybridization buffer with 1 µg/ml antisense probe at 60°C

2.3.3.2. Blocking and Antibody incubation

- 10 minutes wash with hybridization buffer at 60°C
- 20 minutes wash with 2XSSC at 60°C (2 times)
- 30 minutes wash with 0.2XSSC at 60°C (2 times)
- 15 minutes wash with Maleic acid buffer (MAB) at room temperature (2 times)
- 1 hour incubation with MAB + 2 % Blocking buffer (BMB) at room temperature
- 1 hour incubaiton with MAB + 2 % Blocking buffer (BMB) + 20 % goat serum at room temperature
- Overnight incubation with MAB + 2 % Blocking buffer (BMB) + 20 % goat serum + anti-digoxigenin antibody (1:2000 dilution) at 4°C

2.3.3.3. Staining

Prestaining washes and staining are done at room temperature

- 1 hour wash with MAB (5 times)
- 5 minutes wash with Alkaline phosphatase buffer (AP) (2 times)

- Incubation in BM-purple AP-substrate + 5 mM Levamisol until staining samples are stained
- Stained samples are fixed in MEMFA

2.4. Real time PCR

The Mx3005® multiplex quantitative PCR Thermal Cycler system from Stratagene was used to monitor the real-time experiments and generated data were collected and analyzed using the MxPro software provided with the system. RNA was extracted from whole embryos and explants using Trizol and cDNA was prepared using Superscript reverse transcriptase (Invitrogen). Primers were designed either manually or using primer3 software based on sequences available in Unigene and quality tested for accuracy. Real-time PCR reactions were prepared using the QuantiTect SYBER Green PCR kit from Qiagen. 10 µl of 2x QuantiTect SYBER Green PCR Master Mix was used for each reaction and primer concentration was optimized for 0.8 µM. PCR conditions were as follows 15 minutes activation at 95 degree, 30 seconds denaturation at 94 degree, 1 minute annealing at 58 degree and 30 seconds extension at 72 degree for 40 cycles. Real time PCR values were normalized according to EF1α. Statistics for real time PCR analysis are done for figures 3.4 and 3.7 but not for the microarray analysis since the samples are pools of different explants.

3. Results

Differential ability of Ptf1a and Ptf1a-VP16 to convert stomach, duodenum and liver to pancreas

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3.1. SUMMARY

Determining the functional attributes of pancreatic transcription factors is essential to understand how the pancreas is specified distinct from other endodermal organs, such as liver, stomach and duodenum, and to direct the differentiation of other cell types into pancreas. In this paper, we examine the functional ability of seven pancreatic transcription factors, both unmodified and VP16 fusions, to convert liver to pancreas: Nkx2.2, Hlxb9, Islet1, Pax4, Pax6, NeuroD and Ptf1a. Using the transthyretin promoter to drive expression in the early liver region/bud of transgenic *Xenopus* tadpoles, we find that only one of these factors, Ptf1a-VP16, is able to convert liver to pancreas. Overexpression of the unmodified Ptf1a on the other hand, has no effect in liver, but is able to convert stomach and duodenum to pancreas. When overexpressed at earlier embryonic stages throughout the endoderm, Ptf1a activity is similarly limited, whereas Ptf1a-VP16 has increased activity. Interestingly, in all instances we find that Ptf1a-VP16 is only capable of promoting acinar cell fates, whereas Ptf1a promotes both acinar and endocrine fates. Lastly, we demonstrate that, similar to mouse and zebrafish, *Xenopus Ptf1a* is essential for the initial specification of both endocrine and exocrine cells during normal pancreas development.

Keywords: *Xenopus*, pancreas, Ptf1a, specification, transdifferentiation, endocrine, exocrine, organogenesis

3.2. Introduction

The vertebrate pancreas has its embryological origin as two endodermal buds developing on the dorsal and ventral side of the duodenum (Edlund, 2002; Kim and MacDonald, 2002). The dorsal bud arises just below the notochord, while the ventral bud develops adjacent to the hepatic diverticulum (Slack, 1995). The fusion of the two buds gives rise to a single mixed gland composed of exocrine and endocrine cells. The exocrine pancreas is a lobulated branched tissue, which includes acinar and ductal cells that secrete and transport digestive enzymes into the duodenum. The endocrine cells are grouped into islets of Langerhans composed of five principal cell types, α, β, δ, ε and PP that secrete glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide hormones into the bloodstream.

In the amphibian *Xenopus laevis*, development of the pancreas proceeds in an almost identical manner to that seen in mammals (Kelly and Melton, 2000). The dorsal bud is the first to appear from a region just below the notochord at stage 35/36. The ventral pancreas derives from two ventral buds adjacent to the liver that fuse at stage 37/38. At stage 39, morphogenetic movements of the gastrointestinal tract reposition the pancreatic rudiments leading to their fusion and formation of a single organ. Signals from the mesoderm are required for pancreatic differentiation, and development of exocrine and endocrine cells occurs in a spatially and temporally distinct manner (Horb and Slack, 2001; Horb and Slack, 2002; Kelly and Melton, 2000). Exocrine cells are initially specified in the ventral pancreas, and endocrine cells in the dorsal. *Amylase* is first detected at stage 40 only in the ventral

pancreas, and expression subsequently spreads to the dorsal pancreas such that by stage 45 it is present throughout the entire pancreas (Horb and Slack, 2002). On the other hand, *insulin* is first expressed at stage 32 in the dorsal pancreatic endoderm; expression in the ventral pancreas is only detected at stage 47 (Horb and Slack, 2002; Kelly and Melton, 2000). In contrast, the other endocrine markers *glucagon* and *somatostatin* are not detected in the pancreas until stage 45; though expression is present in the stomach at earlier stages.

Several classes of transcription factors are involved in the specification and differentiation of both endocrine and exocrine lineages (Habener et al., 2005). Although the number of these transcription factors is significant, their precise roles in the pancreatic transcriptional cascade and the downstream targets they regulate remain unknown. Two of the earliest acting transcription factors are *Pdx1* and *Ptf1a*. *Pdx1* is a ParaHox gene that is expressed at the earliest stages in the dorsal and ventral pancreatic buds as well as in the duodenum (Wright et al., 1989); at later stages it is highly expressed in beta cells, with lower levels also found in acinar cells and all rostral duodenal cells (Jonsson et al., 1995). Mice lacking *Pdx1* do not develop a pancreas (Jonsson et al., 1994; Offield et al., 1996) and mutations in the human homologue, *Ipf1*, are associated with pancreatic agenesis (Stoffers et al., 1997).

Ptf1a is a bHLH gene that is expressed in early pancreatic progenitors (dorsal and ventral buds) (Kawaguchi et al., 2002), but in adults is only expressed in acinar cells (Krapp et al., 1996). The early induction of *Ptf1a* in the dorsal pancreas was shown to require interactions with endothelial cells (Yoshitomi and Zaret, 2004),

while *Fgf10* is necessary to maintain this dorsal expression (Jacquemin et al., 2006). Loss-of-function studies in mice have demonstrated that *Ptf1a* is essential for acinar cell development and plays an important role in endocrine cell development as well (Kawaguchi et al., 2002; Krapp et al., 1998); in humans, PTF1A gene mutations are associated with pancreatic and cerebellar agenesis (Sellick et al., 2004). Similarly, morpholino knockdown studies in zebrafish and more recently in *Xenopus* have shown that *Ptf1a* is required for development of all acinar cells and a subset of endocrine cells (Afelik et al., 2006; Lin et al., 2004). It is however, only expressed in a subset of pancreatic progenitors in the left ventrolateral endoderm, and not in the dorsal posterior endoderm (Lin et al., 2004; Zecchin et al., 2004). Several reports have suggested that *Ptf1a* may function as a master regulator of pancreatic cell fate. For example, pancreatic cells lacking *Ptf1a* switch their fate and become duodenal (Kawaguchi et al., 2002), and loss of Hes1 leads to the generation of ectopic *Ptf1a* expression in stomach, duodenum and bile duct resulting in ectopic pancreas formation (Fukuda et al., 2006). Similarly, overexpression of *Ptf1a* in *Xenopus* embryos leads to an expansion of the pancreatic region, but only within the *Pdx1*-expression domain, whereas combined overexpression of *Pdx1* and *Ptf1a* is sufficient to promote acinar cell fates in posterior endoderm (Afelik et al., 2006). Altogether, these results suggest that *Ptf1a* plays a central role in the decision to become stomach, duodenum, bile duct or pancreas.

Transdifferentiation is the conversion of one differentiated cell type to another (Okada, 1991; Slack and Tosh, 2001). At the molecular, level transdifferentiation is associated with a change in the expression of master regulatory genes (Tosh and

Slack, 2002), of which *Pdx1* is considered to be the pancreatic master gene. Although it is expressed in and required for the development of an endodermal domain broader than the pancreas alone, *Pdx1* can be considered to be part of the transcription factor program associated with the adoption of pancreas fate because it is expressed very early in the outgrowth of the anlagen of this organ, and because there is such an early abrogation of pancreas development in Pdx1-/- mutants. In agreement with this, *Pdx1* has been shown to convert liver to pancreas (Meivar-Levy and Ferber, 2006). Overexpression of *Pdx1* in liver activates expression of pancreatic endocrine and exocrine markers (Ferber et al., 2000; Kojima et al., 2003; Miyatsuka et al., 2003; Tang et al., 2006b), and is sufficient to prevent STZ induced hyperglycemia (Ber et al., 2003; Ferber et al., 2000; Sapir et al., 2005; Zalzman et al., 2003). We also showed that expression of a super-active form of *Pdx1* (*Pdx1*-VP16) in both human HepG2 cells and *Xenopus* transgenics converts liver into pancreatic tissue containing both exocrine and endocrine cell types (Horb et al., 2003; Li et al., 2005). As seen with Pdx1, these Pdx1-VP16 expressing liver cells are capable of functioning as beta cells to restore euglycemia in diabetic mice (Cao et al., 2004; Imai et al., 2005; Kaneto et al., 2005; Tang et al., 2006a). Apart from *NeuroD*, which was shown to induce islet neogenesis in the liver (Kojima et al., 2003), the functional ability of other pancreatic transcription factors to convert liver to pancreas has not been fully explored.

We now report our results examining whether the ability to convert liver to pancreas is a general characteristic of every pancreatic transcription factor or limited to those that function as master regulators of early cell fate as opposed to those that

might be involved in specific lineage decisions made after the acquisition of a general pancreas fate. We find that only one of these, *Ptf1a*, is able to promote ectopic pancreas formation in the endoderm. We demonstrate that *Ptf1a* and *Ptf1a-VP16* have differential activities in converting endodermal organs to pancreas, dependant on the timing of overexpression. Furthermore, we find that *Ptf1a-VP16* is only capable of promoting an acinar cell fate, while the unmodified *Ptf1a* promotes both acinar and endocrine cell fates. Last, we investigate the role of *Ptf1a* in normal *Xenopus* pancreas development and find that large-scale knock-down of *Ptf1a* function affects the initial specification of both exocrine and endocrine cells. Taken together, our results establish *Ptf1a* as being both necessary and sufficient for endocrine and exocrine pancreatic cell fate.

3.3. MATERIALS AND METHODS

3.3.1. *Xenopus* transgenics and transgene construction

All Xenopus transgenic constructs were cloned into the *TTR-VP16:Elas-GFP* vector that was derived from *TTR-Xlhbox8-VP16:Elas-GFP* (Horb et al. 2003). Each transgene was constructed as follows and confirmed by sequencing. *TTR-XNkx2.2:Elas-GFP - XeNK-2* (gift from R. Grainger) cut *NotI*(blunt)-*XhoI* and cloned into *TTR-VP16:Elas-GFP* (blunt-*XhoI*) *TTR-XNkx-2.2-VP16:Elas-GFP- XNkx-2.2* was PCR isolated with 5' *XhoI* site in primer and cloned in frame to the C-terminus of VP16 (*XhoI*) in *TTR-VP16:Elas-GFP*. *TTR-Pax4:Elas-GFP-* mouse *Pax4* cDNA (kind gift of Takashi Yamaoka (Matsushita et al. 1998)) cut *NotI*(blunt)-*XhoI* and cloned into *ClaI*(blunt)-*XhoI* of *TTR-Xlhbox8-VP16:Elas-GFP*. *TTR-VP16-Pax4:Elas-GFP- mPax4* was PCR isolated with 5' *XhoI* site in primer and cloned in frame into the *XhoI* site of *TTR-VP16:Elas-GFP*. For *TTR-XHlxb9:Elas-GFP*, we first cloned the full length *XHlxb9* as only a partial sequence had been isolated previously. The 5'end of *XHlxb9* was isolated using a 5' degenerate primer (ATGGARAARWSNAARAAYTT- MEKSKN) based on mouse and human HLXB9 sequences and an internal primer based on the published *XHlxb9* (AF072382). This was ligated to the 3'end of *XHlxb9* that was cloned by PCR and was confirmed by sequencing. *TTR-XHlxb9:Elas-GFP- XHlxb9* was cut *NotI(blunt)-XhoI* and cloned into *TTR-Xlhbox8-VP16:Elas-GFP StuI-XhoI*. *TTR-XHlxb9-VP16:Elas-GFP- XHlxb9*

was PCR isolated with 3' *ClaI* site in primer and cloned in frame into the *ClaI* site of *TTR-VP16:Elas-GFP* by PCR. *TTR-Pax6:Elas-GFP- Pax6* (gift of Jane Wu) was cut *EcoRI*(blunt)-*XhoI* and cloned into *ClaI*(blunt)-*XhoI* of *TTR-Xlhbox8-VP16:Elas-GFP*. *TTR-VP16-Pax6:Elas-GFP- Pax6* was PCR isolated with 5' *XhoI* site in primer and cloned in frame into *XhoI* site of *TTR-VP16:Elas-GFP*. *TTR-NeuroD:Elas-GFP-NeuroD* (gift from Jackie Lee) was cut *ClaI-XbaI*(blunt) and cloned into *ClaI-XhoI*(blunt) of *TTR-Xlhbox8-VP16:Elas-GFP*. *TTR-NeuroD-VP16:Elas-GFP- NeuroD* was PCR isolated with 3' *ClaI* site in primer and cloned in frame into *ClaI* site of *TTR-VP16:Elas-GFP*. *TTR-Islet1:Elas-GFP- Islet1* (gift of D. Melton) was cut *EcoRI*(blunt)-*XhoI* and cloned into *TTR-Xlhbox8-VP16:Elas-GFP* cut *ClaI*(blunt)-*XhoI*. *TTR-VP16-Islet1:Elas-GFP- Islet1* was PCR isolated with 5' *XhoI* site in primer and cloned in frame into *XhoI* site of *TTR-VP16:Elas-GFP*. *TTR-VP16-mPtf1a:Elas-GFP- mPtf1a* was cut *EcoRI* (mung bean nuclease) and cloned into *TTR-VP16:Elas-GFP* cut *XhoI* (blunt). *TTR-mPtf1a:Elas-GFP- mPtf1a* was cut *XbaI* (blunt)-*XhoI* and cloned into *TTR-Xlhbox8-VP16:Elas-GFP* cut *StuI-XhoI*. *IFABP-mPtf1a:Elas-GFP-TTR-mPtf1a:Elas-GFP* was cut *KpnI* (blunt) and ligated into *IFABP-GFP* cut *BamHI* (blunt). Same procedure followed for *IFABP-VP16-mPtf1a:Elas-GFP*. *TTR-Xptf1a:Elas-GFP- Xptf1a* in *pCR-Script* cut *NotI-ClaI* (blunt) and cloned into the *TTR-Xlhbox8-VP16:Elas-GFP* vector was cut *ClaI-XhoI* (blunt). *Xptf1a-VP16- Xptf1a* was isolated as above and cloned into *VP16-N* vector cut *ClaI* (blunt). *TTR-Xptf1a-VP16:Elas-GFP- Xptf1a-VP16* was cut *NcoI-XbaI* (blunt) cloned into *TTR-Xlhbox8-VP16:Elas-GFP* as above. *Xenopus laevis* transgenics were produced as previously described (Horb et al. 2003). In later stages of our work, we switched the protocol for

creating transgenics to the I-SceI meganuclease method, with no change in activity (Pan et al., 2006; Thermes et al., 2002). All functional portions (TTR-GeneX;Elas-GFP) of the transgenes described above were cloned in between two I-SceI sites.

