Regulation of Pancreas Development in *Xenopus laevis*

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submitted by

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born in

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Göttingen, 2006

Affidavit

Herewith I declare, that I prepared the PhD thesis

“Regulation of Pancreas Development in *Xenopus laevis*”

on my own and with no other sources and aids than quoted

Fong Cheng Pan .....

Date of submission .....

List of Publications

1. Chen, Y., Pan, F.C., Brandes, N., Afelik, S., Sölter, M., and Pieler, T. 2004. Retinoic acid signalling is essential for pancreas development and promotes endocrine at the expense of exocrine cell differentiation in *Xenopus*. *Dev. Biol*. **271**: 144-160

2. Pan, F.C., Chen, Y., Löber, J., Henningfeld, K., and Pieler, T. 2006. *I-SceI* meganuclease-mediated transgenesis in *Xenopus. Dev. Dyn.* **235**, 247-252

3. Pieler, T., Pan, F.C., Afelik, S. and Chen, Y. 2006. Molecular Genetics of Liver and Pancreas Development. *In Cell Signaling and Growth Factors in Development: From Molecules to Organogenesis (ed. K. Unsicker and K. Krieglstein),* **Vol. 2**. pp. 823-840. Wiley-vch, Weinheim.

4. Pan, F.C., Chen, Y., Bayha, E., Grapin-Botton, A., and Pieler, T. Retinoic acid patterning of dorsal pre-pancreatic endoderm during gastrulation involves interaction with BMP signalling. (to be submitted)

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Abstract

How and when the vertebrate endoderm is first subdivided into discrete progenitor cell populations that will give rise to the different major organs, including pancreas and liver, is only poorly understood. We have used *Xenopus laevis* as a model system to characterize these early endodermal patterning events as mediated by RA. Our findings show that RA signalling is required for pancreas but not liver specification as early as during gastrulation. Using embryonic explant experiments, our results support the notion that RA acts both directly on the endoderm and indirectly via the mesoderm to specify the dorsal endoderm towards a pancreatic fate. Inhibition of BMP signalling, by noggin, and additional mesodermal factor(s) is also critically required for dorsal endoderm patterning in conjunction with RA during gastrulation. We also demonstrate that *Sonic Hedgehog*, a known inhibitor of pancreas development in the mouse and chick, is negatively regulated by RA in the dorsal prepancreatic endoderm. Furthermore, RA is found to promote endocrine at the expense of exocrine differentiation in the dorsal pancreas via inhibition of Notch signalling. Conversely, RA enhances exocrine gene expression in the ventral pancreas while inhibiting liver development in a concentration dependent manner.

We also established a new method to generate transgenic frog using *I-SceI* meganuclease. We obtained transgenesis efficiency of 10% or more under optimal conditions and a high rate of germ-line transmission rate. *I-SceI* meganuclease approach thus provides a simple and highly efficient tool for generating transgenic frogs also for the study of later phases of pancreas development.

1. Introduction

1.1 Pancreas morphology and its morphogenesis

The name pancreas derives from the Greek roots 'pan' meaning 'all' and 'creas' meaning 'flesh' (Slack, 1995). It is a vital organ controlling glucose homeostasis and food digestion in vertebrate. The pancreas is a compound gland, which can be functionally and morphologically divided into two parts, the exocrine compartment and the endocrine compartment (Figure 1.1A). The exocrine compartment, including acinar and duct cells, comprises 95% - 99% of the pancreas. The acinar cells secrete digestives enzyme that promote food digestion and absorption in the gut (Slack, 1995). The ductal cells form the epithelial lining of the branched tubes that flush the enzymes made by the acini into the duodenum. The ductal cells also secrete bicarbonate to neutralise stomach acidity (Grapin-Botton, 2005) (Figure 1.1B).

The endocrine compartment, which plays a role in monitoring blood glucose levels and releases hormones into the bloodstream to maintain proper glucose homeostasis, makes up 1% - 2% of the pancreas. It contains five hormone-producing cell-types: glucagon producing alpha(α)-cells, insulin-producing beta(β)-cells, somatostatin-producing delta(δ)-cells, pancreatic polypeptide-producing PP-cells, and the more recently identified ghrelin producing epsilon(ε)-cells. These cells are grouped into clusters, defined as the islets of Langerhans in mammals, birds, reptiles and amphibians including *Xenopus*, or as Brockmann bodies in fish. In rodents, the β-cells form the core of the islet and they are surrounded by the other cell types (Slack, 1995; Prado et al., 2004) (Figure 1.1 C).

Fate mapping analysis and studies using vegetal explants in *Xenopus* suggest that the pancreas originates from the dorsal endoderm of gastrula (st10) embryo, and that the suprablastoporal cells (dorsal to the lip) give rise to the dorsal pancreas and the ventral pancreas derives from the subblastoporal cells (Gamer & Wright, 1995; Henry et al., 1996; Zorn et al., 1999; Chalmers & Slack, 2000; Kelly & Melton, 2000).

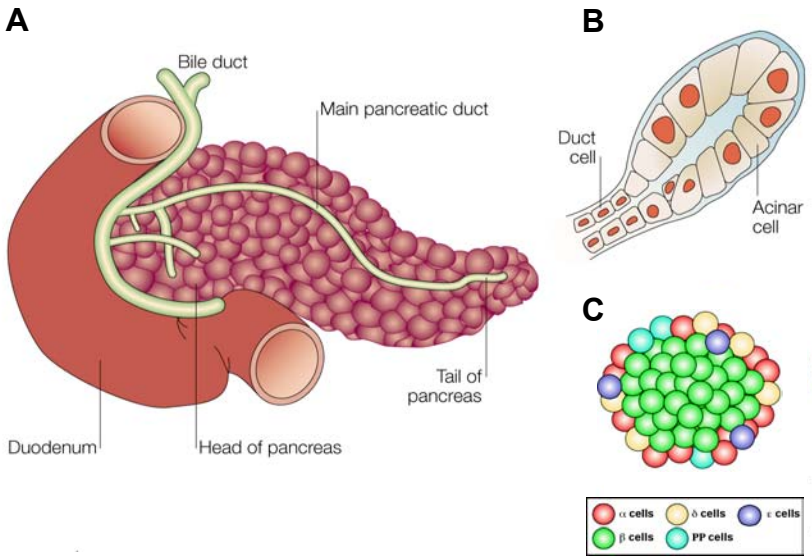

Figure 1.1 Morphology of the pancreas (A) The adult pancreas is located adjacent to the duodenum. (B) The exocrine compartment contains the acinar cells and duct cells. (C) The endocrine portion of the pancreas consisting five hormone-producing cell-types organised into the islets of Langerhans (adapted from Edlund, 2002 with modifications).

During embryogenesis, the embryonic pancreas evaginates from the primitive gut epithelium of the foregut endoderm as one dorsal and two ventral buds. In *Xenopus*, this budding process becomes evident at stage 35/36 for the dorsal anlage in the dorsal gut wall and at stage 37/38, the two ventral anlagen appear more anteriorly and adjacent to the liver diverticulum. At stage 40, gut rotation leads to the juxtaposition and subsequent fusion of the three primordia, which are located predominantly in the left half of the tadpole and which are embedded between the stomach and the duodenum (Kelly & Melton, 2000) (Figure 1.2A).

Pancreas morphogenesis in *Xenopus*, as in the mouse, follows two phases of development. The first phase is known as the “primary transition” or “precursor phase” and it is marked by the change in shape of the pancreatic domain of the gut as a consequence of the proliferation of the pancreatic precursor cells. This occurs between stage 28 and stage 38 and is marked by the expression of the precursor markers, *XlHbox8* and *Ptf1a/p48*, as well as by the first wave of insulin expression, which is only confined to the dorsal pancreas until stage 45

(Pieler & Chen 2005; Slack, 1995). The secondary transition initiates at stage 39, involves growth and differentiation of the pancreas precursor cells into functional acini as marked by the expression of exocrine genes, *XPDIp* and trypsinogen. In *Xenopus*, the other pancreatic hormone-expressing endocrine cells, and the second phase of insulin-expressing cells, appear relatively late at stage 46/47 (reviewed in Pieler and Chen, 2005) (Figure 1.2B).

In the mouse, the primary transition occurs between E9.5 and E13.5, in which glucagon and insulin-expressing cells that do not contribute to the mature islets can be detected. The differentiation of acinar cells and hormone positive cells that will form the mature islets marks the secondary transition and occurs between E14.5 and E16.5 (Wilding and Gannon, 2004).

Pancreatic fate commitment and specification occurs in a stepwise manner. Initially, early endoderm is patterned to generate a domain that is competent to form pancreas. Subsequent development of this prepancreatic endodermal domain requires interaction with the neighbouring tissues to induce pancreas-specific differentiation. These processes will be discussed in detail in the following sections.