F0 Elas-GFP transgenics were generated using the Elas-GFP transgene as described (Beck and Slack, 1999). F1 offspring were generated by crossing F0, a female Elas-GFP adult, with a wild type male, whereas F2 offspring were generated by fertilizing F1 female transgenic eggs with F1 transgenic sperm *in vitro*. Germline transmission of the Elas-GFP transgene from these F1 adults is found in 75% of the offspring when transgenic eggs are fertilized with F1 transgenic male sperm (n>300). When transgenic sperm is used to fertilize wild type female eggs, only 45% of the embryos are transgenic.

3.3.2. Isolation of *Xptf1a*

Degenerate PCR primers used to amplify a partial fragment of the bHLH region of *Xptf1a* were based on the following peptides: PTLPYEKR, 5'-TCCCCACCCTGCCCtaygaraarmg-3' for the forward primer and ENEPPFEFV, 5'-CACGAACTCGAAAGGGggytcrttytc-3' for the reverse primer. The PCR product was cloned into the *pCR-Script* vector, and the sequence was used to identify a *Ptf1a* open reading frame from the *X. tropicalis* genome assembly. Based on the *X. tropicalis Ptf1a* sequence the following primers were designed to amplify the 5'end of *X. laevis Ptf1a* from stage 42 whole gut cDNA: forward 5'-

CCGGCACCATGGAAACGGT-3' and reverse primer 5'-ATCCTCAGGAGTCCACACT-3'. The PCR product was cloned into the *pCR-Script* vector and ligated to the bHLH region of *Xptf1a* previously isolated by cloning into the *Not1-Bsu36I* sites. The 5' UTR of *Xptf1a* was cloned using 5' RACE (BD Biosciences). 5' ready cDNA was prepared from stage 42 *Xenopus laevis* whole tadpoles (gift of G. Andelfinger). Two different reverse primers were designed: the first primer was designed 720 bp from the start site 5'-ATCCTCAGGAGTCCACACT-3' and the second 500 bp from the start site 5'-TGAGGAAGTTAATGTAGC-3'. The PCR product was cloned into the *pCRII* vector (Invitrogen).

For cloning the *X. laevis* intron, we designed the following primers 60 bp upstream and downstream of the predicted site of the intron: forward 5'-GTACAGTCCGATCTGCCGCT-3' and reverse 5'-CTCAGTTGCTTCTCATCAGT-3'. We expected the *X. laevis* intron to be approximately 500 bp in size since the *X. tropicalis* and mouse introns are 477 bp and 330 bp in length, respectively. We amplified the *X. laevis* intron from stage 42 whole gut cDNA; this can be accomplished since *X. laevis* cDNA frequently contains intronic sequence. A single band of 832 bp was amplified and cloned into the *pCRII* vector and sequenced; the intron being 712 bp long. Interestingly, when compared to the *X. tropicalis* intron only one region of 43 bp was similar in sequence, showing 84% nucleotide identity (data not shown). The accession number for the complete cDNA sequence including 5'UTR is AY372268.

3.3.3. Embryological assays and whole mount *In situ* hybridization

Antisense morpholino oligonucleotides were designed by Gene Tools, LLC. MO1 5'-CAACTGCTCCAGGACCGTTTCCATG-3' targets the initiation codon of *Xptf1a*. MO2 5'-ACGTTGGACTTACTTGTGCCCCGG-3' targets the exon-intron boundary. Synthetic mRNA transcripts were synthesized by SP6 *in vitro* transcription (mMessage machine, Ambion). Whole-mount *in situ* hybridizations with single probes were performed as described using BM Purple (Horb et al., 2003). Antisense digoxigenin probe for *Xptf1a* was synthesized from *Xptf1a* in *pCR-Script* linearized with *SacII* and transcribed with T7 RNA polymerase. Probes for other pancreatic markers were prepared as previously described (Horb et al., 2003).

3.3.4. Real time PCR

The Mx3005® multiplex quantitative PCR Thermal Cycler system from Stratagene was used to monitor the real-time experiments and generated data were collected and analyzed using the MxPro software provided with the system. RNA was extracted from whole embryos and explants using Trizol, cDNA prepared using Superscript reverse transcriptase (Invitrogen). Primers were designed based on sequences available in Unigene and quality tested for accuracy. Real-time PCR reactions were prepared using the QuantiTect SYBER Green PCR kit from Qiagen. 10µl of 2x QuantiTect SYBER Green PCR Master Mix was used for each reaction and primer concentration was optimized for 0.8 µM. PCR conditions were as follows

15 min activation at 95 degree, 30 sec denaturation at 94 degree, 1 min annealing at 58 degree and 30 sec extension at 72 degree for 40 cycles. Real time PCR values were normalized according to EF1α.

3.4. Results

3.4.1. *Ptf1a-VP16* is sufficient to convert liver to pancreas

Previously, we demonstrated that Pdx1, when fused to VP16, was sufficient to convert liver to pancreas (Horb et al., 2003), but whether this attribute was a general characteristic of every pancreatic transcription factor or limited to those that function as master regulators of early cell fate was not explored. To address this, we examined the transdifferentiation activity of several pancreatic transcription factors using *Xenopus* transgenics. Both unmodified and VP16 fusions of seven different pancreatic transcription factors, Hlxb9, NeuroD, Islet1, Pax4, Pax6, Nkx2.2 and Ptf1a, were expressed in *Xenopus* tadpole liver (Table 3.1). Except for *Pax4* and *Ptf1a*, all constructs were generated with *Xenopus* cDNAs; for both *Pax4* and *Ptf1a* we used the mouse cDNAs as the *Xenopus* genes had yet to be cloned. (As outlined below we also cloned and tested *Xenopus Ptf1a*.) We confirmed all fusion proteins were in frame by *in vitro* transcription/translation and western blot analysis with a VP16 antibody (data not shown). We created transgenic tadpoles overexpressing each of these pancreatic transcription factors using the double transgene construct previously described with Pdx1-VP16 (Horb et al., 2003). Briefly, each gene was cloned downstream of the liver transthyretin (TTR) promoter and the resultant transgenic tadpoles examined for ectopic Elas-GFP expression. In *Xenopus*, the TTR promoter directs expression to the liver, stomach, and duodenum after stage 44 as we previously

demonstrated (Fig. 3.4I,J in (Horb et al., 2003)); this is after organ bud formation and cell fate specification, but prior to complete maturation (Yan et al., 1990). The pancreas-specific elastase promoter controls GFP expression providing a real-time marker of pancreatic cell fate and showing that the transgene is not silenced due to integration-site effects (Beck and Slack, 1999; Horb et al., 2003). Though elastase is an acinar-specific gene product, the elastase promoter element (A+B+C) used here is the 213 bp fragment of the complete promoter that is active in both endocrine and exocrine cells as it lacks the endocrine repressive element (Kruse et al., 1993). We defined positive transdifferentiation activity as ectopic expression of GFP in transgenic tadpole liver and/or stomach/duodenum (Horb et al., 2003); ectopic GFP expression representing a generic pancreatic fate. Of these fourteen constructs (VP16 fusions and unmodified) we found only two, *Ptf1a-VP16* and *Ptf1a*, were able to promote ectopic activation of Elas-GFP (Table 3.1). Since those pancreatic transcription factors defined as having no activity in our assay are known regulators of endocrine cell differentiation, it remained possible that ectopic formation of endocrine pancreatic cells did occur in the absence of elastase promoter activation. To rule out this possibility, we examined transgenic whole guts expressing these endocrine transcription factors for ectopic insulin expression. We did not detect ectopic insulin expression outside the pancreas in any of these transgenics (data not shown). Nevertheless, it still remains possible that some of these endocrine pancreatic transcription factors may have some ability to promote ectopic differentiation of other endocrine cells (alpha, delta), but this was not explored. Since positive results were

obtained with *Ptf1a*, we decided to perform a more detailed characterization of the phenotypes associated with overepxression of *Ptf1a* and *Ptf1a-VP16*.

Transgenic TTR-GeneX;Elas-GFP	Liver Elas-GFP	St/Duo Elas-GFP	Ectopic Insulin	n	% TD
Nkx2.2-VP16	---	---	---	26	0
HlxB9-VP16	---	---	---	24	0
Islet-VP16	---	---	---	21	0
Pax4-VP16	---	---	---	28	0
Pax6-VP16	---	---	---	17	0
NeuroD-VP16	---	---	---	31	0
Ptf1a-VP16	+++	---	---	36	44
Ptf1a	---	+++	+++	77	27

Table 3.1 Transdifferentiation ability of pancreatic transcription factors. Both VP16 and unmodified constructs were tested, but only the VP16 fusions are shown here. All transgenes were cloned downstream of the TTR promoter. % TD- Percent transdifferentiation activity; defined as ectopic expression of Elas-GFP outside the normal pancreatic region.

In transgenic *TTR-mPtf1a-VP16:Elas-GFP* tadpoles ectopic GFP fluorescence is detected in the liver after stage 45 (Fig. 3.1B), whereas in *TTR-mPtf1a:Elas-GFP* transgenics ectopic activation of Elas-GFP is detected in the stomach and duodenum and not in the liver (Fig. 3.1C). Prior to this stage Elas-GFP expression is only found in the pancreas, and initial development of liver, pancreas, stomach and duodenum occurs normally in both *Ptf1a* and *Ptf1a-VP16* transgenics. In *Ptf1a-VP16* transgenic tadpoles, we find only partial conversion of the liver, similar to that seen in *Pdx1-VP16*

transgenics (Horb et al., 2003), whereas in *Ptf1a* transgenics we find complete conversion of the stomach and duodenum (see below). The proportion of *Ptf1a* and *Ptf1a-VP16* transgenic tadpoles showing ectopic GFP expression was 27% and 44%, respectively (Table 3.1). This was quite different from the results we previously obtained with *Pdx1-VP16*, where we obtained 61% transgenics with ectopic pancreas (Horb et al., 2003). The reason for these differences is unclear, but may reflect the fact that the *Pdx1* acts earlier than *Ptf1a* in pancreas development. These results demonstrate that the ability to bring about transdifferentiation of liver to pancreas is specific to *Pdx1-VP16* and *Ptf1a-VP16* and does not arise simply from the use of the strong transactivator VP16 with any pancreatic transcription factor. Furthermore, it demonstrates that the ability to convert other organs to pancreas is not a general characteristic of every pancreatic transcription factor, but is limited to those that act during initial stages of cell fate specification (perhaps as master regulatory genes).

To characterize the ectopic pancreatic tissue in the livers of *Ptf1a-VP16* transgenics, we determined which pancreatic cell types (endocrine or exocrine) were present. Whole guts were isolated from transgenic tadpoles at stage 46-47 and the expression of both acinar and endocrine markers was examined by whole mount *in situ* hybridization. Abundant expression of *amylase* RNA was detected throughout the ectopic pancreas (Fig. 3.1E). We did not detect expression of *insulin* or *glucagon*, although occasionally a single positive cell was detected in the ectopic pancreatic tissue (Fig. 3.1H,K). To confirm that our results represented a transdifferentiation event, we examined *Ptf1a-VP16* transgenics for loss of liver tissue. We found a large decrease in expression of the liver differentiation marker transthyretin (Fig. 3.2B). In the example

shown only a small piece of liver tissue remains fused with the ectopic pancreas. In agreement with our previous results with *Pdx1-VP16,* we found different phenotypes of liver to pancreas transdifferentiation, from an almost complete loss of liver tissue to partial conversions (data not shown). Histological analysis confirmed ectopic pancreas fusion with liver in *Ptf1a-VP16* transgenics (Fig. 3.3B). In conclusion, these results demonstrate that *Ptf1a-VP16* is able to cause transdifferentiation of liver to pancreas, but only to acinar cells and not endocrine cells.

Since the mouse Ptf1a protein shares only 60% overall amino acid identity with *Xenopus* Ptf1a (see below), we decided to examine whether *Xenopus Ptf1a* had similar transdifferentiation activity. We therefore cloned *Xenopus Ptf1a* and created a *Xenopus Ptf1a-VP16* fusion and overexpressed it in transgenic tadpole liver. Identical to that seen with mouse *Ptf1a*, *Xenopus Ptf1a-VP16* was sufficient to promote pancreatic acinar, and not endocrine, fate in liver (data not shown). To determine whether the placement of VP16 is important we created both N- and C-terminal fusions with Ptf1a, but found no difference in activity (data not shown). These results establish that placement of the VP16 activation domain is not important, and that the mouse and *Xenopus* proteins behave similarly.

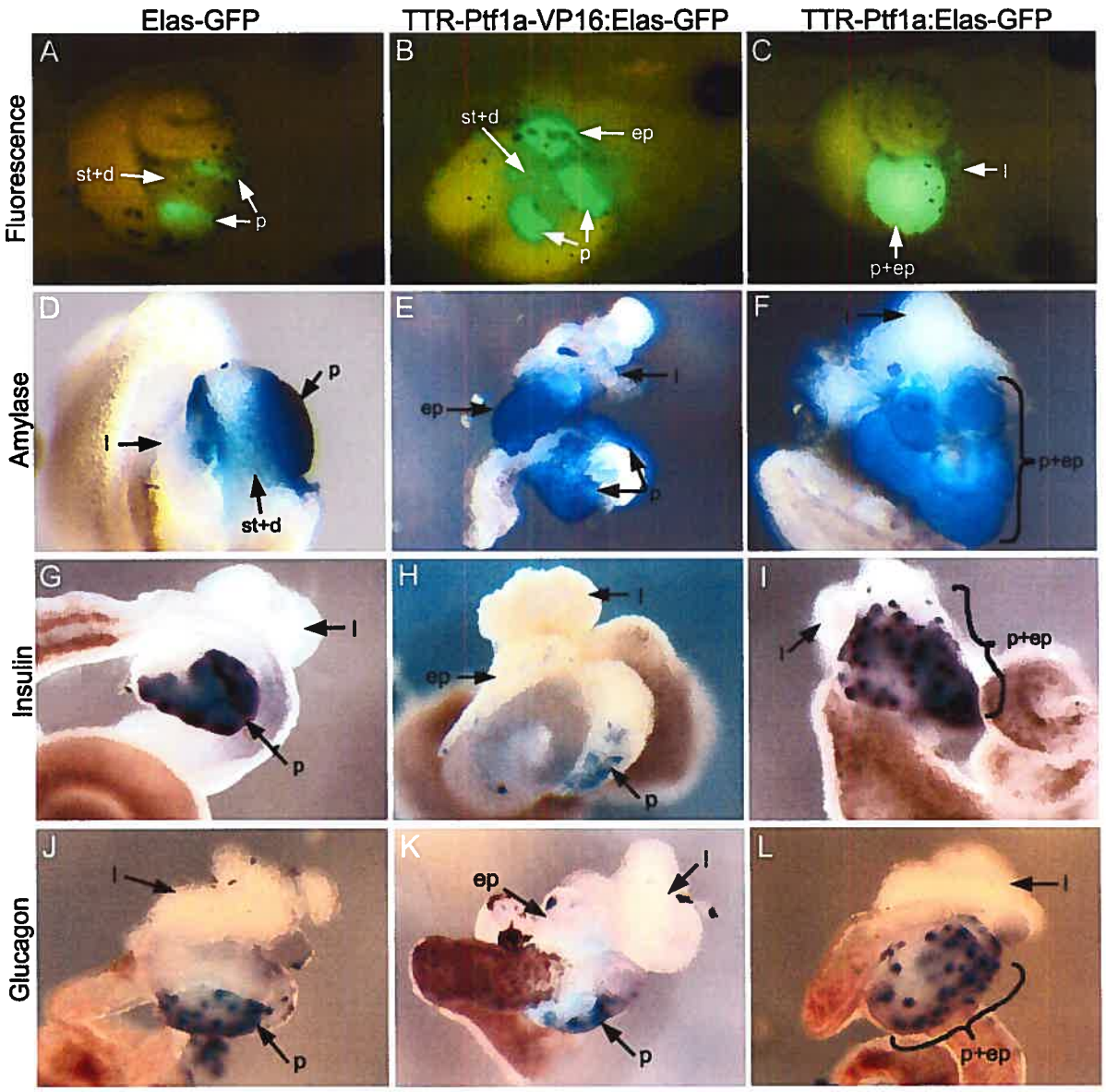

Elas-GFP
TTR-Ptf1a-VP16:Elas-GFP
TTR-Ptf1a:Elas-GFP
Fluorescence
Amylase
Insulin
Glucagon
A
B
C
D
E
F
G
H
I
J
K
L
st+d
ep
p
l
p+ep

Figure 3.1 Transgenic overexpression of *Ptf1a* and *Ptf1a-VP16* promotes ectopic pancreas formation. (A) Control St.44 Elas-GFP transgenic tadpole showing GFP expression only in the pancreas (p). No expression is seen in the stomach and duodenum (st+d). (B) St.45 TTR-Ptf1a-VP16:Elas-GFP transgenic tadpole. Ectopic expression (ep) of Elas-GFP is seen opposite the pancreas in the liver. Stomach and duodenum are normal. (The punctate GFP expression in between the ectopic and normal pancreas does not represent epithelial cells in the stomach or duodenum, but are cells released from the ectopic pancreas due to mechanical abrasion during processing of samples for photography.) (C) St.45 TTR-Ptf1a:Elas-GFP transgenic tadpole. No stomach or duodenum is evident and instead ectopic GFP is detected throughout this region (p+ep). The liver (l) is normal. (D-F) *Amylase* RNA expression in stage 46/47 whole guts. (E) Ectopic expression (ep) is seen in the livers of TTR-Ptf1a-VP16 tadpoles. (F) In TTR-Ptf1a transgenics ectopic *amylase* expression is now seen in the stomach and duodenum that is fused with the normal pancreas (p+ep) and not in the liver. (G-I) *Insulin* RNA expression in stage 46/47 whole guts. (G,H) Expression of *insulin* is only seen in the pancreas of control and TTR-Ptf1a-VP16 transgenics. No expression is seen in the ectopic pancreas (ep). (I) In TTR-Ptf1a transgenics insulin expression is detected in the ectopic pancreatic tissue encompassing the stomach, duodenum and pancreas (p+ep). The normal pancreas is seen at the tip of the arrow, while the ectopic pancreas is just to the right. The shape of this ectopic pancreatic tissue resembles the normal stomach. The liver (l) is normal.

3.4.2. The unmodified *Ptf1a* converts duodenum and stomach to pancreas

The ectopic Elas-GFP expression seen in *TTR-Ptf1a:Elas-GFP* transgenics derives from the stomach and duodenum, and not from the liver as seen in *Ptf1a-VP16* transgenics (Fig. 3.1A-C). Upon dissection of whole guts from transgenic tadpoles after stage 46, we found no evidence of stomach or duodenum, while the esophagus, intestine and liver appeared normal. Instead, we found a large region of pancreatic tissue in the posterior foregut resulting from fusion of the normal pancreas with ectopic pancreatic tissue generated from the stomach and duodenum (Fig. 3.1C). We examined *TTR-Ptf1a* transgenics for ectopically expressed pancreatic differentiation markers. Interestingly, we detected expression of both endocrine and exocrine markers, *amylase, insulin* and *glucagon,* within the ectopic pancreatic tissue emanating from the region of the stomach, duodenum and pancreas (Fig. 3.1F,I, L). This is in contrast to that seen in *Ptf1a-VP16* transgenics, where no endocrine markers were detected in the ectopic pancreatic tissue. These results demonstrate that overexpression of the unmodified Ptf1a after organogenesis of stomach, duodenum and liver is sufficient to promote both ectopic pancreatic endocrine and exocrine cell fates in the stomach/duodenum, but not in the liver.

To confirm transdifferentiation of stomach and duodenum to pancreas, we examined whether there was loss of the stomach/duodenum marker *frp5* in Ptf1a transgenic guts. We found an almost complete absence of *frp5* expression in whole guts isolated from TTR-Ptf1a transgenics as compared to control and TTR-Ptf1a-VP16 transgenics (Fig. 3.2F). We did detect a small amount of *frp5* staining at the

position corresponding to reappearance of the gut tube ("d"), at the posterior end of the ectopic pancreatic tissue (Fig. 3.2F). We next examined whether liver differentiation occurred normally, and found normal expression of the liver differentiation marker *transthyretin* in Ptf1a transgenics (Fig. 3.2C). This was in contrast to the reduced expression found in Ptf1a-VP16 transgenics (Fig. 3.2B), but in agreement with the lack of ectopic Elas-GFP expression in the livers of Ptf1a transgenics (Fig. 3.1C). Serial histological analysis of whole guts isolated from TTR-Ptf1a transgenics confirmed the loss of stomach and duodenum, and replacement with pancreatic tissue (Fig. 3.3C-F). In anterior sections, the esophagus is evident, whereas in more posterior sections no gut tube is present, and the entire region is replaced with pancreatic tissue (Fig. 3.3E). In anterior sections, both the liver and pancreas can be seen clearly (Fig. 3.3C); in more posterior sections, the pancreas expands to encompass the whole region of the gut tube that should have developed as stomach and duodenum (Fig. 3.3D,E). We have determined that the ectopic pancreatic tissue replaces the stomach and duodenal tube for a distance of 168μm (Fig. 3.3C-F). In conclusion, these results confirm that unmodified *Ptf1a* is sufficient to cause transdifferentiation of stomach/duodenum to pancreas, but has no effect in liver cells.

We next examined whether *Ptf1a* and *Ptf1a-VP16* transdifferentiation activity is limited to stomach, duodenum and liver by testing their ability to convert more posterior intestinal cells into pancreas using the intestinal fatty acid binding (IFABP) promoter (Sweetser et al., 1988). In *Xenopus* transgenics, the IFABP promoter is active throughout the entire intestine posterior to the duodenum beginning at stage

44, but not in the colon (Beck and Slack, 1999). Transgenic tadpoles were generated bearing the transgenes *IFABP-mPtf1a:Elas-GFP* or *IFABP-VP16-mPtf1a:Elas-GFP*, and examined for ectopic intestinal GFP expression. However, at no time (up to stage 48/49) did we observe ectopic GFP fluorescence in the intestine (data not shown). These results demonstrate that the ability of *Ptf1a* and *Ptf1a-VP16* to convert other cell types into pancreas is limited to the posterior foregut derivatives stomach/duodenum and liver, respectively.

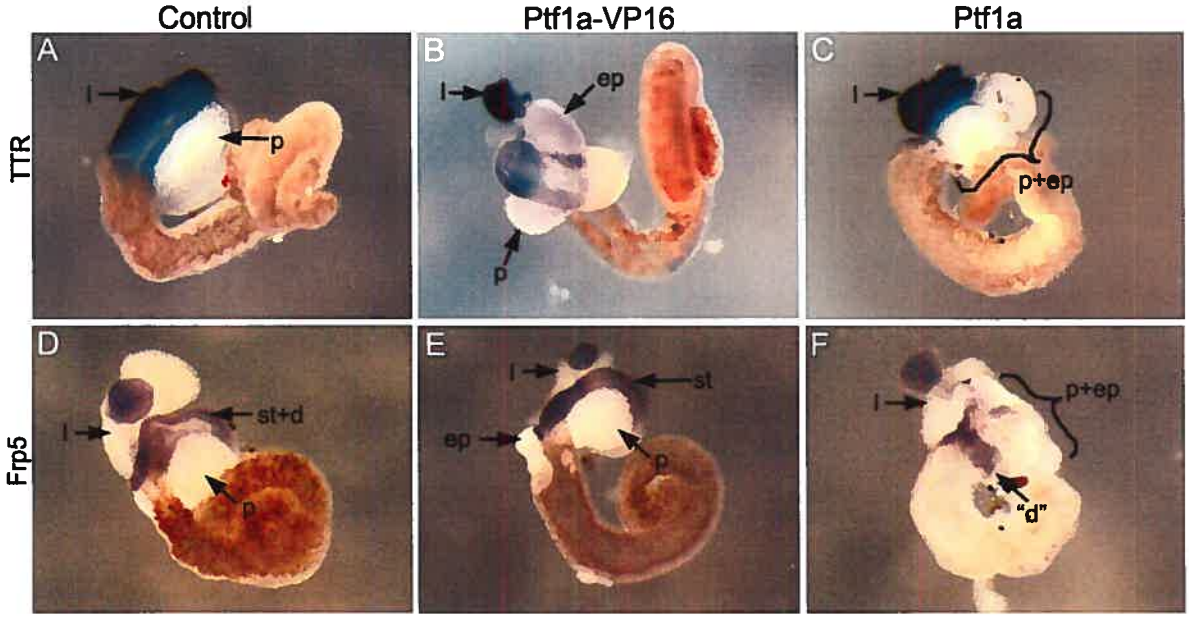


Figure 3.2 Effects of transgenic overexpression of *Ptf1a-VP16* and *Ptf1a* on liver, stomach and duodenum. (A-C) Liver differentiation marker, *transthyretin* expression in stage 46/47 whole guts. (A) Control showing normal domain of *TTR* expression. Notice the size of the liver. (B) *Ptf1a-VP16* transgenic whole gut. Only a small domain of *transthyretin* expression is evident, located adjacent to the ectopic pancreas. (C) In *Ptf1a* transgenics *transthyretin* expression is normal. (D-F) Expression of the stomach differentiation marker *frp5* in stage 46/47 whole guts. (M) *Frp5* expression in control whole guts extends from the duodenum through the stomach region (st+d), (E) *Ptf1a-VP16* transgenic whole gut- *frp5* expression is normal. (F) *Ptf1a* transgenic whole gut. Almost no *frp5* expression is detected. A small region at the posterior end of the pancreas and ectopic pancreas does express *frp5* and may be marking a small remnant of the duodenum ("d").