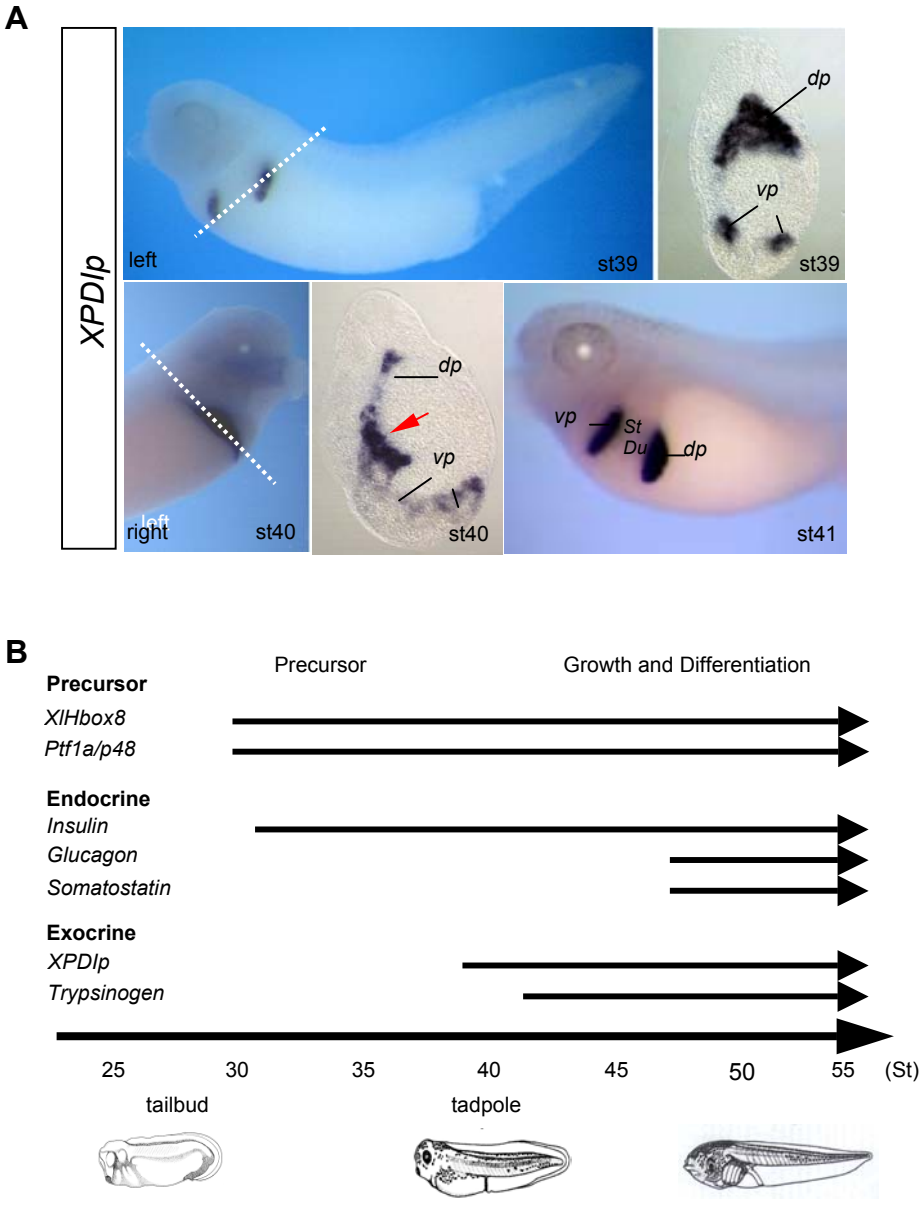

Figure 1.2 *Xenopus* pancreas development and temporal gene expression profiles. (A) The fusion of the dorsal and ventral pancreas anlagen is demarcated using the exocrine marker, *XPDIp*, which is expressed both in the dorsal and ventral pancreatic buds starting at stage 39. dp, dorsal pancreatic bud; vp, ventral pancreatic bud; St, stomach; Du, duodenum. (adapted from Afelik et al., 2004, with modification). (B) Temporal expression profiles of pancreatic genes in *Xenopus* embryos during two distinct phases of development (adapted from Pieler & Chen, 2005 with modifications).

1.2 Endoderm formation and fate determination

Endoderm, one of the three primary germ layers formed during early embryogenesis, gives rise to the epithelial lining of the respiratory tract and the gastrointestinal tract as well as to it associated organs, including liver and pancreas. Most of the studies on endoderm formation and patterning are largely carried out in *Xenopus* and zebrafish. In *Xenopus*, the endoderm derives from the cells located at the vegetal pole of the embryo (Dale & Slack, 1987). Single-cell transplantation experiments have shown that the fate of vegetal pole blastomeres becomes restricted to endoderm around the mid-blastula stage. Heasman and colleagues (1984) have shown that a single vegetal blastomere from morula (stage 6) or mid-blastula stage embryos transplanted to an ectopic environment will adopt the fate of their new neighbours, indicating that they are not yet determined to an endodermal fate. However, vegetal blastomeres from late blastula or early gastrula stage embryos will differentiate into endoderm derivatives even when they are transplanted into an ectopic environment, suggesting that the fate of vegetal pole blastomeres becomes progressively determined to endoderm (Wylie et al., 1987). Endoderm determination also requires cell-cell communication, since vegetal blastomeres isolated and cultured *in vitro* will adopt an endodermal fate only when an appropriate cell mass is present (Wylie et al., 1987).

VegT, a maternally derived T-box transcription factor localised to the vegetal cortex of the *Xenopus* egg, is a crucial regulator of endoderm development. Embryos develop from maternal VegT-depleted eggs lack endoderm and the mesoderm extends into the vegetal region (Zhang et al., 1998). VegT has been shown to promote the expression of nodal-related TGF-β signalling molecules (Xnrs, derriere) and several endoderm-specific transcription factors (Mix-type homeobox transcription factors, GATA 5 and XSox17α) in a cell-autonomous manner (reviewed in Fukuda & Kikuchi, 2005). Sox7, another maternal factor recently reported, is also required for endoderm development. Zhang and colleagues (2005) has shown that the ability of VegT to induce endodermal genes appears to depend on Sox7 activity.

On the basis of the sequential activation of these endodermal factors, Yasuo and Lemaire (1999) proposed a two-step model for endoderm fate determination. The initial phase involves cell autonomously activation of early endodermal genes (XSox17α and Mix.1) by maternal determinants, including VegT, during mid-blastula transition. The second step takes place during late blastula stages and leads to the activation of genes such as Mixer and GATA4 and to the reinforcement of the expression of early endodermal genes (XSox17α, Mix.1, Xnrs), and this second phase requires cell-cell interactions.

1.3 Regional specification of the endoderm

Regional specification within the endoderm provides positional information along the anterior-posterior (A-P), dorsal-ventral (D-V) and left-right (L-R) axes and is marked by expression of region-specific markers (Horb & Slack, 2001). Using the amphibian *Cynops pyrrhogaster*, Okada had demonstrated that the endoderm would differentiate only in the presence of mesoderm and that also the regional character of the differentiated endoderm is determined by the mesoderm (reviewed in Okada, 1960).

However, early studies in *Xenopus* using vegetal explants isolated from early blastula stage embryos have shown that these vegetal explants express regional markers cell-autonomously, also in the absence of mesoderm. Dorsal vegetal explants express the anterior endodermal marker, *XlHbox8*, whereas ventral vegetal explants express posterior endodermal markers, such as IFABP. These studies also pointed to an important role for TGF-β (activin and Vg1) and FGF signalling in the patterning of the A-P axis of the endoderm. Blocking TGF-β and FGF signalling using appropriate dominant negative receptors abolished expression of *XlHbox8*, whereas IFABP was not affected; the activities of these signalling molecules was also demonstrated to depends on cortical rotation (Gamer & Wright, 1995; Henry et al., 1996). Zorn and colleagues (1999) have shown that the anterior endomesoderm, as marked by the expression of Xhex and cerberus, is specified by a combined action of maternal Wnt/β-catenin signals, resulting from cortical rotation, and TGF-β signalling. These early

studies had pointed out that, in the absence of mesoderm, endoderm is regionally specified in an autonomous fashion before gastrulation.

Whereas all the above studies in *Xenopus* are based on results obtained from vegetal explants isolated from late blastula stage embryos, a more recent analysis by Horb and Slack (2001) claims the presence of mesodermal precursor cells in those vegetal explants. The same study also shows that endoderm devoid of mesoderm, as isolated from neurula and tailbud stage embryos, does not express any regional marker and that this endoderm acquires stable regional specification only in the presence of adjacent mesodermal tissues until tailbud stage.

In the mouse, endoderm acquires an initial A-P patterning during gastrulation, when the endodermal cells migrate through the primitive streak. This patterning is revealed by the expression of region-specific endodermal markers long before gut tube formation. FGF4, a soluble factor from the mesoderm, can induce the differentiation of endoderm in a concentration-dependent manner and has recently been shown to be required for establishing gut domains along the A-P axis in a paracrine manner (Wells & Melton, 2000; Dessimoz et al., 2006).

In zebrafish, RA and BMP signalling are required for the regionalization of the endoderm along its A-P axis. Stafford and Prince (2002) showed that addition of RA to zebrafish embryos posteriorizes the gut and transforms the anterior endoderm into liver and pancreas fates. Upon reduction of RA signalling, either with RAR antagonists or using raldh2 mutant fish, the pharyngeal endoderm expands posteriorly, and liver-pancreas development is inhibited. The critical time period in which RA signalling can cause endodermal fate transformation is near the end of gastrulation. Using *swirl* (BMP2$^{-/-}$) and *chordino* (chordin$^{-/-}$) zebrafish mutants, Tiso and colleagues (2002) showed that BMP signalling is involved in regulating the A-P patterning of zebrafish endoderm by controlling her5 expression. They found that the anterior endoderm is expanded and the pancreatic domain is reduced in *swirl* mutants, and the phenotype is opposite to the one of *chordino* mutants.

1.4 Signals from the mesodermal derivatives are essential for pancreas development

The specification of pancreatic progenitors within the dorsal and ventral endodermal epithelium proceeds in two phases of mesodermal interactions and exposure to different stimuli from the mesodermal derivatives. Endoderm that will form the dorsal pancreatic bud lies at the midline of the embryo and has prolonged initial contact with the notochord and subsequently with the dorsal aorta, both of which have been shown to be essential sources of signals for pancreas specification and differentiation (Kim et al., 1997; Lammert et al., 2001). The endoderm that will give rise to the ventral pancreatic bud interacts with the lateral plate mesoderm and, later, the cardiac and septum transversum mesoderm (Rossi et al., 2001; Deutsch et al., 2001) (Figure 1.3). During the budding process, the pancreatic mesenchyme condenses around the pancreatic bud and is essential for growth, morphogenesis and differentiation of the pancreas (reviewed in Edlund, 2002).

1.4.1 Notochord signals are required for pancreas development

Embryonic manipulations in chick have revealed that premature separation of notochord from the dorsal prepancreatic endoderm abolishes dorsal pancreatic gene expression, while co-culture of endoderm with isolated notochord restores pancreatic gene expression (Kim et al., 1997). Interestingly, notochord is not able to induce pancreatic gene expression in gastrula stage endoderm or non-pancreatic somite-stage endoderm, suggesting that the notochord signals are permissive rather than instructive (Kim et al., 1997; Wells & Melton, 2000). In the midgestation mouse, Sonic Hedgehog (*Shh*) is expressed at high levels in stomach and duodenal endoderm, but is excluded from pancreatic endoderm. Signals from the notochord to the endoderm are required to suppress *Shh* expression in the pancreatic endoderm. Removal of notochord causes ectopic *Shh* in the dorsal pancreatic anlagen inhibiting dorsal pancreas development, while the ventral pancreas develops normally (Kim et al., 1997; Hebrok et al., 2000). Ventral pancreas endoderm, though, has no contact with the notochord but does not express Shh (Deutsch et al., 2001). Purified activin-βB and FGF2,

mimic the notochord signals and are able to induce pancreatic gene expression in isolated prepancreatic endoderm (Hebrok et al., 2000).