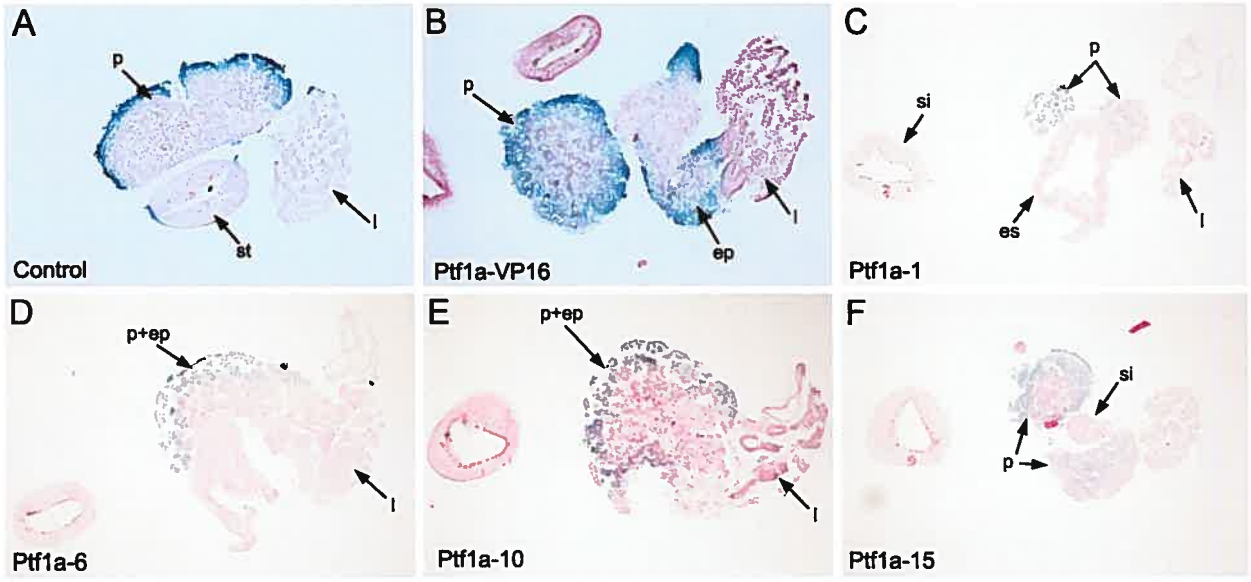
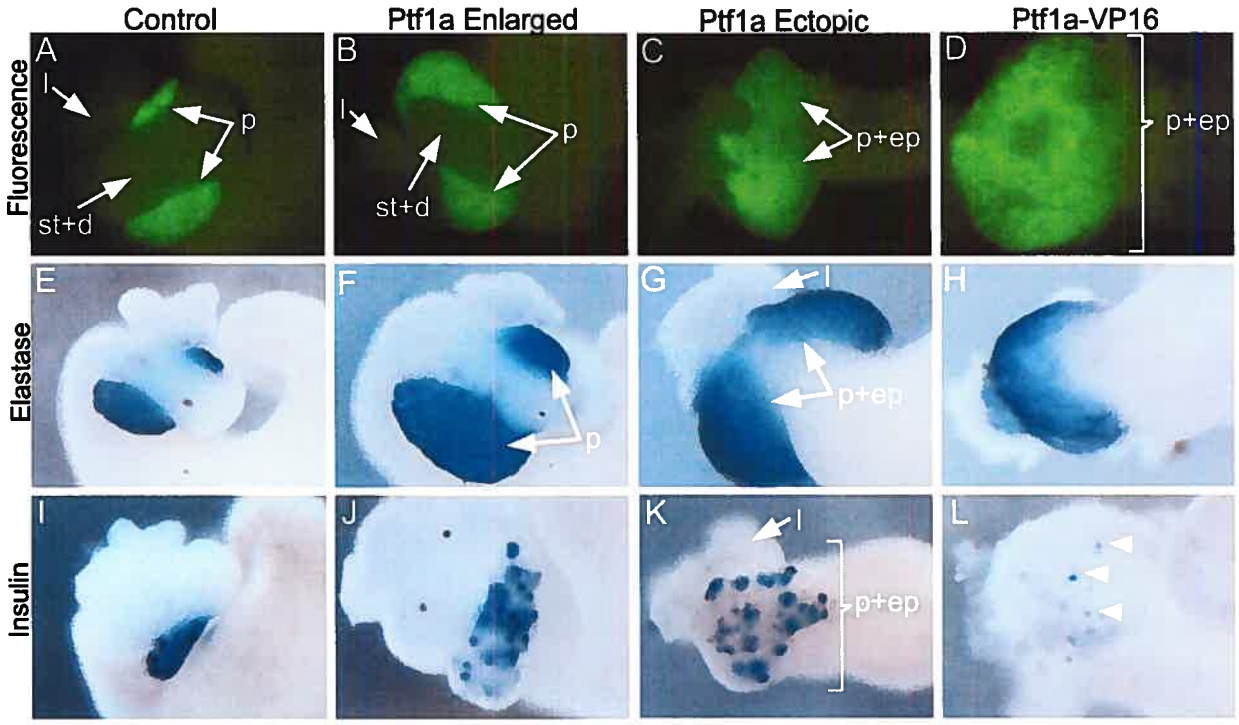


Figure 3.3 Histological analysis of Ptf1a and Ptf1a-VP16 transgenic whole guts. (A) Control whole gut section stained for amylase showing normal stomach (st), pancreas (p) and liver(l). (B) Section from Ptf1a-VP16 transgenic whole gut stained with amylase. The ectopic pancreas (ep) is seen fused with the liver, and the normal pancreas adjacent but separate. (C-F) Single whole gut from TTR-Ptf1a:Elas-GFP transgenic tadpole was processed for histology after staining for amylase expression. (C-F) 12µm serial sections. The posterior small intestine (si) is evident in every section on the left side for orientation. (C) Section #5- esophagus (e), pancreas (p) and liver (l) are all present. (D) Section #11- the pancreas has almost completely replaced the stomach and duodenum (p+st/d). A small portion of remaining stomach tube ("st") is evident, but contains no recognizable stomach cells and the tube is open. Part of the liver (l) can still be seen. (E) Section #18- no gut tube is evident, and a large ectopic pancreas (p+st/d) is found in place of the stomach and duodenum. (F) Section #24- the small intestine (si) first reappears, with pancreatic tissue and liver still present. We measured the lack of stomach and duodenum from section 10-24 to be 168µm in total.

3.4.3. *Ptf1a* and *Ptf1a-VP16* have similar capabilities in embryonic endoderm, prior to organogenesis

To determine whether *Ptf1a* and *Ptf1a-VP16* activity is dependant on the differentiation state of endodermal cells, we tested their ability to ectopically activate the Elas-GFP promoter at earlier embryonic stages, prior to organogenesis. *Ptf1a* mRNA was injected into the four vegetal blastomeres of 8 cell transgenic F2 Elas-GFP *X. laevis* embryos (see Materials and Methods). The *Elas-GFP* transgene is active from stage 32 and GFP fluorescence can be observed as early as stage 38 in all pancreatic cells (Beck and Slack, 1999; Horb et al., 2003). Overexpression of *Ptf1a* mRNA in vegetal blastomeres, which was expected to diffuse broadly throughout the endodermal progenitors, leads to ectopic activation of the Elas-GFP transgene only in the stomach and duodenum (Fig. 3.4C). No ectopic expression was detected either in the liver or posterior endoderm, similar to that seen in transgenics. We also observed a second milder phenotype of enlarged pancreas, which contained a recognizable stomach and duodenum of approximately normal size, in contrast to the absence of these tissues as noted above (Fig. 3.4B). To confirm that ectopic activation of the Elas-GFP transgene is indeed indicative of pancreatic differentiation, we examined injected embryos for expression of *elastase* and *insulin* using whole mount *in situ* hybridization. In *Ptf1a*-injected embryos, we found ectopic expression of both *elastase* and *insulin* in the stomach and duodenum (Fig. 3.4G,K). In the enlarged pancreata, we also found increased expression of both *insulin* and *elastase* (Fig. 3.4F,J). We confirmed the expression level changes of *insulin* and

amylase in *Ptf1a*-injected embryos using real time PCR. Four individual embryos (control and *Ptf1a*-injected) were processed for real time PCR. In agreement with our in situ results, we found a 1.4 fold increase in *insulin* expression and a 2.3 fold increase in *amylase* expression (Fig. 3.4M). The other endocrine markers *glucagon* and *somatostatin* are not expressed in the developing *Xenopus* pancreas until stage 45; prior to this stage however, both are expressed in stomach and duodenal endocrine cells. To examine *glucagon* and *somatostatin* expression in *Ptf1a*-injected embryos, we collected whole guts at stage 45. Overall, we found reduced levels of both genes, which may be indicative of the loss of stomach and duodenum (Fig. 3.5B,E). We did detect relatively normal levels of expression of *glucagon* and *somatostatin* in the ectopic pancreas, but unlike *insulin* expression we did not detect increased expression of these genes (Fig. 3.5). These results demonstrate that overexpression of *Ptf1a* in early endoderm is sufficient to promote pancreatic acinar and beta cell development, and repress stomach and duodenal endocrine cell development.


Control
Ptf1a Enlarged
Ptf1a Ectopic
Ptf1a-VP16
Fluorescence
Elastase
Insulin
A
B
C
D
E
F
G
H
I
J
K
L
l
p
st+d
p+ep

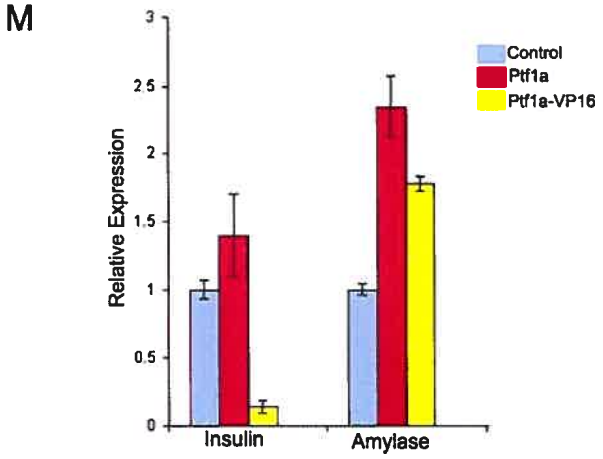

M
Relative Expression
3
2.5
2
1.5
1
0.5
0
Control
Ptf1a
Ptf1a-VP16
Insulin
Amylase

Figure 3.4 Overexpression of *Ptf1a* and *Ptf1a-VP16* mRNA promotes ectopic and enlarged pancreas formation. Stage 42-44 dissected whole guts are shown in each image. (A) Control F2 Elas-GFP transgenic tadpole whole gut. (B) *Ptf1a*-injected embryos showing expanded GFP fluorescence. Stomach (st), duodenum (d) and liver (l) are normal, but the pancreas (p) is enlarged. (C) Ectopic pancreas formation in Ptf1a-injected embryo. The pancreas, stomach and duodenum (p+ep) form a large ectopic pancreas expressing Elas-GFP. (D) *Ptf1a-VP16* injected embryo showing ectopic GFP fluorescence throughout the anterior endoderm. (E-H) *In situ* hybridization for *elastase* expression on isolated whole guts from (E) Control, (F,G) *Ptf1a*-injected embryos and (H) *Ptf1a-VP16* injected embryos. Notice expanded and ectopic expression of *elastase* in *Ptf1a* and *Ptf1a-VP16* injected embryos. (I-L) *In situ* hybridization for *insulin* in (I) Control, (J,K) *Ptf1a*-injected embryos and (L) *Ptf1a-VP16* injected embryos. More insulin-expressing cells are detected in Ptf1a-injected whole guts, while there is a large decrease in insulin-expressing cells in Ptf1a-VP16 injected whole guts (arrowheads). (M) Real time PCR analysis of endocrine and exocrine markers in Ptf1a and Ptf1a-VP16 injected embryos for amylase and insulin expression in control, Ptf1a-injected and Ptf1a-VP16 injected whole embryos at stage 35. Each bar is an average of 4 individual whole tadpoles. Amylase expression is increased in both, while insulin expression is decreased in Ptf1a-VP16 and increased in Ptf1a-injected embryos. Purple-control tadpoles, red-Ptf1a mRNA injected tadpoles, yellow-Ptf1a-VP16 injected tadpoles. Error bars correspond to standard error = standard deviation/$\sqrt{n}$ (n=number of samples).

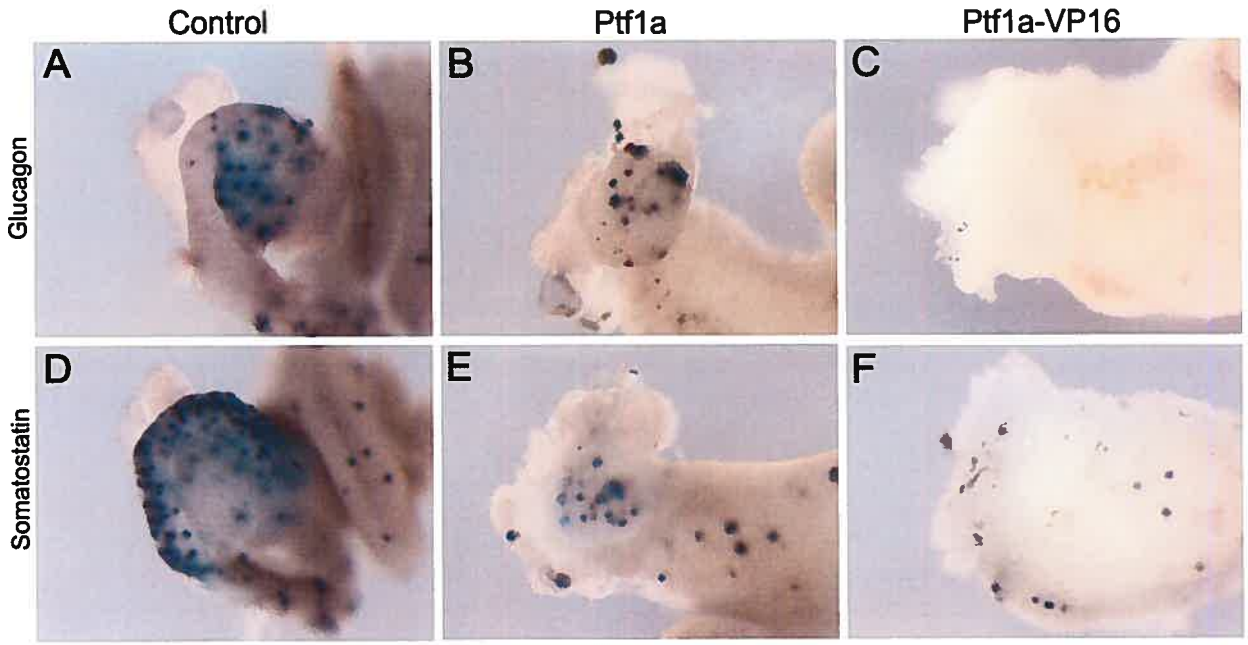


Figure 3.5 Development of *glucagon* and *somatostatin* expressing cells in *Ptf1a* and *Ptf1a-VP16* injected embryos. (A-C) In situ hybridization for *glucagon* expression in control, *Ptf1a* and *Ptf1a-VP16* injected embryos at stage 45. (B) Stomach and duodenal expression of *glucagon* is absent, while pancreatic *glucagon* expression appears slightly reduced in *Ptf1a*-injected ectopic pancreas phenotype. (C) No *glucagon* expression is detected in *Ptf1a-VP16* injected embryos. (D-F) *Somatostatin* expression. (E) Overall *somatostatin* expression is reduced in *Ptf1a*-injected embryos. Pancreatic expression appears normal, while stomach and duodenal expression is completely absent. (F) *Somatostatin* expression is completely absent in *Ptf1a-VP16* injected embryos.