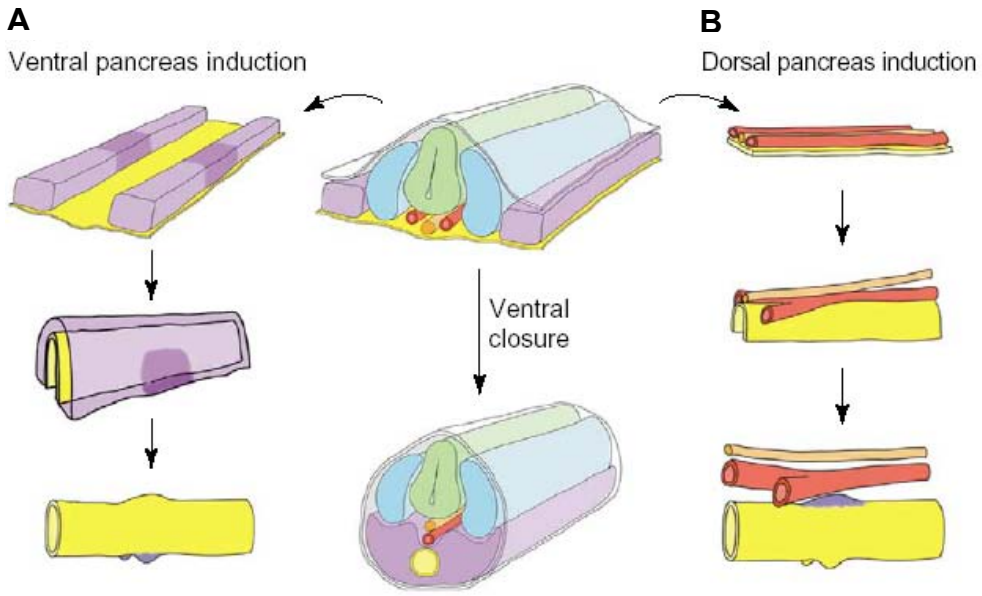

Figure 1.3 Schematic representation of mesenchyme-to-epithelial signalling in the context of dorsal and ventral pancreas induction in the mouse. (A) Signals from lateral plate mesoderm (purple) induce pancreatic differentiation in ventral endoderm. Signals from notochord (orange) at E8.5, and dorsal aorta (red) at E9.5 are required for dorsal pancreas induction. Endoderm, yellow; endothelium, red; notochord, orange; lateral plate mesoderm, purple; neural tube, green; somite, light blue (adapted from Kumar & Melton, 2003).

1.4.2 Vascular endothelial signalling and pancreas development

Lammert and colleagues (2001) have shown that the endothelial cells of the forming aorta and vitelline veins are required to promote pancreatic development. *Xenopus* embryos lacking an aorta failed to express pancreatic genes. Co-culture of mouse dorsal prepancreatic endoderm with aorta was sufficient to induce both *Pdx1* and insulin expression. Furthermore, it was also shown that driving VEGF-A expression under the *Pdx1* promoter leads to islet hyperplasia and ectopic insulin-expressing cells, as well as to the formation of bud-like structures in the stomach. Later, by deleting VEGF-A in mouse pancreas, Lammert and colleagues also showed that endocrine cells signal back to the adjacent endothelial cells to induce the formation of a dense network

of fenestrated capillaries in islet and that this factor is required for fine tuning of blood glucose regulation (Lammert et al., 2003).

A more recent study by the same group revealed that β-cells, which normally do not form a basement membrane, use VEGF-A to attract endothelial cells that will form capillaries with a basement membrane next to the β-cells. They found that laminin, a basement membrane protein, acts as the endothelial signal to promote insulin expression. They also show that β-cells require β1-integrin to respond to laminin (Nikolova et al., 2006).

Another group working on the same issue, using *Flk*$^{-/-}$ (VEGFR knockout) mice lacking endothelial cells, has shown that the emergence of the dorsal pancreatic bud and maintenance of *Pdx1* expression require interaction with aortic endothelial cells, but the initial induction of *Pdx1* expression does not. They also showed that aorta endothelial cells are required to induce Ptf1a expression in the dorsal pancreas (Yoshitomi & Zaret, 2004). Later, they revealed that the aortic endothelial signals are relayed via the dorsal mesenchyme. The aortic endothelial cells are crucial in promoting the survival of the dorsal mesenchyme, and FGF10 signalling from the dorsal mesenchyme is required to promote the initial differentiation of the dorsal pancreas, as well as the induction of Ptf1a expression (Jacquemin et al., 2006).

1.4.3 Signals from the pancreatic mesenchyme are required for growth, morphogenesis and differentiation of the pancreas

Early studies by Golosow and Grobstein (1962) and Wessel and Cohen (1967) have showed that the development of the pancreas is dependent on epithelio-mesenchymal interactions. In their studies, they showed that E11 pancreatic epithelium that was freed from the adjacent mesenchyme and cultured alone failed to grow in comparison with the epithelium cultured in the presence of mesenchyme, which grew and lobulated. Recombination of the naked pancreatic epithelium with the non-pancreatic mesenchyme also promoted the growth of pancreatic epithelium *in vitro*, suggesting that the mesenchymal signal is permissive.

Epidermal growth factor (EGF) and fibroblast growth factor (FGF) have been shown to define the mesenchymal signals that can stimulate growth and morphogenesis of the pancreas. EGF stimulate growths of E13.5 rat pancreatic epithelium that had been depleted of mesenchyme (Cras-Meneur et al., 2001). Mice lacking functional ErbB1 and ErbB3 (EGF receptors) have perturbed pancreatic development, providing further support for a role of EGF in promoting growth and morphogenesis of the pancreas (Miettinen et al., 2000; Erickson et al., 1997).

FGF1, 7 and 10, which are expressed in the pancreas during development, are able to induce pancreatic epithelial cell proliferation in the absence of its surrounding mesenchyme *in vitro* (Miralles et al., 1999). FGFR2IIIb, which mediates the effects of these FGF ligands, is also expressed in the pancreatic epithelium. Driving the expression of a dominant negative form of this receptor under the control of the ubiquitous metallothionein promoter, results in a pancreas that shows abnormal growth and a greatly reduced number of acinar cells. Furthermore, $FGF10^{-/-}$ mouse exhibit an underdeveloped pancreas (Ohuchi et al., 2000; Bhushan et al., 2001). Two recent studies have shown that FGF10 from the mesenchyme is needed to maintain Notch activation in the pancreatic epithelium and thereby stimulates proliferation and suppresses differentiation of the pancreatic progenitor cells (Norgaard et al., 2003; Hart et al., 2003).

The dorsal pancreatic mesenchymes express a LIM homeodomain protein, Isl1, and N-cadherin. Mutant mice lacking either of these factors do not form dorsal mesenchyme and have dorsal pancreas development blocked (Ahlgren et al., 1997; Esni et al., 2001). Conversely, ventral pancreas development is not affected in these mutant mice. Later, Edsbagge and colleagues (2005) have revealed that the pancreatic phenotype of N-cadherin deficient mice was secondary to the cardiac/vascular defect. Using an *in vitro* pancreatic explant assay, they were able to show that *sphingosine-1-phosphate* (*S1P*)-mediated G-protein coupled signalling rescues formation of the dorsal pancreas in N-cadherin deficient mice *in vitro*, by specifically triggering the proliferation of the dorsal pancreatic mesenchyme.

Various studies have shown that signals derived from the mesenchyme are also involved in the control of pancreatic cell differentiation and regulate the proportion of exocrine versus endocrine cells. Removal of pancreatic mesenchyme impairs pancreatic exocrine development but promotes endocrine development (Golosow & Grobstein, 1962; Rutter et al., 1978; Gittes et al., 1996; Miralles et al., 1998). Miralles and colleagues (1998 & 1999) showed that mesenchymal signals, which can be mimicked by follistatin (a TGF-β antagonist) and FGFs (FGF1, 7 and 10), promote exocrine cell differentiation. Conversely, endocrine differentiation is inhibited in the presence of mesenchyme. Activin-mediated TGF-β signalling has been shown to promote endocrine development in an autocrine manner (Kim et al., 2000). Mice harboring a null mutation in type II activin receptors, ActRIIA and ActRIIB, display islet hypoplasia.

1.4.4 The role of lateral plate mesoderm, cardiac mesoderm and septum transversum in ventral pancreas development.

The lateral plate mesoderm that lies directly under the presumptive ventral pancreatic endoderm is a source of signals that establishes the ventral pancreatic domain. Using *in vitro* quail-chick tissue recombination assays, Kumar and colleagues (2003) have shown that ventral prepancreatic endoderm receives instructive signals from the lateral plate mesoderm (LPM) and that the pancreatic fate is determined at 6-somites stage prior to the expression of *Pdx1*. Transplantation of the specified prepancreatic endoderm to a rostral endodermal region does not abolished *Pdx1* expression, whereas grafting the same explants to the caudal region respecified the grafted endoderm to a more caudal fate. They also revealed that these instructive signals from the LPM, which might be BMP, RA or activin, pattern the endoderm in a posterior-dominant fashion (Kumar et al., 2003).

Liver and ventral pancreas develop from a population of bipotential precursor cells in the ventral foregut endoderm (Deutsch et al., 2001). It has been well studied in chick (Le Douarin, 1964) and mouse (Gualdi et al., 1996) that interaction with cardiac mesoderm is required for proper hepatic differentiation. Jung et al. (1999) have shown that FGFs can induce hepatic differentiation in

endodermal explants in the absence of cardiac mesoderm, whereas in the absence of FGF signalling from the cardiac mesoderm, ventral foregut endoderm does no assume a hepatic fate. Instead, pancreas fate is initiated suggesting that the default fate of ventral foregut endoderm is to be pancreas (Deutsch et al., 2001).

Rossi and colleagues (1998) further showed that BMP signalling from the septum transversum mesenchyme is necessary to induce liver genes and to exclude a pancreatic fate in the ventral foregut endoderm. BMPs regulate the levels of *GATA4* transcription factor and act in parallel to FGFs from the cardiac mesoderm to induce liver fate (Rossi et al., 1998).

1.5 Pancreas determination

Once the endoderm region is committed to the pancreas fate, it starts to express several transcription regulators (*HlxB9*, *Pdx1*, *Ptf1a/p48*), which mark the pancreatic precursor cells. A precise combination of the expression of these transcription factors is critical in establishing the dorsal and ventral pancreatic domain since some of these factors are also express in tissues adjacent to pancreas.

HlxB9 is a homeodomain factor that expresses transiently in the pancreatic progenitor cells and later restricted to the β-cells. Its expression precedes *Pdx1* expression. Mice lacking *HlxB9* display dorsal pancreas agenesis and dorsal bud initiation is not observed. Conversely, ventral pancreas develops normally until later stages, when *HlxB9* is required for β-cells maturation (Harrison et al., 1999; Li et al., 1999). Expression of *HlxB9* needs to be controlled temporally since overexpression of *HlxB9* from the *Pdx1* promoter is detrimental to pancreas development (Li & Edlund, 2001).

Pdx1, another homeodomain protein, was the first gene shown to be cell-autonomously required for formation of the pancreas in mice and humans (reviewed in Jensen, 2004). *XlHbox8*, the *Xenopus* homologue of mouse *Pdx1*, is expressed in the anterior endoderm that is fated to become pancreas,

stomach and duodenum (Wright et al., 1989). Pancreas development arrests after initial budding in *Pdx1* null mice, resulting in an animal with no pancreas (Jonsson et al., 1994; Offield et al., 1996). The phenotype of mice lacking *Pdx1* demonstrates that *Pdx1* is necessary for growth of the pancreatic bud, but not for initial induction of bud formation. Lineage tracing analysis showed that *Pdx1* expressing precursor cells give rise to all three pancreatic cell types later in the development (Gu et al., 2002). *Pdx1* is also expressed in the β-cells and in a subset of δ-cells that appear during the secondary transition (Øster et al., 1998) and it is found to be one of the regulators of insulin gene expression (Ohlsson et al., 1993). *Pdx1* haploinsufficiency (*Pdx1*$^{+/-}$) affects β-cells function, in that mice display reduced insulin secretion and Glut2 expression (Brissova et al., 2002). *Pbx1*, a member of the TALE family of homeodomain factors, modulates the activity of *Pdx1*. *Pbx1* is capable of forming heterodimers with *Pdx1 in vitro*, and it is known to confer DNA binding specificity to its partner. The *Pdx1*/*Pbx1* complex has been shown to be important in the different activities of Pdx1 in endocrine and exocrine cells respectively (Swift et al., 1994)

Ptf1a/p48 is one of the B-type bHLH transcription factors in the trimeric complex of Pancreas transcription factor (*Ptf*). It was originally identified as an exocrine specific transcription factor. *Ptf1a/p48* null mice have no detectable exocrine pancreatic tissue, while the dorsal endocrine cells develop and migrate to the spleen (Krapp et al., 1996). However, by recombination-based lineage tracing, Kawaguchi and colleagues (2002) were able to demonstrate that *Ptf1a/p48* is expressed at early stages in the progenitors of pancreatic ducts, exocrine and endocrine cells, and support the specification of precursors of all three pancreatic cell types. In their study, they found that in the absence of *Ptf1a/p48*, ventral pancreas fails to form and adopts a intestinal fate suggesting that *Ptf1a/p48* is responsible for specification of the ventral pancreas. In *Xenopus*, overexpression of *Ptf1a/p48* together with *XlHbox8* leads to the conversion of duodenal fate to pancreas fate and to the formation of a giant pancreas containing a normal proportion of exocrine and endocrine cell types (Afelik et al., 2006 in press), indicating that *Ptf1a/p48* is required in the nascent pancreatic buds for the commitment of the precursors to all three major cell lineages.

1.6 Exocrine and endocrine fate segregation

Exocrine and endocrine cells start to differentiate during the secondary transition. Exocrine and endocrine fate determination, similar to generation of neurons during neurogenesis, is mediated by lateral inhibition via Notch signalling. In this system, cells expressing the ligands Delta and Serrate activate the Notch receptor on neighbouring cells, leading to cleavage of the intracellular domain of the activated Notch receptor. Notch-ICD interacts with the *RBP-Jκ* to activate the expression of bHLH Hairy/Enhancer of Split (*Hes)* genes. Activation of the *Hes* genes leads to suppression of primary target genes, such as neurogenin (reviewed in Edlund, 2001).

In mice, all four Notch genes (Notch 1-4), its ligands *Serrate 1* and *2*, as well as the Notch target gene *Hes1* have been shown to differentially expressed in the mesenchymal and epithelial cells of the developing pancreas (Lammert et al., 2000; Jensen et al., 2000a). Mice deficient for *Delta-like gene 1* (*Dll1*), intracellular mediator RBP-Jκ or the repressor *Hes1*, all showed accelerated endocrine cell differentiation at the expense of the pool of the progenitor cells (Apelqvist et al., 1999; Jensen et al., 2000a). A similar phenotype was observed with mice overexpressing the intracellular domain of Notch3, acting as a repressor of Notch signalling (Apelqvist et al., 1999).

However, recent studies by Hald et al. (2003) and Murtaugh et al. (2003) have suggested a new role for Notch signalling in pancreas development. By using the same strategy, these two group have found that misexpression of activated form of Notch1 receptor (Notch1-IC) in *Pdx1*-expressing progenitor cells prevents differentiation of both exocrine and endocrine lineages. The progenitors cells remain trapped in the undifferentiated state. When Notch1-IC is misexpressed in differentiated exocrine pancreas, mature acinar cells are replaced by a nestin-positive precursor cell population invoking a dedifferentiation process (Miyamoto et al., 2003). Taken together, these data suggest that Notch signalling controls multiple steps in the context of pancreas development.

1.6.1 Exocrine differentiation

Exocrine differentiation is marked by the initial expression of exocrine differentiation markers, such as amylase, trypsinogen and carboxypeptidase A. In the mouse, exocrine differentiation starts at E13.5 (reviewed in Jensen, 2004) whereas in *Xenopus,* it starts at stage 39, when expression of *XPDIp*, a pancreas-specific protein disulfide isomerase, is first evident (Afelik et al., 2004). Compared to endocrine differentiation, exocrine differentiation has obtained less attention. As mentioned above, *Ptf1a/p48* has been shown to play a role in exocrine pancreas development. Inhibiting the function of *Ptf1a/p48*, either in mice or in frog, causes a loss of exocrine pancreas (Krapp et al., 1996; Afelik et al., 2006). Mist1, another bHLH transcription factor, has been shown to be strongly expressed in the exocrine pancreas. Mist null mice display a loss of differentiated exocrine cells leading, to exocrine lesions that undergo a regeneration process. The cells in these lesion co-express ductal and exocrine markers, suggesting that Mist1 is required for maintenance of a stable exocrine fate (Pin et al., 2001).

1.6.2 Endocrine differentiation

Endocrine differentiation is already apparent as the pancreas begins to bud. During these early stages, from E9.5 to E12.5 in the mouse, the majority of the endocrine cells formed are glucagon-positive α-cells. During the secondary transition, the rate of endocrine differentiation, especially β-cells, increases. Newly formed endocrine cells delaminate from the epithelium and migrate into the mesenchyme to form aggregates.

Neurogenin3 (Ngn3), a bHLH protein, is a key regulator of endocrine development. It is exclusively expressed in the endocrine progenitor cells and is subsequently downregulated during differentiation (Apelqvist et al., 1999; Jensen et al., 2000b; Gu et al., 2002). The pancreas of Ngn3 mutant mice appears normal at first glance, but histological analysis shows a complete absence of endocrine cells (Gradwohl et al., 2000). Conversely, overexpression of Ngn3 throughout the pancreatic epithelium causes endocrine hyperplasia at

the expense of progenitor cells (Apelqvist et al., 1999; Schwitzgiebel et al., 2000). Furthermore, misexpression of Ngn3 is sufficient to induce endocrine differentiation throughout the gut epithelium (Grapin-Botton et al., 2001). Promoter studies have shown that the Ngn3 promoter contains multiple binding sites for the Hes1 repressor, and for several other transcription factors such as HNF1, Foxa1, Foxa2 and HNF6 (Jacquemin et a., 2000; Lee et al., 2001). Genetic studies in mouse further support the idea that HNF6 might be the upstream activator of Ngn3 expression (Jacquemin et al., 2000).

NeuroD/Beta2, a neuroendocrine bHLH factor, has been shown to be induced by Ngn3 and is expressed in the post-mitotic endocrine cells (Naya et al., 1995). In NeuroD/Beta2 null mice, pancreatic islet development is severely impaired. The islet number is reduced, and a significant portion of the β-cells undergoes apoptosis prior to birth (Naya et al., 1997). NeuroD/Beta2 expression is autoregulated, suggesting a role in the stabilization of the endocrine fate (Yoon et al., 1998). NeuroD/Beta2 might play a role in promoting endocrine precursor cell cycle exit, since its expression precedes other post-mitotic markers such as Pax6 and Islet1 (Jensen et al., 2000b).

Determination of the individual endocrine cell fate to become α-cell, β-cell, δ-cell, PP-cell or ε-cell is likely to depend on the transcription factor code specific for each cell type. Many transcription factors have been identified in this context. These include Nkx2.2, Nkx6.1, Pax4, Pax6, Arx4, Foxa1, Foxa2, HNF4, MafA and Islet1 (reviewed in Jensen, 2004). Regulation of temporal and spatial expression of these transcription factors is critical for the endocrine cell subtype specification. A simplified model for the role of these transcription factors in endocrine cell subtype determination is depicted in Figure 1.4.

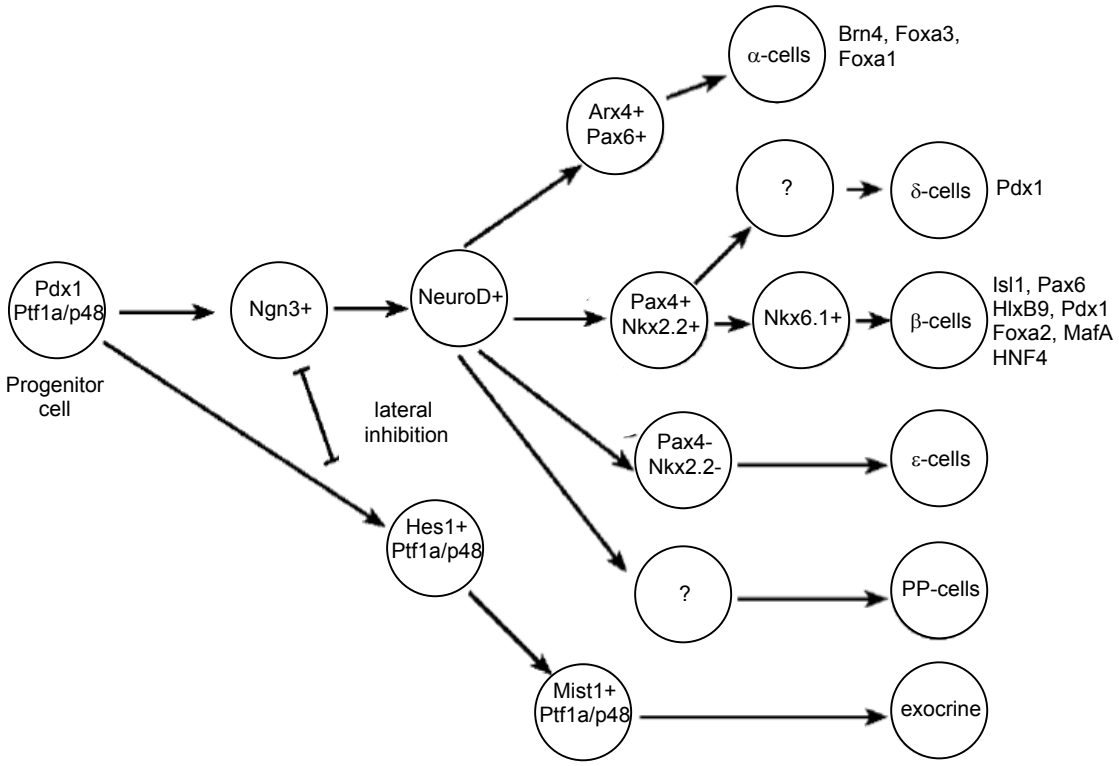

Figure 1.4 A simplified model for the role of various transcription factors in establishing the endocrine cell types fate in the developing pancreas. The proposed position for each transcription factor is based on its timing of expression, timing of predominant functional role, or both. Question marks indicate that the gene(s) involved in a particular lineage is not known (adapted from German, 2003 and Jensen, 2004 with modifications).