To determine what effects *Ptf1a* has on development of adjacent endodermal organs, we examined *Ptf1a*-injected embryos for expression of *Frp5* (Pilcher and Krieg, 2002) and *Hex* (Newman et al., 1997), markers of stomach/duodenum and liver respectively. In *Ptf1a*-injected embryos, we found normal expression of *Hex*, but little to no *frp5* expression was detected, indicating that stomach and duodenum did not develop properly, while liver development was normal (Fig. 3.6C,G). In the enlarged pancreas phenotype both *frp5* and *Hex* expression was normal (Fig. 3.6B,F). We next examined *Ptf1a*-injected embryos for excessive histone H3 expression to determine if this activity is due to an overproliferation of pancreatic cells. However, we did not detect any extra histone H3 staining above control (data not shown). These results indicate that *Ptf1a* is sufficient to respecify early stomach/duodenal cells to pancreas, prior to their differentiation, but has no effect in the developing liver bud or posterior endoderm, identical to that seen in transgenics.

We next examined whether *Ptf1a-VP16* activity is similarly restricted in early endoderm as in transgenics. Similar to that seen with the unmodified *Ptf1a*, overexpression of *Ptf1a-VP16* within the entire endoderm resulted in ectopic Elas-GFP expression only in the anterior endoderm (Fig. 3.4D); no ectopic expression was detected in posterior endoderm in agreement with the results obtained in transgenic overexpression. In these embryos, we found Elas-GFP expression to encompass a much greater area than that seen with *Ptf1a*, suggesting that *Ptf1a-VP16* was now able to respecify liver, stomach and duodenum into pancreas (Fig. 3.4). We confirmed the loss of liver, stomach and duodenum by examination of *Hex* and *frp5* expression (Fig. 3.6D,H). To determine which pancreatic cell types *Ptf1a-VP16* promoted, we examined

endocrine and exocrine gene expression in stage 40 injected embryos. In agreement with the transgenic overexpression data, we found abundant expression of *elastase* throughout the Elas-GFP domain (Fig. 3.4H). However, to our surprise almost no *insulin* expression was detected (Fig. 3.4L). To determine more quantitatively the effects of *Ptf1a-VP16* on *insulin* expression, we examined initial *insulin* expression at stage 35 using real time PCR. In agreement with the results seen in stage 40 whole guts, we found an 86% decrease in *insulin* expression in *Ptf1a-VP16* injected embryos at early stage 35 when beta cells are first specified (Fig. 3.4M). In contrast, in *Ptf1a*-injected embryos, we found a 40% increase in *insulin* expression at this same early stage. We also examined *Ptf1a*-injected embryos for activation of acinar gene products. By real time PCR we found a 1.8 fold increase in *amylase* expression in *Ptf1a-VP16* injected embryos (Fig. 3.4M). In conclusion, these results demonstrate that *Ptf1a-VP16* has greater activity in early endoderm than at later organogenesis stages, but that its capability is similarly restricted to promoting only acinar cell fate. In addition, the fact that insulin is down-regulated by *Ptf1a-VP16* suggests that *Ptf1a-VP16* alters the differentiation program of endocrine precursors, such that they differentiate along the acinar lineage.

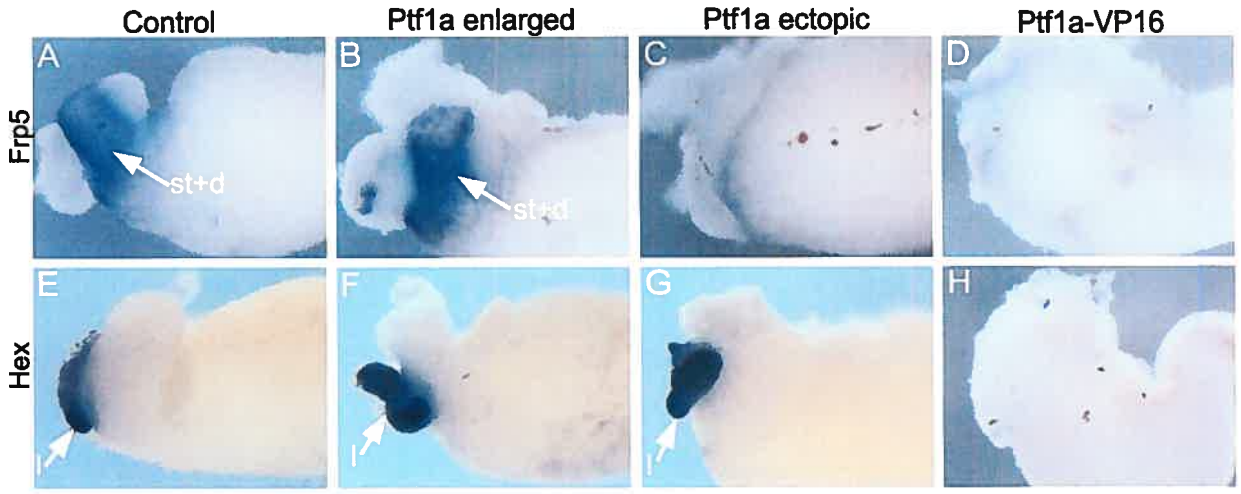


Figure 3.6 Effects of Ptf1a and Ptf1a-VP16 mRNA overexpression on organogenesis of liver, stomach and duodenum. (A-D) *In situ* hybridization for *frp5* expression in control, *Ptf1a*-injected embryos and *Ptf1a-VP16* injected embryos. (B) Normal expression is seen in the enlarged pancreas phenotype from Ptf1a-injected embryos, (C) whereas reduced expression is seen in the ectopic pancreas phenotype from *Ptf1a*-injected embryos. (D) No frp5 expression is detected in whole guts from *Ptf1a-VP16* injected embryos. (E-H) *In situ* hybridization for *Hex* expression in Control, *Ptf1a*-injected embryos and Ptf1a-VP16 injected embryos. (F,G) Normal expression of *Hex* is detected in both enlarged and ectopic phenotypes from *Ptf1a*-injected embryos. (H) No Hex expression is detected in whole guts from *Ptf1a-VP16* injected embryos.

3.4.4. *Xenopus Ptf1a* is essential for both exocrine and endocrine pancreas development

To examine the functional role of *Ptf1a* in normal *Xenopus* pancreas development, we isolated the *X. laevis Ptf1a* cDNA. The deduced protein sequence of *Xenopus* Ptf1a shares approximately 60% amino acid identity with other species, with highest similarity found in the bHLH DNA binding domain (data not shown). Similar to that seen by Afelik et al., we find Ptf1a to be first expressed at neurula stages in the midbrain-hindbrain region. Within the pancreatic endoderm *Ptf1a* is first detected in both dorsal and ventral pancreatic anlagen at stage 32 prior to overt morphogenesis and differentiation (data not shown). The expression of *Ptf1a* in both dorsal and ventral pancreatic buds prior to differentiation suggests that it may play a fundamental role in early cell fate specification of both endocrine and exocrine cells.

To determine if *Ptf1a* is essential for early pancreas development, we used antisense morpholinos to block translation and splicing of *Ptf1a*. A morpholino overlapping the translation start site of *Ptf1a* was designed (MO1) based on 5'UTR sequence, which we cloned using 5' rapid amplification of cDNA ends (see materials and methods). A second morpholino (MO2) was also designed to the exon-intron boundary to inhibit splicing of the *Ptf1a* RNA (see materials and methods). We examined the efficacy of this second morpholino by determining what effects it had on splicing of the *Ptf1a* transcript. Injection of MO2 prevented splicing of the primary *Ptf1a* transcript leading to inclusion of the single intron (Fig. 3.7R). We were still able to detect some of the proper splice transcript in MO2-injected embryos suggesting

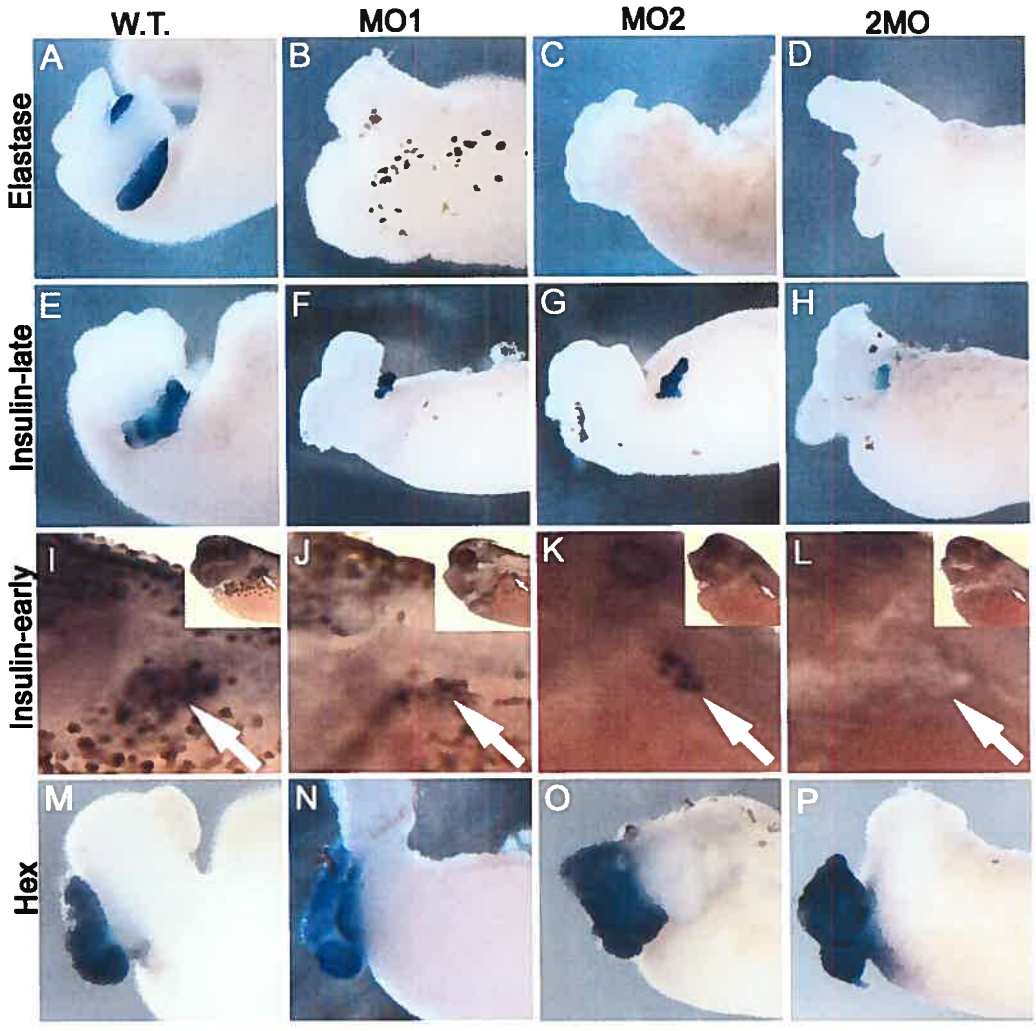
that the single morpholino was unable to completely inhibit Ptf1a translation; this may explain the reason for a more severe phenotype being found with co-injection of both morpholinos. To specifically target loss of *Ptf1a* to the region of the embryo where it is selectively expressed in the pancreatic endoderm, and avoid any indirect effects of reducing protein levels in neural tissue, another site of robust *Ptf1a* expression, we injected *Ptf1a* morpholinos into all four vegetal blastomeres of 8 cell *Xenopus* embryos. The antisense morpholinos are fluorescently labeled thus allowing us to identify rapidly whether the injection was properly targeted to the anterior endoderm. Embryos were then allowed to develop to tadpole stages and analyzed for any abnormalities.

Embryos injected with 40 ng of either MO1 or MO2 developed normally through gastrula, neurula and tail bud stages. However, at tadpole stages the gut developed abnormally with almost no recognizable pancreas (Fig. 3.7). At stage 45, approximately 50% of injected embryos have a small dorsal nub of tissue remaining, while the other 50% seem to completely lack any obvious pancreatic outgrowth (data not shown). Expression of acinar differentiation markers was completely lost (Fig. 3.7A-C), while insulin expression was reduced substantially (Fig. 3.7E-G). Interestingly, even in those embryos with no discernable pancreatic tissue, several insulin-expressing cells can be detected. These results would therefore seem to suggest that *Ptf1a* is not essential for endocrine cell development. Alternatively, it is possible that since we are looking at *insulin* expression four to five days after morpholino injection the presence of endocrine cells may be due to a dilution of the morpholino allowing newly generated beta cells to develop. To address this

possibility, we examined the *insulin* expression at the earlier tadpole stage 32 when it is first detected. Similar to the results at later stages, we found *insulin* expression reduced, but not completely absent (Fig. 3.7Q), thus arguing against a dilution of the morpholino. It is possible that reduction of *Ptf1a* function more significantly affects exocrine (acinar) differentiation, and perhaps that lower levels still allow the derivation of endocrine cells from the uncommitted progenitor cells.