1.7 Retinoic acid signalling and pancreas development

Retinoic acid (RA), a vitamin A metabolite, is involved in vertebrate morphogenesis, growth, cellular differentiation and tissues homeostasis (reviewed in Mark et al., 2006). The vitamin A that is obtained from the diet is stored in the liver in the form of retinyl esters before it is hydrolysed to retinol when the body is in need. Cells that require RA take up retinol and convert it through two enzymatic reactions. In the first step, retinol is reversibly converted to retinal by retinol dehydrogenases (ROLDH), and retinal is subsequently oxidized, irreversibly, to RA by retinaldehyde dehydrogenase (RALDH) (reviewed in Maden, 2001). Among the three RALDH isotypes identified in vertebrates, RALDH2 is the earliest and most broadly expressed one during embryogenesis, and it exhibits the greatest substrate specificity (Niederreither et al., 1997). RA is further broken down to several inactive metabolites, such as 4-

oxo-RA, 4-OH-RA, 18-OH-RA, and 5,18-epoxy-RA, by a cytochrome P450 enzymes known as CYP26. In *Xenopus,* the homologues of these enzymes, xRALDH2 and xCYP26A1, have been well characterized (Hollemann et al., 1998; Chen et al., 2001). Both genes are expressed during gastrulation in a complementary, non-overlapping manner. xRALDH2 is strongly expressed in the involuting mesoderm and xCYP26A1 is expressed predominantly in the prospective anterior neural plate.

There are two forms of RA which are biologically active, all-trans-RA and 9-cis-RA, which act by means of different receptors. Once RA has been synthesized inside the cell, it enters the nucleus and binds to two classes of functionally and structurally distinct nuclear receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). Both classes of receptors exist in the form of three different isotypes (α, β, and γ) and numerous isoforms form by differential promoter usage or alternative splicing. These RARs and RXRs show highly dynamic and complex expression patterns during embryogenesis. The ligand for RXRs is 9-cis-RA, whereas RARs bind to both 9-cis-RA and all-trans-RA. The receptors form dimers and bind to the RA response element (RARE) of the RA target genes, regulating their expression (reviewed in Mark et al., 2006).

RA is required for patterning of the *Xenopus* embryo along its A-P axis during early embryogenesis. Exogenously applied RA produces a concentration-dependent truncation of anterior, and enhancement of posterior structures in *Xenopus* embryos (Durston et al., 1989; Sive et al.; 1990) through its influence on the mesoderm and ectoderm (Ruiz i Altaba and Jessell, 1991; Sive and Cheng, 1991). RA is also involved in the anterior-posterior patterning of the developing CNS (reviewed in Chen et al., 2004). Given the established role of RA in A-P patterning of the mesoderm and ectoderm, studies on the influence of RA in A-P patterning of the endoderm have emerged only recently. Wendling et al. (2000) and Matt et al. (2003) showed that RA signalling is needed to pattern the pharyngeal endoderm. In *Xenopus*, application of RA to tailbud stage embryos affects morphogenesis of liver, stomach and intestine (Zeynali and Dixon, 1998). Moriya et al., (2000a, b), in two separate studies, have shown that

Xenopus dorsal lip and animal cap explants transiently treated with activin and RA are directed to a pancreatic fate.

In the zebrafish, RA is required to regionalize the endoderm and it is essential for liver and pancreas development at the end of gastrulation. Upon downregulation of RA signalling, either by using RA antagonists or raldh2 mutant fish, liver and pancreas development is inhibited. Exogenous application of RA causes anterior expansion of pancreatic gene expression (Stafford and Prince, 2002). A more recent study by the same group has shown that RA signals directly to zebrafish endoderm to specify insulin-expressing β-cells (Stafford et al., 2006). In chick, RA is also required for the patterning of the endoderm and for pancreas development (Kumar et al., 2003; Stafford et al., 2004). In the mouse, two different groups showed that RA generated by RALDH2 in the mesoderm is required for dorsal pancreas development at a stage preceding *Pdx1* expression. Dorsal pancreas is lost in Raldh2 null mice whereas liver development is not affected (Motlokov et al., 2005; Martin et al., 2005).

1.8 Aim of this study

In the context of this study, we aimed at defining the role of RA signalling in liver and pancreas development in the African clawed frog, *Xenopus laevis*. In the first part of the study, we investigate the effect of increasing and decreasing levels of RA on liver and pancreas development. In the second part, we concentrate on the molecular mechanisms that enable RA to pattern the gastrula endoderm that will give rise to the pancreas. We also establish a new method for generating transgenic frog embryos using *I-SceI* meganuclease approach adapted from fish, providing a simple and highly efficient tool for studies on later phases of pancreas development, such as transdifferentiation, gain-/loss-of function or promoter analyses.

Chapter I: Retinoic acid signalling activities in the context of liver and pancreas development in *Xenopus*

Yonglong Chen[1], Fong Cheng Pan[1], Nadia Brandes, Solomon Afelik, Marion Sölter, and Tomas Pieler, **Retinoic acid signaling is essential for pancreas development and promotes endocrine at the expense of exocrine cell differentiation in Xenopus**, *Developmental Biology.,* 2004, 271, 144–160

[1] equal contribution

Statement of contribution:

Animal caps isolation and cultivation, RNA isolation, normal and quantitative RT-PCR, time course treatment assays with BMS453 and RA, whole mount in situ hybridization on animal caps and injected embryo, quantification of insulin expressing cells, vibratome sectioning, data collection.

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Retinoic acid signaling is essential for pancreas development and promotes endocrine at the expense of exocrine cell differentiation in *Xenopus*

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Abstract

How and when the vertebrate endoderm is first subdivided into discrete progenitor cell populations that will give rise to the different major organs, including pancreas and liver, are only poorly understood. We have used *Xenopus laevis* as a model system to characterize these events, since it is particularly suited to study the early embryonic patterning in vertebrates. Our experimental results support the notion that retinoic acid (RA) functions as an essential endodermal patterning signal in *Xenopus* and that it acts as early as during gastrulation. As a result of RA treatment, the expression of Sonic Hedgehog (Shh), a known inhibitor of pancreas development in other vertebrate systems, is negatively regulated in the dorsal prepancreatic endoderm. Furthermore, RA is found to promote endocrine at the expense of exocrine differentiation in the dorsal pancreas, correlating with a specific inhibition of Notch signaling activities in this territory. Conversely, RA enhances exocrine marker gene expression in the ventral pancreas.

Keywords: BMS453; Endocrine; Exocrine; Retinoic acid; *Xenopus laevis*

Introduction

The endodermal germ layer gives rise to a number of different organs, including liver and pancreas. In amphibia and higher vertebrates, the pancreas is derived from three different primordia, one dorsal and two ventral pancreatic buds. During embryonic development, one of the two ventral buds regresses, and gut rotation results in the juxtaposition of dorsal and ventral buds, which fuse to form one organ. The mature pancreas consists of a number of different cell types, organized in form of two principal functional units, the endocrine and the exocrine pancreas (reviewed in Edlund, 2002; Kumar and Melton, 2003; Slack, 1995). The liver arises from the same population of precursor cells as the ventral pancreas (reviewed in Zaret, 2002).

Lineage tracing analysis in *Xenopus* suggests that the pancreas originates from the dorsal endoderm of stage 10.5 gastrulae (Chalmers and Slack, 2000). These early endodermal precursor cells form in a cell-autonomous manner under the control of the maternal transcription factor VegT and the dorsalizing activity of β-catenin. On the basis of results obtained with vegetal explants from blastula stage embryos, it has been suggested that this early embryonic endoderm is already prepatterned along the dorsal–ventral body axis, as evident from the differential expression of endodermal marker genes such as *XlHbox8* (Gamer and Wright, 1995; Henry et al., 1996), as well as *XHex* and cerberus (Zorn et al., 1999) in dorsal but not ventral vegetal explants. A more recent report provides strong evidence that specification of the endoderm along the anteroposterior body axis relies on the inductive activity of adjacent mesodermal tissue in tailbud stage embryos (from stage 25 onwards) and that mesodermal precursor cells may also be present in vegetal explants (Horb and Slack, 2001). The molecular identity of the signals involved in these early endodermal patterning

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events is not exactly defined, but transforming growth factor β signaling has been found to exert a positive regulatory function in the vegetal explant system (Gamer and Wright, 1995; Henry et al., 1996; Zorn et al., 1999).

The dorsal pancreatic bud forms at stage 35/36, followed by the ventral rudiments at stage 37/38. The earliest pancreatic differentiation marker is insulin, which is confined to the dorsal pancreatic anlage during early development (Kelly and Melton, 2000). Exocrine differentiation, as first detected at stage 39 by expression of *XPD*lp, occurs in both dorsal and ventral pancreas (Afelik et al., 2004). The dynamic movements of the gastrointestinal tract during stage 40 shift the dorsal pancreatic primordium towards the ventral one, allowing for the fusion of the two pancreatic lobes to complete by stage 40 (Afelik et al., 2004; Kelly and Melton, 2000).

Several transcription regulators (such as Pdx1, Hlxb9, p48/Ptf1a) have been described, which identify pancreatic progenitor cells before expression of the pancreas differentiation markers can be detected (reviewed in Edlund, 2002). A critical combination of multiple of these transcription regulators is thought to be necessary to control the initial pancreas specification, as some of these factors are also expressed outside of the pancreatic anlage. A second, partially overlapping wave of transcription factor activity operates in the differentiation of the individual exocrine and endocrine cell types. Several of these factors (such as p48, Hes1, ngn3, and NeuroD) appear to be directly linked to the Notch signal transduction cascade. It has been demonstrated in the mouse that Notch activation suppresses endocrine differentiation and may be involved in maintaining the undifferentiated state of pancreatic precursor cells (Apelqvist et al., 1999; Hald et al., 2003), while the ngn3-induced gene cascade seems to promote endocrine cell differentiation (Gu et al., 2002).

The development of the pancreas has been found to be under the control of signaling molecules secreted from neighboring tissues. TGFβ and FGF signals from the notochord seem to exert a function in repressing Sonic Hedgehog (Shh) expression in the dorsal pancreatic endoderm. Ectopic expression of Shh in this same domain has been shown to impair pancreas formation, while overall inhibition of the Hedgehog-induced signal transduction pathway leads to heterotopic pancreas development (reviewed in Hebrok, 2003). Circumstantial evidence also indicates a restrictive role for Shh in ventral pancreas development (reviewed in Zaret, 2002). Furthermore, it was also demonstrated that the endothelium of blood vessels that are in transient, direct contact with the dorsal and both ventral pancreatic primordia also functions as an essential signaling source for pancreas development (Lammert et al., 2001). However, the molecular identity of the signal(s) involved remains to be elucidated.

Retinoic acid (RA), which operates via direct binding to a number of different nuclear receptors that are expressed as various isoforms in complex patterns during embryogenesis, is a well-characterized signaling molecule that acts in anteroposterior patterning of neuroectoderm and mesoderm in vertebrates (reviewed in Maden, 2001). More recent data indicate that RA may also be involved in the regionalization of the embryonic endoderm. Specifically, Stafford and Prince (2002) have revealed that RA signaling at the end of gastrulation is required for both liver and pancreas specification in the zebrafish system. Spatial and temporal characteristics of RA synthesis in the developing embryo are primarily controlled by the activity of two metabolic enzymes; CYP26 inactivates RA, and RALDH2 is involved in the synthesis of RA (reviewed in Chen et al., 2004). We have previously characterized these two activities in *Xenopus* embryos; the expression characteristics observed for the two enzymes during *Xenopus* gastrulation (Chen et al., 2001; Hollemann et al., 1998) suggest that RA signaling may also be relevant for patterning of the dorsal endoderm that gives rise to liver and pancreas.

In this study, we have investigated the role of RA signaling in pancreas and liver development in *Xenopus*. We find RA to be essential for aspects of pancreas but not liver formation. Inhibition of RA signaling at gastrula stages results in an expansion of Shh expression into the prospective dorsal pancreatic endoderm, correlating with a loss of dorsal exo- and endocrine pancreas development. The ventral pancreas is only moderately affected under these conditions. Conversely, application of excessive RA during gastrulation expands the endocrine cell population at the expense of exocrine cells in the dorsal pancreas, correlating with a negative regulation of Notch signaling activity. In contrast, increased RA levels expand exocrine marker gene expression in the ventral pancreas, while development of the liver is inhibited. These findings reveal a link between RA-mediated prepatterning of the primitive endoderm with Shh and Notch signaling as regulators of pancreas development. Furthermore, they also define a scenario for pancreas development in *Xenopus* that is closely related to the situation in the mouse but significantly different from the one in zebrafish.

Materials and methods

Embryo cultivation

RA (all-*trans*-RA, Sigma), BMS453 (a gift from Bristol Myers Squibb), and all-*trans*-retinal (Sigma) were first prepared as 10-mM stock solutions in 100% ethanol (RA and retinal) and dimethyl sulfoxide (DMSO, BMS453) and then diluted into desired concentrations with 0.1 × MBS (at least 1:1000 dilution). Corresponding amount of ethanol and DMSO was added to control embryos.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was done in principle as described (Harland, 1991) with modifications as

reported in Hollemann et al. (1999). The probes were prepared as follows: *XlHbox8* (RT–PCR cloning into pCS2+, GenBank accession no. X16849), cut with *Cla*I, transcribed with T7 RNA polymerase; insulin (RT–PCR cloning into pGEMT, GenBank accession no. M24443), cut with *Not*I, transcribed with T7 RNA polymerase; glucagon (Horb and Slack, 2002), cut with *Not*I, transcribed with T7 RNA polymerase; somatostatin (from Marko Horb, GenBank accession no. AY372267), cut with *Not*I, transcribed with T7 RNA polymerase; *XPD*lp (Afelik et al., 2004), cut with *Bam*HI, transcribed with T7 RNA polymerase; *Xtn*Ic (Drysdale et al., 1994), cut with *Not*I, transcribed with T7 RNA polymerase; *XHex* (Newman et al., 1997), cut with *Bam*HI, transcribed with T7 RNA polymerase; *Cyl18* (Chen et al., 2003), cut with *Cla*I, transcribed with T3 RNA polymerase; X-Shh (Ekker et al., 1995), cut with *Xba*l, transcribed with T3 RNA polymerase; XCYP26A1 (Hollemann et al., 1998), cut with *Eco*Rl, transcribed with T7 RNA polymerase; XRALDH2 (Chen et al., 2001), cut with *Bam*Hl, transcribed with T7 RNA polymerase; *xHB9* (Saha et al., 1997), cut with *Xba*l, transcribed with T3 RNA polymerase; PTF1-p48 (degenerative RT–PCR cloning into pGEMT), cut with *Sal*l, transcribed with T7 RNA polymerase; NeuroD (Lee et al., 1995), cut with *Xba*l, transcribed with T7 RNA polymerase; *ESR*10 (Li et al., 2003), cut with *Sal*I, transcribed with T7 RNA polymerase; *Hoxb-1* (Godsave et al., 1994), cut with *Pvu* II, transcribed with SP6 RNA polymerase; the cDNA inserts of *XPTB* (Chen et al., 2003), trypsinogen, and elastase (both from an adult pancreas cDNA library) in pBK-CMV vector were amplified by PCR with a pair of primers in the vector (forward: 5′ CGCGCCTGCAGGTCGACACTA-3′ and reverse: 5′-GCAAGGCGATTAAGTTGGGTA-3′). Since the reverse primer is downstream of the T7 promoter in the vector, the PCR products were directly applied to the in vitro transcription reaction with T7 RNA polymerase.

Embryo microinjection and animal cap explants

For whole-mount in situ hybridization analysis, 5 pmol of XRALDH2 morpholino antisense oligonucleotide (5′CGCTTTGGACTATCCCCTTGTCTCT, Gene Tools, LLC) and 2 ng of XCYP26A1 (Hollemann et al., 1998) and XRALDH2 (Chen et al., 2001) mRNAs were injected at the 1- or 2-cell stage. For the animal cap assay, embryos were injected as described by Chen et al. (2003). Animal caps were dissected from stage 9 embryos, cultured in 0.5 × MBS with or without RA treatment at stage 11 for 1 h and harvested for RT–PCR analysis when control siblings had reached the appropriate stages.

RT–PCR

Total RNA from whole embryos and animal caps was isolated by use of the RNeasy kit (Qiagen). All RNA samples were controlled by 35 cycles of a histone H4-specific PCR reaction for DNA contamination. RT–PCR was carried out using the Gene Amp RNA PCR core kit (Perkin-Elmer). The sequences of primers used in the PCR reactions and cycle numbers are listed below:

Xenopus histone H4 (24 cycles, Niehrs et al., 1994), forward: 5′-CGGGATAACATTCAGGGTATCACT-3′ and reverse: 5′-ATCCATGGCGGTAACTGTCTTCCT-3′; *XPD*lp (30 cycles, Afelik et al., 2004), forward: 5′-GGAGGAAAGAGGGACCAA-3′ and reverse: 5′-GCGCCAGGGCAAAAGTG-3′; *XlHbox8* (32 cycles), forward: 5′-AATCCACCAAATCCCACACCT-3′ and reverse: 5′-GCCTCAGCGACCCAATAGAA-3′; insulin(32 cycles, Henry et al., 1996), forward: 5′-ATGGCTC-TATGGATGCAGTG-3′ and reverse: 5′-AGAGAA-CATGTGCTGTGGCA-3′; *XHex* (30 cycles), forward: 5′-GGTTCCAGAACAGAAGAG-3′ and reverse: 5′-CCTTTGTCGCCTTCAATG-3′; transthyretin (32 cycles), forward: 5′-GGAATCCCCGCTGCCAATC-3′ and reverse: 5′-ATGAGAAGGAGTAGGGGGTGA-3′; trypsinogen (30 cycles, Afelik et al., 2004), forward: 5′-CTGGCTGGGGCAACACTCTC-3′ and reverse: 5′-TAGCCCCAGGACACCACACC-3′.

Quantification of insulin-expressing cells

In stage 34–36 embryos, most insulin-expressing cells are dispersed in the dorsal pancreatic endoderm, just underneath notochord. After whole-mount in situ hybridization and carefully removing neural tube, notochord, and somites, insulin-positive cells were counted under the stereomicroscope. Insulin-expressing cells in stage 40 embryos were counted in vibratome sections after whole-mount in situ hybridization.

Vibratome sectioning

Vibratome sections (30 μm) were prepared as described previously using the Leica VT1000S vibratome (Hollemann et al., 1998).

Results

RA signaling is required for exocrine and endocrine cell differentiation in the dorsal pancreatic primordium

All-*trans*-RA has profound effects on the anteroposterior patterning of *Xenopus* embryos (Durston et al., 1989). RA treatment before the end of gastrulation results in a loss of anterior neural structures but not in an overt phenotype on gut development (Fig. 1A, panels 3 and 7). BMS453 is a synthetic RA antagonist that is specific for RA receptors α and γ (Matt et al., 2003; Schulze et al., 2001). BMS453-treated *Xenopus* embryos do not exhibit

gross morphological abnormalities during early development, except for a slight enlargement of the head, as well as a mild compression of the anteroposterior body axis. However, at tadpole stages of development, BMS453-treated embryos exhibit severe gut malformations with a loss of gut coiling and formation of edema (Fig. 1A, panels 2 and 6). These effects can be partially rescued by simultaneous application of a high dose of RA (Fig. 1A, panels 4 and 8), suggesting that BMS453 specifically inhibits RA signaling in *Xenopus* embryos in respect to gut development.

To monitor early events in pancreas, liver, and intestinal development, we applied a panel of differentiation markers to RA- or BMS453-treated tailbud and tadpole stage embryos. *XPD*lp is expressed in the exocrine portion of both dorsal and ventral pancreas, first visible by whole-mount in situ hybridization at stage 39 (Afelik et al., 2004; Fig. 1B, panel 5); in the dorsal pancreas, BMS453 treatment completely ablates expression of *XPD*lp (Fig. 1B, panel 6). Next, we analyzed the expression of trypsinogen and elastase, which are activated later than *XPD*lp; at stage 41, trypsinogen and elastase start to be expressed in the ventral pancreas, extending into the dorsal pancreas at stage 43 (Horb and Slack, 2002). BMS453 treatment efficiently blocks expression of trypsinogen (Fig. 1B, panel 14) and elastase (not shown). Thus, inhibition of RA signaling has similar effects on early and late exocrine markers of the dorsal exocrine pancreas in *Xenopus* embryos.