To determine if an incomplete block of *Ptf1a* was responsible for the partial inhibition in endocrine cell development, we co-injected both morpholinos. To accurately compare our results, we injected the same total amount of both morpholinos (20 ng each) used with each single morpholino. 20 ng of MO1 and 20ng of MO2 (2MO) were co-injected into the vegetal blastomeres of eight cell embryos. Similar to that seen with each single morpholino, no pancreatic outgrowth was evident; *elastase* expression was absent (Fig. 3.7D), and insulin expression was reduced at stage 42 (Fig. 3.7H). Interestingly, in contrast to the single morpholino injections, expression of insulin was completely absent at early stage 32 demonstrating that Ptf1a function is essential for initial specification of both endocrine and exocrine cells (Fig. 3.7L). We confirmed *insulin* expression differences quantitatively by real-time PCR analysis of MO1-, MO2-, 2MO-injected and control embryos at stage 35. Four individual embryos were examined for each injection. There was an average decrease in *insulin* expression of 3.5 fold for single MO1- and MO2-injected embryos, whereas in 2MO-injected embryos there was an 18 fold reduction (Fig. 3.7Q). In all cases, single or double morpholino, we found liver development occurred normally as marked by expression of *Xhex* (Fig. 3.7M-

P). The loss of *insulin* expression at early stages and subsequent re-expression at later stages suggests that in *Xenopus Ptf1a* is essential for the initial specification of both endocrine and exocrine cells, while its function is dispensable for the generation of the later-appearing insulin cells.


W.T.
MO1
MO2
2MO
Elastase
Insulin-late
Insulin-early
Hex
A
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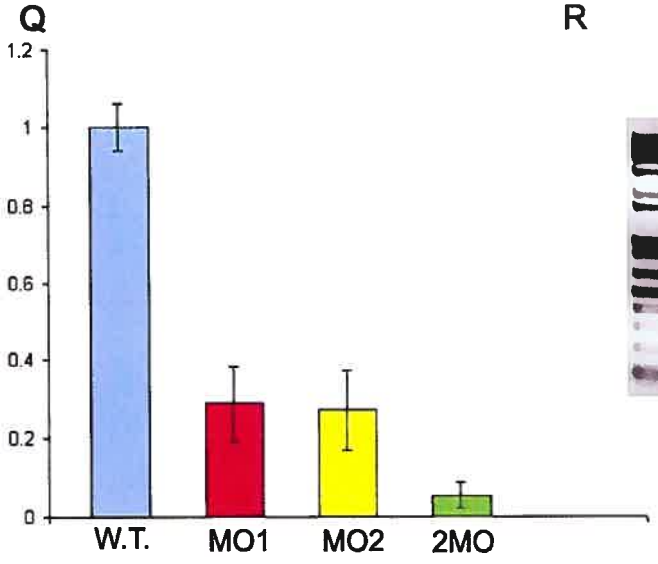
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1
0.8
0.6
0.4
0.2
0
W.T.
MO1
MO2
2MO

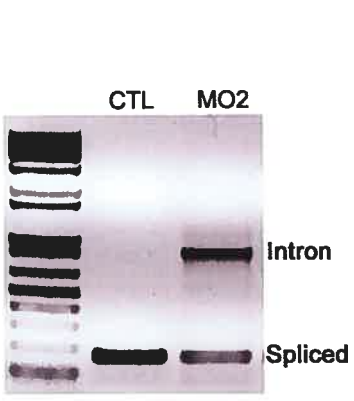
R
CTL
MO2
Intron
Spliced

Figure 3.7 Elastase expression is absent in *Ptf1a* morphants, while insulin expression is reduced at late stages, but absent at early stages. (A-D) Whole mount *in situ* hybridization for *elastase* RNA expression in control and *Ptf1a*-MO dissected whole guts at stage 42. *Elastase* expression is not detected in either single or double morpholino injected guts. (E-H) Whole mount *in situ* hybridization for *insulin* RNA expression in control and *Ptf1a*-MO whole guts. (F,G) *Insulin* expression is decreased in both single morpholino injections. (H) In embryos injected with both morpholinos together *insulin* expression is also reduced, but still present. (I-L) Initial insulin expression is lacking in *Ptf1a* morphants. *Insulin* RNA expression by whole mount in situ hybridization in control and *Ptf1a-MO* injected embryos at stage 35. Inset in each panel is the low power view. (I) Control tadpole showing normal punctate *insulin* expression in the dorsal pancreas (arrow). (J,K) *Insulin* expression is reduced in single morpholino injected embryos, but (L) completely lacking in double morpholino injected embryos. (M-P) Expression of the liver marker *Hex* is normal in both single and double *Ptf1a* morpholino injected embryos. (Q) Real time PCR analysis of *insulin* expression in control and *Ptf1a*-MO injected embryos at stage 35 confirms *insulin* reduction in single and double morpholino injected embryos. Each bar is an average of four individual tadpoles. There is a 50-60% reduction in single morpholino tadpoles (40ng), but an almost complete absence in the double morpholino tadpoles (20ng each). Error bars correspond to standard error = standard deviation/$\sqrt{n}$ (n=number of samples). (R) RT-PCR analysis of splicing in control and MO2-injected embryos showing the inhibition of splicing by MO2. Loading control was established with *EF1α* (not shown). Primers were designed flanking the single *Ptf1a* intron.

3.4.5. Mouse *Ptf1a* mRNA rescues pancreatic agenesis caused by *Xptf1a* morpholino

To determine if the phenotype caused by the *Ptf1a* morpholino is directly related to inhibition of endogenous *Ptf1a*, we attempted to rescue the loss of pancreatic tissue and acinar differentiation markers by co-expressing mouse *Ptf1a* mRNA. Injection of 25 ng of MO2 leads to a complete loss of ventral pancreatic tissue and persistence of a dorsal nub in 50% of injected embryos (Fig. 3.8B). Co-injection of 300 pg of *mPtf1a* mRNA rescued the loss of pancreatic tissue and acinar gene expression. 50% of rescued embryos developed a normal sized pancreas with elastase expression (Fig. 3.8C). The other 50% had a slightly enlarged pancreas with elastase expression, similar to the *Ptf1a* overexpression phenotype (Fig. 3.8D). These results clearly demonstrate that the *Ptf1a* morpholino phenotype is indeed due to inhibition of the endogenous *Xenopus Ptf1a* mRNA.

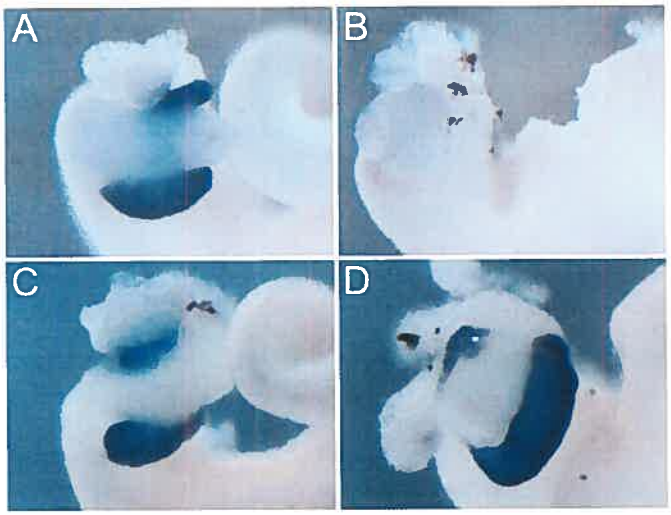

Figure 3.8 Mouse *Ptf1a* rescues the *Ptf1a*-morpholino induced phenotype. (A) Control whole gut showing elastase expression in the pancreas. (B) In *Ptf1a-MO* whole guts elastase is absent. (C,D) Pancreatic tissue and elastase expression is restored in *Ptf1a-MO* embryos co-injected with *mPtf1a*.

3.5. Discussion

Despite the numerous studies pursuing loss-of-function studies with pancreatic transcription factors, few have addressed the sufficiency of these factors in activating the pancreatic differentiation program in other cell types. In this paper, we have used *Xenopus* transgenics and mRNA overexpression to examine the functional ability of seven different pancreatic transcription factors in converting liver to pancreas, and found that only one of these, Ptf1a-VP16, is sufficient. We also show that, while the unmodified Ptf1a has no effect in liver, it is sufficient to reprogram stomach/duodenum to pancreas. Last, we have investigated the role of *Xenopus laevis Ptf1a* in early pancreatic cell fate specification and found that it is essential for proper development of both endocrine and exocrine pancreatic lineages. These results establish Ptf1a as a central player in directing pancreatic cell fate determination being both necessary and sufficient.

3.5.1. *Ptf1a* is a master regulator of pancreatic cell fate

Much of the previous work on converting other cell types to pancreas has focused on *Pdx1* since in its absence the pancreas does not form. Indeed, we showed that *Pdx1-VP16* is sufficient to convert liver to pancreas (Cao et al., 2004; Horb et al., 2003; Li et al., 2005). The ability of other PTFs to convert liver to pancreas has not been explored. Using our transgenic transdifferentiation assay, we

find that only one other transcription factor, Ptf1a, has any activity to ectopically activate the Elas-GFP transgene in *Xenopus* transgenics. The ability to convert liver to pancreas would therefore seem to be limited to only those transcription factors that function during the initial stages of pancreas development, prior to the division of endocrine and exocrine progenitors. The fact that loss of *Ptf1a* or *Pdx1* leads to pancreas agenesis, while loss of the other PTFs affects only certain endocrine lineages supports this notion. We have not however thoroughly investigated every pancreatic transcription factor, and it will be of interest to determine if this concept holds true for other early acting lineage restricted transcription factors, such as ngn3. Furthermore, it is possible that a whole series of combinations of the various factors (twos, threes, etc.) might reveal interesting conversion phenotypes consistent with the ability of *Ptf1a* and *Pdx1* to act early to engage these suites of transcription factors in the correct order/combinations.

Our examination of the sufficiency of *Ptf1a* in converting other endodermal cells into pancreas suggests that the addition of VP16 (though enhancing transcriptional activation) actually restricts Ptf1a activity, and has revealed differential responses to this transcription factor among distinct foregut tissues. Several lines of evidence support this contention: 1) the unmodified Ptf1a is sufficient to convert stomach/duodenum to pancreas at organogenesis stages, whereas Ptf1a-VP16 has no such ability; 2) Ptf1a-VP16 can only promote acinar cell fate, while the unmodified Ptf1a promotes both endocrine and acinar cell fates; and 3) even at earlier stages, prior to differentiation and organogenesis, Ptf1a-VP16 only promotes acinar cell fates. These ideas do not however address the fact that the

unmodified Ptf1a is itself limited in function, such that it is unable to exert any phenotypic effect in liver cells at any stage. We simply suggest that the addition of VP16 restricts Ptf1a target specificity and/or protein interactions to only those promoting acinar and not endocrine differentiation. Therefore, the ability of Ptf1a to promote either endocrine or acinar cell fates seems to be determined by its partner protein interactions at different times during embryogenesis. This implies that the intrinsic activity of Ptf1a when expressed as a VP16 fusion is to convert liver, and endoderm-generally, to acinar fates, while a version that does not "bypass" its normal transcriptional complex build-up is able to promote both acinar and endocrine cell fates. The finding that Ptf1a can only convert posterior foregut tissues to pancreas would therefore suggest that other coactivators are essential for Ptf1a to bring about a fate change. Indeed, the fact that PTF1a is part of a heterotrimeric complex and that it is regulated differently depending on its interaction with partner proteins supports this argument (Beres et al., 2006; Esni et al., 2004).

Identification of the different protein partners and downstream targets of Ptf1a and Ptf1a-VP16 will help elucidate how pancreatic progenitors are directed down an endocrine or acinar lineage. The fact that Ptf1a-VP16 is able to promote acinar cell fate in early anterior endoderm and later liver cells (but not stomach/duodenum) suggests that the same protein partners are present throughout the anterior endoderm as in the liver, and not in the posterior endoderm or the stomach and duodenum. Of interest along these lines is the finding that neither Ptf1a nor Ptf1a-VP16 overexpression in early embryos has any effect on gastrulation and neurulation. In fact, we only see a phenotype after stage 30. Based on results with

other transcription factors in *Xenopus*, one would have thought that overexpression of a VP16 fusion would lead to early developmental defects. The simplest interpretation is that the necessary cofactors for Ptf1a are only present after stage 30. However, since Ptf1a is a bHLH protein it should interact with other bHLH proteins at all stages and interfere with their function. Alternatively, since we are only injecting the mRNA into vegetal blastomeres, it may be that the early endoderm lacks the necessary bHLH cofactors. In seeming agreement with this, overexpression of Ptf1a in ectoderm inhibits neurogenesis at early stages (Obata et al., 2001).

3.5.2. Two pathways to generate endocrine cells: *Ptf1a* dependent and independent

Our results concur with previous data in zebrafish and mouse demonstrating that *Ptf1a* is an essential regulator of acinar cell development, whereas it is only required for the development of a subset of endocrine cells. In zebrafish *Ptf1a* morphants no exocrine pancreatic tissue develops, while early endocrine cell development is normal. The development of a late-appearing population of endocrine cells however does require *Ptf1a* (Lin et al., 2004). Similarly, in *Ptf1a* mutant mice, there is a complete loss of acinar cells, with a dramatic reduction in all endocrine cell types (Kawaguchi et al., 2002; Krapp et al., 1998). Here, we demonstrate that in *Xenopus* the initial specification of both exocrine and endocrine

cells is inhibited in *Ptf1a* morphants, whereas a late-appearing population of insulin-positive cells is unaffected. Our results are however in conflict with the recent study examining *Xenopus Ptf1a* function during normal development (Afelik et al., 2006). In their study, they come to the opposite conclusion that *Ptf1a* function is not necessary for early insulin-expressing cells, but rather is essential for late-appearing endocrine cells. In agreement with their data, we also found normal development of early insulin-expressing cells in single *Ptf1a* morpholino injections. In contrast however, we found that early-appearing insulin cells did not develop in our double *Ptf1a* morphants. Furthermore, in both our single and double *Ptf1a* morphants the late-appearing insulin population always develops. The main difference between our studies and theirs is that we have used a combination of two morpholinos to knock-down *Ptf1a* function. In agreement with this our results demonstrating that the second splice morpholino effectively inhibits splicing of the Ptf1a transcript also revealed that splicing was not completely inhibited (see Fig. 3.7). This suggests that either single morpholino, whether inhibiting translation or splicing, is not completely effective in blocking *Ptf1a* and that a combination of both is more effective. This explains why we found effects on the early insulin-expressing population, whereas the previous study did not. In combination, the results from all three species would therefore suggest that there are two pathways to generate endocrine cells: a *Ptf1a*-dependent and a *Ptf1a* independent pathway. This is entirely consistent with the existence of a lower number of endocrine cells in the pancreatic rudiment formed in Ptf1a-null mouse embryos.