As a molecular marker for endocrine pancreas development, we made use of insulin, which is exclusively expressed in the dorsal pancreas of *Xenopus* embryos until late tadpole stages (Horb and Slack, 2002; Kelly and Melton, 2000; Fig. 1B, panels 1, 9, and 13). Inhibition of RA signaling by BMS453 totally ablates insulin-positive cells (Fig. 1B, panels 2, 10, and 14). Thus, RA signals appear to be required not only for dorsal exocrine, but also for dorsal endocrine pancreas development.

Increased RA signaling expands the dorsal endocrine cell population at the expense of exocrine cells

In gain of function experiments, we studied the effect of RA on exocrine and endocrine cells in the dorsal pancreas. Excess of RA results in a dose-dependent reduction of exocrine cells, as demarcated by *XPD*lp and trypsinogen expression in the dorsal pancreas, leading to a complete loss at 5 μM RA (Fig. 1B, panels 7 and 15; Table 1). In contrast, titration of RA in the range of 1 to 5 μM RA results in a gradually increasing number of insulin-positive endocrine cells from an average of 25 in control embryos to a maximum of 50 to 55 at RA concentrations around 5 μM (Fig. 1B, panel 3; Table 1). Thus, RA stimulates the development of endocrine cells and blocks the formation of exocrine cells in a dose-dependent manner.

RA inhibits Notch signaling in the dorsal pancreas

Since earlier findings had indicated that Notch signaling promotes exocrine versus endocrine differentiation, we tested the effect of altered RA signaling on different elements of the Notch signaling pathway (Fig. 2). Expression of the Notch receptor itself, as well as of its downstream target *ESR*10 (Fig 2A, panels 3 and 6, as well as data not shown) is markedly reduced in the prospective exocrine cells upon application of excess RA. Conversely, expression of NeuroD (Fig. 2B, panels 3, 6, and 9) in the prospective endocrine cells is significantly expanded under the same conditions. If RA signaling is blocked by treatment with BMS453, expression is ablated for these genes (Figs. 2A and B, panels 2, 5, and 8). Thus, the expansion of the endocrine cell population in response to increased RA signaling seems to occur at the expense of exocrine cell development via inhibition of Notch signaling activity.

Ventral pancreas development is only moderately influenced by RA signaling

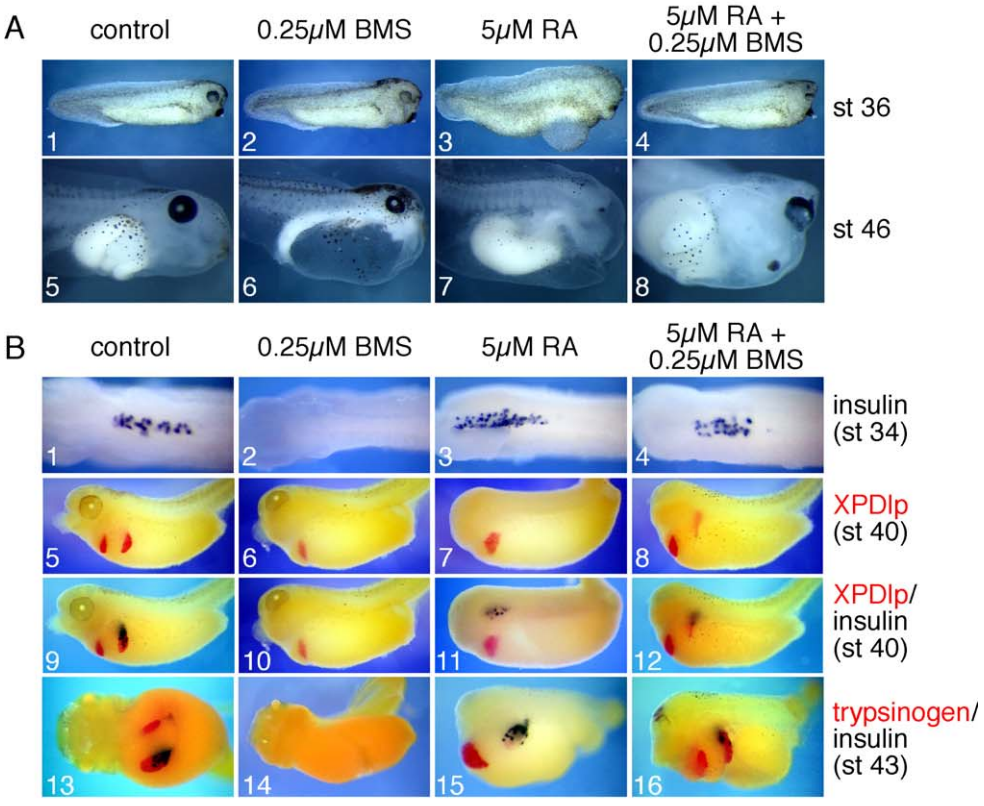
Next, we investigated the contribution of RA signaling on cell differentiation in the ventral pancreas. While RA treatment resulted in a dose-dependent reduction of exocrine marker gene expression in the dorsal pancreas, expression of the same genes appears to be stimulated in the ventral pancreas (Fig. 1B, panels 7, 11, and 15). Conversely, application of BMS453 exerts an inhibitory influence on the expression of exocrine markers in the ventral pancreas. While a very weak and delayed expression of trypsinogen (compare Fig. 1B, panel 14, with Fig. 1C, panel 8) and elastase (not shown) is observed upon inhibition of RA signaling, *XPD*lp expression is reduced to low but still detectable levels at both stages 43 and 48 (Fig. 1B, panels 6 and 10, and Fig. 1C, panels 2, 4, and 6).

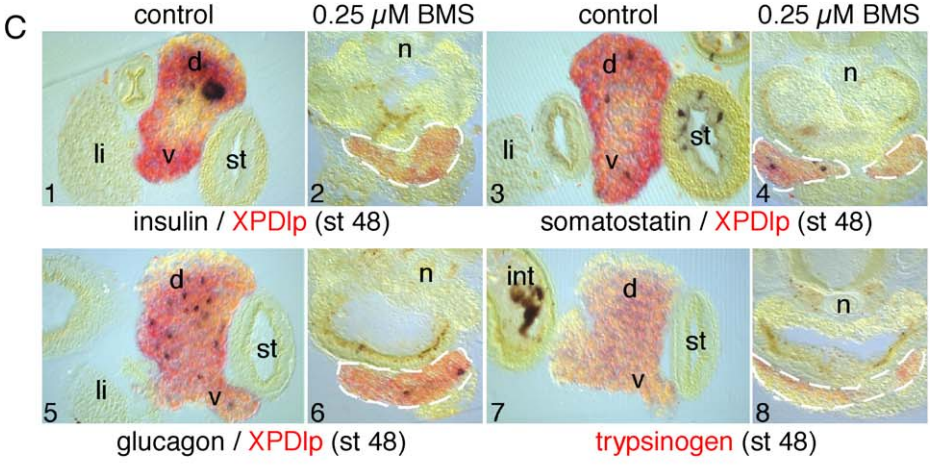
We also analyzed the expression of endocrine marker genes, such as glucagon and somatostatin, which are activated later in development in both dorsal and ventral pancreatic domains (Horb and Slack, 2002; Kelly and Melton, 2000). Insulin expression remains undetectable in the ventral portion of the pancreas in the latest stages (stage 48) tested here (Fig. 1B, panels 9–16, and Fig. 1C, panels 1 and 2). In contrast, somatostatin and glucagon expression in the ventral portion of the pancreas is detectable in both control and BMS453-treated embryos (Fig. 1C, panels 3–6). As RA-treated embryos hardly survive beyond stage 46, we were not able to analyze if RA can also increase endocrine differentiation in the ventral pancreas. Thus, the effects on pancreas differentiation upon modulation of RA signaling can be summarized as follows: inhibition of RA signaling by BMS453 treatment inhibits exocrine and endocrine differentiation completely in the dorsal but only partially in the ventral

pancreas. Conversely, application of excess RA selectively increases the dorsal endocrine cell population at the expense of the dorsal exocrine cells, while, in the ventral pancreas, exocrine differentiation seems to be stimulated (see also Fig. 1D).

Increased RA signaling ablates liver gene expression

The ventral pancreas develops from a precursor cell population that also gives rise to the liver. To investigate the effect of RA signaling on liver development, we used *XHex* as





D

		BMS453	RA
dorsal	endocrine	-	+
	exocrine	-	-
ventral	endocrine	(-)	NA
	exocrine	(-)	+

NA, not analyzed; +, stimulated; -, repressed; (-), partially inhibited.