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4. Discussion

In this thesis I report the cloning, expression pattern and functional role of *Xenopus Ptf1a* during embryonic development of the pancreas. Like *Pdx1* and *Sox9*, *Ptf1a* is one of the earliest transcription factors expressed in dorsal and ventral pancreatic endoderm prior to over morphogenesis and differentiation. We used *Xenopus* transgenic and mRNA overexpression to show that amongst several transcription factors, *Ptf1a-VP16* is the only one sufficient to convert liver to pancreas, while the unmodified *Ptf1a* is sufficient to reprogram stomach/duodenum to pancreas. On the other hand, we performed loss of function experiments using morpholino antisense oligonucleotides to show that *Ptf1a* is essential for proper development of both endocrine and exocrine pancreatic lineages. These results establish *Ptf1a* as a central player in directing pancreatic cell fate determination in *Xenopus*.

4.1. Ptf1a is a master regulatory gene of pancreatic cell fate

The term "Master Gene" or "Master Switch" was first attributed to *Pdx1* because it is one of the earliest genes to be expressed during pancreas development. In addition *Pdx-VP16* has been shown to be sufficient to initiate a full pancreatic program (Horb et al. 2003). Previous loss and gain of function experiments showed that *Ptf1a* plays a fundamental role in pancreas development

and placed *Ptf1a* at the same level with *Pdx1* in the hierarchy of pancreatic transcription factors. These results along with our current analysis establish *Ptf1a* as a master regulatory gene that participates in the specification of all pancreatic lineages (Li et al. 2005). During *Xenopus* embryogenesis, *Ptf1a* is expressed very early in the endodermal pancreatic progenitor cells. Our knock down analysis show that loss of *Ptf1a* activity leads to pancreas agenesis. This contrasts with results from other transcription factors that affect only certain endocrine lineages. In addition, our overexpression analysis show that amongst several transcription factors *Ptf1a* is the only one that is sufficient to convert other endodermal cells into pancreas. These results support the idea that the notion of master regulatory gene is limited to those transcription factors that act in the initial stages of embryogenesis prior to overt morphogenesis and differentiation. However, we have not done an exhaustive analysis of all pancreatic transcription factors and it will be interesting to determine if this concept is applicable for early acting lineage specific transcription factors such as *Ngn3*.

4.2. Different protein partners promote different activities for Ptf1a

Like *Pdx1*, only a superactive form of *Ptf1a* fused to the activation domain *VP16* is able to convert liver to pancreas. Unlike *Pdx1-VP16* that converts liver to both endocrine and exocrine pancreatic cells (Horb et al. 2003), *Ptf1a-VP16* is only able to promote acinar cell fate in the liver. Even when expressed at earlier stages

prior to differentiation and organogenesis *Ptf1a-VP16* promotes only acinar cell fate. This result suggests that the activity of *Ptf1a* is restricted to the exocrine cell population as has been described in previous studies (Krapp et al. 1996; Krapp et al. 1998; Lin et al. 2004; Zecchin et al. 2004). However, we have also shown that the unmodified *Ptf1a* specifies both exocrine and endocrine pancreatic fates in the region of the stomach and duodenum. The fact that the unmodified *Ptf1a* by itself has no effect on the liver and that addition of the activation domain *VP16* promotes transdifferentiation of liver to pancreas suggests that other protein partners are needed with *Ptf1a* to activate pancreatic genes in the liver. Moreover, the fusion of *Ptf1a* to *VP16* seems to restrict the activity of *Ptf1a* in the liver and to limit its interaction to exocrine specific activators promoting acinar but not endocrine differentiation. Therefore, the ability of *Ptf1a* to promote either endocrine or acinar cell fate seems to be determined by its partner protein interactions at different times during embryogenesis.

The presence of *Pdx1* in the duodenum and the pancreas but not in the liver suggests that *Pdx1* and *Ptf1a* may function together to specify the pancreas. Evidence from our work and from previous studies showed that *Ptf1a* and *Pdx1* are both essential for the initiation of the pancreatic program within the endodermal cells. The robust linkage between pancreatic development and the expression of the two transcription factors implies the presence of a cross talk between the two molecules. The nature of this interaction is still unknown; it could be that the two transcription factors activate different pathways which converge to initiate the pancreatic program. Another possibility is that *Ptf1a* and *Pdx1* interact physically as

co-activator to induce the same molecules. However we could not pull down *Pdx1* and *Ptf1a* together by co-immunoprecipitation experiments (Horb, personal communication).

4.3. Two pathways to generate β-cells: Ptf1a dependent and independent

Previous loss of function experiments targeting *Ptf1a* in mice, zebrafish and *Xenopus* have established *Ptf1a* as one of the key transcriptional regulators of exocrine pancreas development. Mice lacking *Ptf1a* do not develop any exocrine tissue, and endocrine cells are reduced and misslocated to the spleen (Krapp et al. 1998). In zebrafish, inhibition of *Ptf1a* also leads to loss of acinar cells and to disrupted development of late-appearing endocrine cells (Lin et al. 2004). A recent study showed a conserved role of *Ptf1a* between *Xenopus* and zebrafish, implicating *Ptf1a* in the specification of the late endocrine population (Afelik et al. 2006). Our results conflict with their study, as we have demonstrated that besides exocrine pancreas agenesis, knock down of *Ptf1a* also inhibits initial specification of endocrine cells, with no effect on the late appearing population of insulin producing cells. The difference between our study and theirs is that we did our knockdown analysis using a combination of two morpholinos targeting both translation initiation and splicing of Ptf1a transcripts. When a single morpholino was used we found normal development of early insulin expressing cells; in contrast double *Ptf1a* morphants do not develop any early insulin cells. Therefore, a single morpholino is

not completely sufficient to block all *Ptf1a* transcripts. This idea is supported by the presence of spliced RNA in MO2 (splicing morpholino) injected embryos (Fig. 3.7). Taken together, these results suggest that the early and late endocrine cell populations are generated through two different pathways: *Ptf1a* dependent and *Ptf1a* independent.

Thus, our model is that the pancreatic nub that persists in *Ptf1a* morpholino injected embryos is due to the fact that this pancreatic population does not require *Ptf1a* and develops normally in its absence. It is also possible that some *Ptf1a* transcripts are still present and promote development of the nub. Two lines of evidence support this possibility: first *Xenopus laevis* is an allotetraploid frog which means that some copies of *Ptf1a* may persist even after morpholino injection (as shown in figure 7R) and second as the embryo develops the morpholino gets diluted in the cells which might reduce the effect of the morpholino and free some *Ptf1a* transcripts form blockage. To eliminate the first possibility and target all copies of *Ptf1a* we used in our experiment two different morpholinos that target both the translation initiation site and the splicing site of *Ptf1a* mRNA and indeed we got a greater insulin reduction in early stage embryos after injection of the double morpholinos. Yet, we can not conclude that later persistent of pancreatic tissue is only due to morpholino dilution because our model supports the presence of a cell population independent of *Ptf1a*.

4.4. *Xenopus* transgenic and mRNA overexpression produce the same phenotype

In our transgenic embryos, overexpression of *Ptf1a* is specifically targeted to the liver, the transgene being driven by the TTR promoter. Therefore, appearance of an ectopic pancreas will result from a conversion of already differentiated liver cells. Injection of *Ptf1a* mRNA into early stage *Xenopus* embryos leads to overexpression earlier in development prior to organogenesis. Therefore, the development of an ectopic pancreatic tissue will result from a change in cell fate of progenitor cells rather than transdifferentiation of more mature cells. We showed in our study that in both transgenic and mRNA injection, overexpression of *Ptf1a* transforms the stomach/duodenum region into pancreas. Our transgenic and mRNA results were also concordant for *Ptf1a-VP16* and ectopic exocrine tissue was expanded to the liver. These results point to the reliability of our techniques and to the specificity of the phenotype. It will be interesting to test these finding *in vitro*, and to study the ability of *Ptf1a* and *Ptf1a-VP16* to transform different liver, stomach and duodenal cell lines to pancreas. On the other hand our overexpression analysis were performed using *Ptf1a* which normally works as a heterodimer with p75 and p64, thus it will be of great interest to check the activities of the whole Ptf1 complex on endodermal organs.

4.5. Different transcription factors specify pancreas, stomach and duodenum

Different signaling molecules act on the gut to prepattern the different organs of the digestive system along the antero-posterior and dorsal-ventral axis. Each endoderm derived organ is specified via the activation of key transcriptional factors. Ectopic activation of *Ptf1a* in all endodermal progenitor cells induces a pancreatic program only in a subset of endodermal cells that will give rise to the stomach and duodenum. *Pdx1* is normally expressed in the pancreas, stomach and duodenum, while *Ptf1a* expression is restricted to the pancreas. We showed by both transgenesis and mRNA overexpression that activation of *Ptf1a* in the endoderm converts stomach and duodenum which normally expresses *Pdx1* to pancreatic tissue. This finding suggests that the pancreas is defined by the presence of both *Pdx1* and *Ptf1a* while the stomach and duodenum are defined by the expression of *Pdx1* with possibly other transcription factors and the absence of *Ptf1a*. Indeed recent results showed that overexpression of *Ptf1a* in *Xenopus* endoderm induces ectopic pancreatic differentiation, but only within the *Xlhbox8* positive region of stomach and duodenum (Afelik et al. 2006). In that study, the ectopic pancreas expresses only exocrine markers while insulin expression is not affected at early and late developmental stages. Our analysis disagrees with this result and both exocrine and endocrine markers are detected in the ectopic pancreas. Other transcription factors such as *Barx1* differentiate between stomach and other part of the intestine (Kim et al. 2005). However, the posterior gut seems to be specified by the absence

of both *Pdx1* and *Ptf1a*. We have not detected any ectopic pancreatic tissue in the posterior endoderm after injection of *Ptf1a* in the endodermal progenitors. This result was not surprising since we know that *Pdx1* is not expressed in that region of the endoderm. Combined expression of *Pdx1* and *Ptf1a* in the endoderm of *Xenopus* induces exocrine pancreatic differentiation in the posterior gut, insulin remains unaffected again (Afelik et al. 2006). Thus, in concert with other transcription factors *Pdx1* specifies the anterior gut: stomach, duodenum and liver, while *Pdx1* and *Ptf1a* together specify the pancreas, and the posterior region of the gut does not require either factor.

4.6. The use of *Xenopus* transgenic for transdifferentiation studies

We have used in this work *Xenopus* transgenesis to study the ability of pancreatic transcription factors to convert endoderm derived organs to pancreas. Only *Ptf1a-VP16* is able to transdifferentiate liver to pancreas, while the unmodified *Ptf1a* converts stomach and duodenum to pancreas. These results and the previous work with *XPdx1*(Horb et al. 2003) illustrate the power of using *Xenopus* transgenic to study pancreatic specification. *Xenopus* transgenics are relatively easy and rapid to produce compared with mice transgenics. One researcher can produce in a single day around 30 transgenics to study organogenesis; in addition *Xenopus* transgenes can be made using tissue specific promoters from other organisms and, with the emergence of *Xenopus* tropicalis genome, it is now easy to clone tropicalis

promoters. In the new era of cell-based therapy to treat diabetes, studying transdifferentiation will help identifying transcription factors to reprogram stem cells and differentiated cells for therapeutic transplantation. Our transgenic transdifferentiation essay seems to be a useful technique to reach this goal. It will be also interesting to complement the transdifferentiation analysis by an mRNA overexpression to see the effect of the same transcription factors on undifferentiated cells. In addition *in vitro* tissue culture permits to check the activity of the identified transcription on mammalian cells.

4.7. The use of Ptf1a and Pdx1 for generating endocrine β-cells *in vitro*

Previous work with *Pdx1* and our current study on *Ptf1a* show that both transcription factors are sufficient to generate functional β-cells in *Xenopus* embryos. *Pdx1* has been also tested in embryonic stem cells and shown to promote high insulin production in the differentiated cells (Miyazaki et al. 2004). It will be interesting to determine if *Ptf1a* alone or in concert with *Pdx1* has similar activity when expressed in embryonic stem cells. Thus combined expression of *Pdx1* and *Ptf1a* might be the perfect tool to generate insulin producing cells *in vitro* for therapeutic use in diabetic patients.

5. Conclusion

In an effort to elucidate the transcriptional regulatory network underlying the molecular mechanisms in pancreas development, we have studied the ability of *Ptf1a* and *Ptf1a-VP16* to convert endoderm derived organ to pancreas. Using *Xenopus* transgenic, we showed that *Ptf1a-VP16* converts liver to pancreas expressing only exocrine markers, while the unmodified *Ptf1a* transforms the stomach/duodenal region to both endocrine and exocrine pancreatic cells. When injected at earlier stages, *Ptf1a* mRNA showed the same phenotype while *Ptf1a-VP16* had a more severe phenotype showing respecification of liver, stomach and duodenum into acinar pancreas. We conclude that *Ptf1a*, in concert with other transcription factors such as *Pdx1*, can respecify anterior endodermal cells into pancreas and that the ability of *Ptf1a* to promote either endocrine or acinar cell fate is determined by its protein partner. Moreover our loss of function experiments using morpholino antisense oligonucleotides show that knocking down *Ptf1a* leads to exocrine pancreas agenesis and loss of early insulin expression while the late insulin expression is not affected. Our results demonstrate that *Ptf1a* is required for the development of both endocrine and exocrine cells and suggest the presence of two different pathways to form β-cells, *Ptf1a* dependent and *Ptf1a* independent. In conclusion, we can say that our work establishes *Ptf1a* as a master switch regulator in *Xenopus* indispensable for the initial specification of all pancreatic lineages.

6. Future directions

6.1. Downstream targets of *Ptf1a*

Our results demonstrate that *Ptf1a* controls initial specification of endocrine and exocrine pancreatic lineages; however it is still unclear which genes *Ptf1a* regulates to establish a particular cell fate. To begin to elucidate the transcriptional regulatory network mediated by *Ptf1a,* we decided to study gene expression changes in both knock down and overexpression of *Ptf1a*. One method to identify these different targets is to compare by microarray analysis tissue lacking or overexpressing *Ptf1a* to wild type. We have already started this project and have the microarray done for pancreatic tissue injected with MO1, 2MO and wild type control pancreas. To perform this experiment we injected the four vegetal blastomeres of eight cell stage embryos with either 40 ng of MO1 or 40 ng of 2MO (20 ng of MO1+20 ng of MO2). Then, we dissected out early pancreatic tissue at stage 34-35, excluding the ectoderm and avoiding neural and heart tissue (Figure 6.1A). At this stage *Ptf1a* expression is already turned on 8 to 10 hours ago and differentiation has just started, so this will be the perfect timing to isolate the greatest number of *Ptf1a* targets that function at early stages of pancreatic cell fate specification. Because all our dissections were not exactly the same, we decided to run each sample in triplicate, so that we can minimize the impact of differential dissections. An average of 20 explants was collected together, and stored for RNA extraction. As result we

have nine different pools of RNA: three controls, three MO1 and three 2 MOs. Before proceeding to the microarray, we have shown by real time PCR a decrease in the expression of *Ptf1a*, *Pdx1* and NeuroD in cDNA prepared from the pools listed above (Figure 6.1B). These results confirm that our dissections are accurate and we thus sent them for cDNA synthesis and hybridization to the Xenopus Affymetrix Genechip.

After hybridization, the microarray data for the nine chips were analyzed using the Robust Multichip Average (RMA) method. RMA results for morpholino injected explants were then compared to the wild type control (Figure 6.2A-B). All probesets with a fold change of 2 and greater are then identified to provide a list of differentially expressed genes. Within this range, 183 genes show decreased expression and 185 show increased expression in the MO1 sample; in the 2MOs sample there are 139 genes with decreased expression and 117 with increased expression.

We went then to validate the microarray results and to confirm the change in expression of different genes identified in the microarray. Quantitative real time PCR is the fastest and most reliable method to examine and compare the expression of the microarray data in wild type and morpholinos injected embryos. We designed primers for 23 genes identified from the microarray results, and confirmed their expression changes in MO1 and 2MO versus the control. For example *Staufen2* shows almost no decrease in the MO1 sample (RMA value 0.27), but a much greater decrease in the 2MOs sample (RMA value 1.27). Similarly, real time PCR analysis shows no change in MO1 and a large decrease in the 2MOs sample (Figure 6.2G).

It is now important to determine if the identified and validated genes are indeed expressed in the pancreas. Whole mount in situ hybridization permits to visualize the expression pattern of these particular genes. We selected six genes for in situ hybridization analysis, only three of them are expressed in the pancreas (Figure 6.2O-Q). Interestingly, *Staufen2* shows expression only in the dorsal pancreas (Figure 6.2O) suggesting that it might be involved in development of endocrine pancreatic lineage. In conclusion, this microarray analysis of tissue lacking *Ptf1a* provides us with a list of interesting genes as candidates for downstream targets of *Ptf1a*. Another useful analysis to do is to compare by microarray tissue overexpressing *Ptf1a* or *Ptf1a-VP16* to wild type pancreatic tissue. The steps described above will be followed to perform the second microarray analysis. However, *Xenopus* embryos will be injected with either *Ptf1a* or *Ptf1a-VP16* mRNA. This analysis will provide information about the differential activity of *Ptf1a* and *Ptf1a-VP16* during pancreas development.

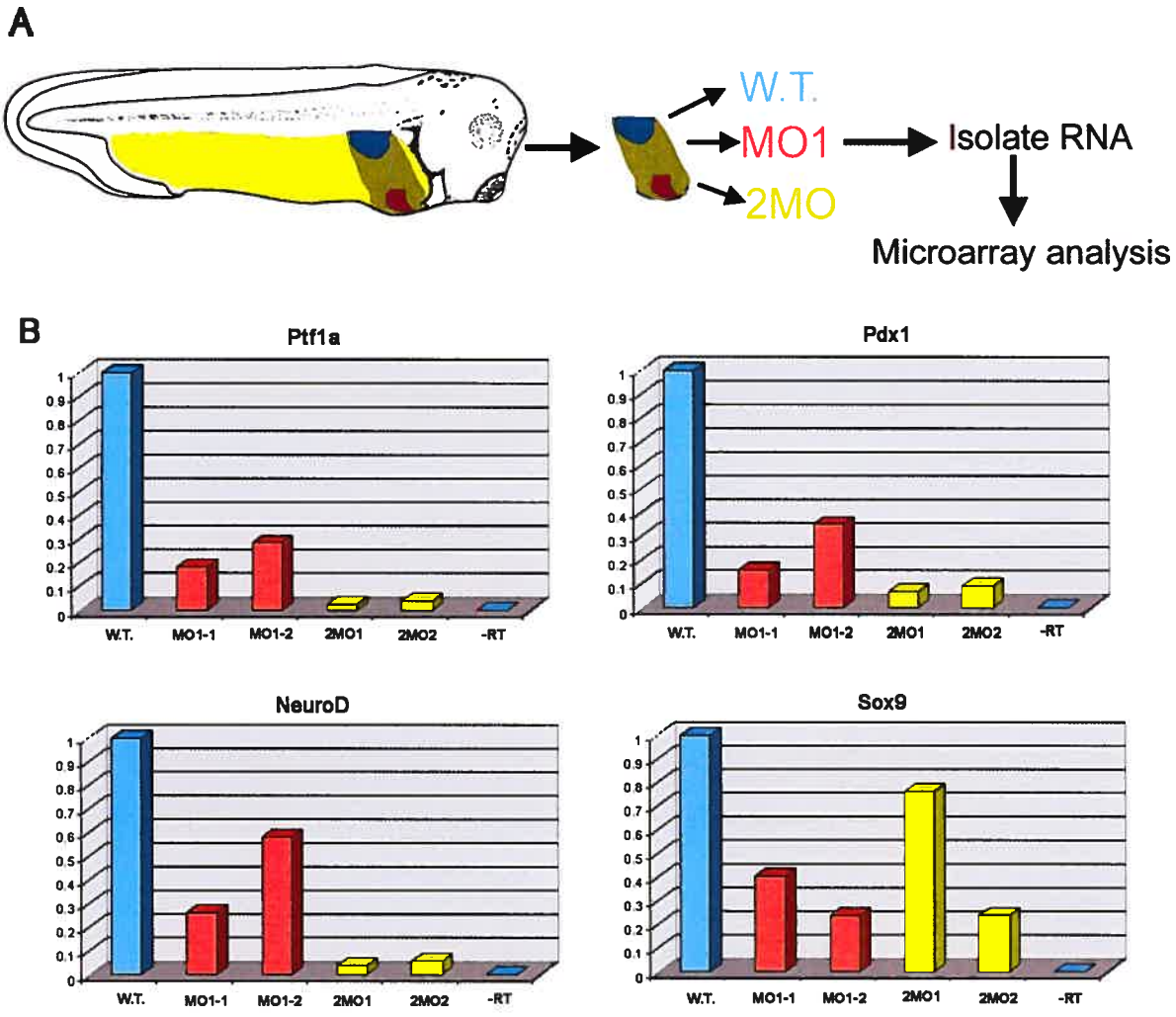

Figure 6.1 Outline of the microarray experiment. (**A**) Diagram illustrating the outline of our experimental plan. Pancreatic tissue was dissected from stage 34-35 embryos avoiding the neural tissue and heart. Control, single morpholino and double morpholino samples were collected from five separate fertilizations. (**B**) Real time PCR analysis of the three sample sets for *Ptf1a*, *Pdx1*, *NeuroD* and *Sox9* showing substantial reduction in *Ptf1a*-MO injected embryos.

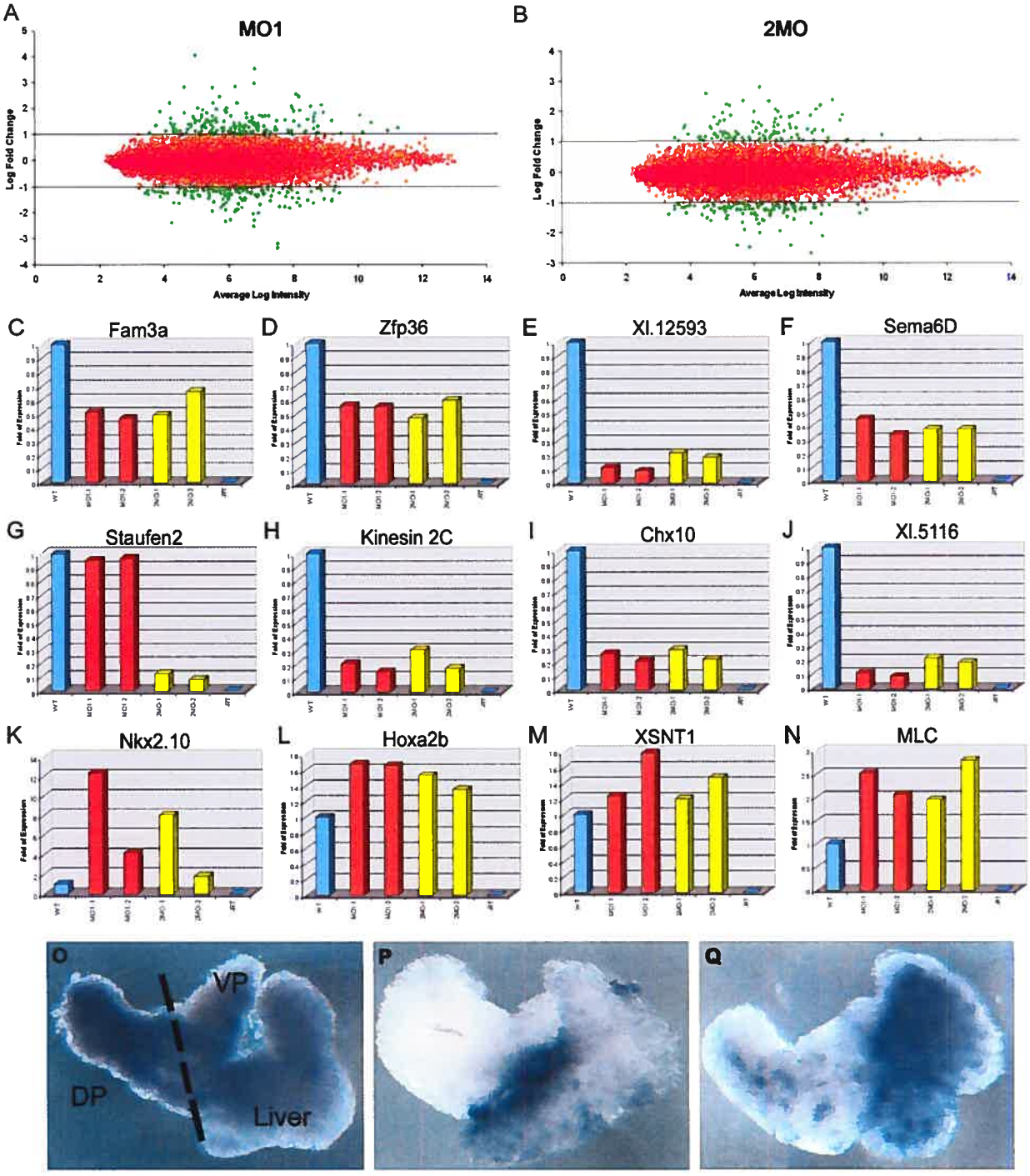
A
MO1
Log Fold Change
Average Log Intensity
B
2MO
Log Fold Change
Average Log Intensity
C
Fam3a
D
Zfp36
E
Xl.12593
F
Sema6D
G
Staufen2
H
Kinesin 2C
I
Chx10
J
Xl.5116
K
Nkx2.10
L
Hoxa2b
M
XSNT1
N
MLC
O
VP
DP
Liver
P
Q

Figure 6.2 Microarray results and validation. (A,B) Scatterplot data for MO1 and 2MO samples against wild type control showing overall gene expression changes obtained by microarray. Horizontal lines are drawn at an RMA value of 1.0. (C-N) Real time PCR confirmation of selected genes identified in microarray screen. (C-G) genes showing decreased expression in the microarray and confirmed by real time PCR. (K-N) Genes showing increased expression in the microarray and confirmed by real time PCR. Blue- control, orange-MO1, yellow-2MO. (O-Q) Whole mount in situ hybridization for three of the genes identified in the microarray in isolated pancreas/liver tissue from stage 42. (O) Staufen2 RNA expression. Notice expression only in the dorsal pancreas (DP). (P) Frz7 RNA expression. Expression is only seen in the ventral pancreas adjacent to the liver. (Q) CD81 RNA expression. Notice punctate expression in both the pancreas and the liver.

6.2. Functional role of *Staufen2* during pancreas development

Staufen2 is a double stranded RNA binding protein that has been shown to be required for localization of different mRNA during development. In Drosophila *Staufen* is required for localization of bicoid and oskar mRNA in the oocyte and for prospero localization in neuroblasts (Ferrandon et al. 1994; StJohnston et al. 1991; Li et al. 1997). In *Xenopus* oocytes, *Staufen1* and 2 are responsible for the localization of *VegT* and *Vg1* to the vegetal cytoplasm (ALLISON et al. 2004). In mammals, *Staufen1* and *2* are implicated in dentritic RNA transport of during neuronal maturation (Roegiers & Jan 2000). Based on the important role of *Staufen* in regulating RNA localization and translation, our preliminary results showing dorsal pancreatic localization of *Staufen2* RNA and substantial decrease of *Staufen2* expression in 2MOs *Ptf1a* morphants suggest that *Staufen2* might be involved in the development of the endocrine pancreatic lineage. To determine if *Staufen2* exhibits any functional role during pancreas development, we will perform loss and gain of

function experiments as we did previously for *Ptf1a*. To knock down *Staufen2* in the developing pancreas, we will inject *Staufen2* morpholino into the dorsal vegetal blastomeres of eight cell stage embryos. Morphants will be dissected at stage 42 and checked for abnormal phenotype. To overexpress *Staufen2* in the prepancreatic endoderm we will inject *Staufen2* mRNA in the dorsal vegetal blastomeres of eight cell stage *Xenopus* embryos. Tadpoles will be dissected and the guts checked for abnormal phenotype. This functional analysis done for *Staufen2* will actually be repeated for other genes selected from the microarray.

The microarray analysis of tissue lacking and overexpressing *Ptf1a* will help us identify the downstream targets of *Ptf1a* and elucidate the transcriptional hierarchy leading to the generation of all pancreatic lineages. Moreover, combined analysis of other pancreatic transcription loss and gain of functions to the microarray results will lead to lead to a map of pancreas development.

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