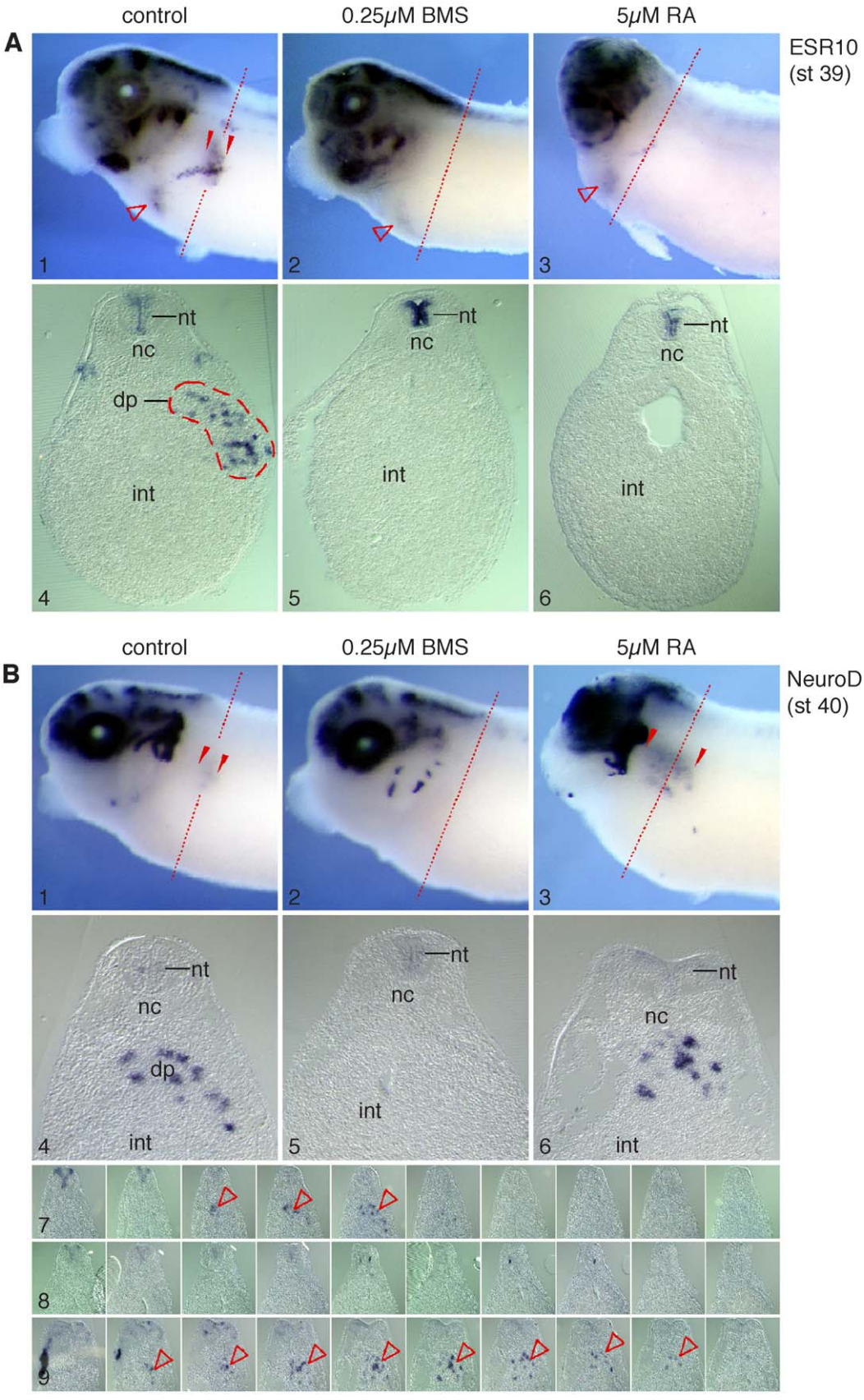
Table 1
Effects of RA and BMS453 on pancreas, liver, and heart marker gene expression

			Insulin		*XPD*lp			Trypsinogen (st 43)			*Hoxb-1* (foregut expression)		*XHex* (liver expression)		Troponin Ic	
						Dorsal	Ventral		Dorsal	Ventral						
Modulators	[μM]	Effects[a]	*n*	%	*n*	%	%	*n*	%	%	*n*	%	*n*	%	*n*	%
RA	1		21		26			24			ND		19		18	
		−2		0.0		8	0.0		0.0	0.0				5.3		5.5
		−1		8.3		46	11		37.5	0.0				5.3		6.7
		0		0.0		46	81		62.5	100				89.4		77.8
		+1		91.7		0.0	8		0.0	0.0				0.0		0.0
		+2		0.0		0.0	0.0		0.0	0.0				0.0		0.0
	2.5		23		24			27			ND		26		18	
		−2		0.0		42	0.0		4	0.0				7.7		22.2
		−1		7.7		42	16		74	0.0				15.4		27.8
		0		0.0		16	34		22	89				76.9		50
		+1		38.5		0.0	50		0.0	11				0.0		0.0
		+2		53.8		0.0	0.0		0.0	0.0				0.0		0.0
	5		66		33			25			35		50		30	
		−3		0.0		45	0.0		32	0.0		0.0		12		10
		−2		0.0		55	0.0		68	0.0		85		16		36.7
		−1		12.1		0.0	18		0.0	0.0		15		28		26.7
		0		0.0		0.0	21		0.0	44		0.0		44		26.6
		+1		21.2		0.0	61		0.0	40		0.0		0.0		0.0
		+2		66.7		0.0	0.0		0.0	12		0.0		0.0		0.0
BMS453	0.25		152		88			30			67		152		38	
		−3		100		100	0.0		100	100		91		0.0		0.0
		−2		0.0		0.0	100		0.0	0.0		9		0.0		0.0
		−1		0.0		0.0	0.0		0.0	0.0		0.0		0.0		0.0
		0		0.0		0.0	0.0		0.0	0.0		0.0		100		100
	10		51		22			25			22		55		23	
		−3		100		100	0.0		100	100		100		0.0		0.0
		−2		0.0		0.0	100		0.0	0.0		0.0		0.0		0.0
		−1		0.0		0.0	0.0		0.0	0.0		0.0		0.0		0.0
		0		0.0		0.0	0.0		0.0	0.0		0.0		100		100
RA + BMS453	5 + 0.25		92		27			34			33		68		36	
		−2		0.0		72	0.0		70	0.0		94		0.0		0.0
		−1		45.7		28	92		30	41		6		0.0		0.0
		0		28.3		0.0	8		0.0	59		0.0		100		100
		+1		19.5		0.0	0.0		0.0	0.0		0.0		0.0		0.0
		+2		6.5		0.0	0.0		0.0	0.0		0.0		0.0		0.0

n indicates number of embryos per experiment; ND, not done.
[a] Effects: −3, complete loss of expression; −2, more than 90% of normal expression levels are lost; −1, slight loss of expression; 0, close to the normal expression levels; +1, slight increase of expression; +2, the expression level is at least twofold increased.

a gene that is strongly but not exclusively expressed in the developing liver (Newman et al., 1997) and *XPTB* that is specific for the liver (Chen et al., 2003). *Hoxb-1* was used as a more anterior foregut endodermal marker that is expressed in posterior pharyngeal endoderm as well as in hindbrain rhombomere 4 (Godsave et al., 1994; Poznanski and Keller, 1997).

Fig. 1. Modulation of RA signaling affects endoderm development. (A) BMS453 treatment affects gut morphology. Stage 11 pigmented embryos were treated with 0.25 μM BMS453 and 5 μM RA, either alone or in combination, for 30 min and collected at stage 36 (panels 1–4, lateral view, anterior towards the right) or stage 46 (panels 5–8, lateral view, anterior towards the right). (B) RA signaling is required for pancreas development. Stage 11 albino embryos were treated with 0.25 μM BMS453 and 5 μM RA, either alone or in combination, for 30 min. (Panels 1–4) Dorsal view of stage 34 embryos stained for insulin expression (anterior towards the left, dorsal structures, such as neural tube, notochord, and somites, were carefully removed after whole-mount in situ hybridization). (Panels 5–8) Lateral view of stage 40 embryos stained for *XPD*lp expression (anterior towards the left). (Panels 9–12) Lateral view of both *XPD*lp (red) and insulin (dark blue) expression in stage 40 embryos. (Panels 13–16) Double-staining analysis of trypsinogen (red) and insulin (dark blue) expression in stage 43 embryos (anterior towards the left) (panels 13 and 14 ventral view; panels 15 and 16 lateral view). Due to the disturbance of the gut movement, the relative positions of dorsal and ventral pancreas in the manipulated embryos are different from those in the control embryo. (C) Both endocrine and exocrine differentiation occurs in the ventral pancreas of BMS453-treated embryos. Gut explants from stage 48 control embryos and whole BMS453-treated embryos with opened belly were used for whole-mount in situ hybridization analysis of different endocrine/exocrine marker gene expression. Thirty-micrometer-thick vibratome sections were prepared after whole-mount in situ hybridization. (Panels 1–6), double-staining with endocrine genes, insulin, somatostatin, and glucagon in blue and the exocrine *XPD*lp in red. (Panels 7 and 8) Single staining of trypsinogen in red. White dashed circles in panels 2, 4, 6, and 8 demarcate the ventral pancreas in BMS453-treated embryos. d indicates dorsal part of the pancreas; int, intestine; li, liver; n, notochord; st, stomach; and v, ventral part of the pancreas. (D) Summary of RA signaling effects on endocrine/exocrine differentiation shown in B and C.


A
control
0.25μM BMS
5μM RA
ESR10
(st 39)
1
2
3
nt
nc
dp
int
4
5
6
B
control
0.25μM BMS
5μM RA
NeuroD
(st 40)
1
2
3
nt
nc
dp
int
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5
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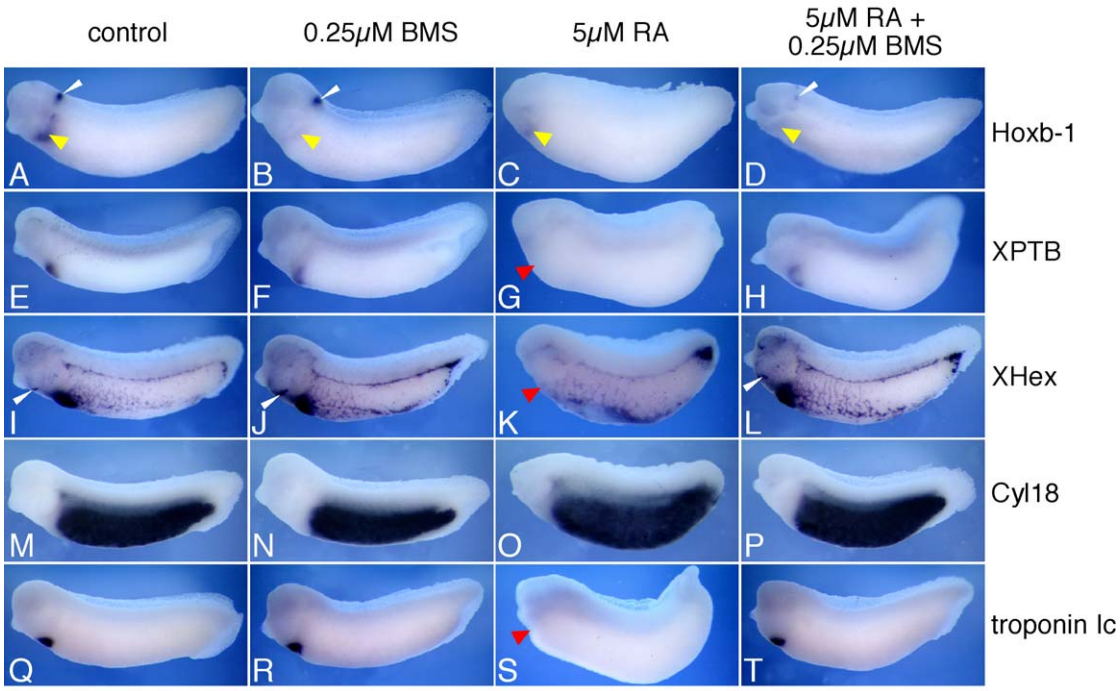


Fig. 3. RA signaling is not required for liver specification. Stage 11 albino embryos were treated with 0.25 μM BMS453 and 5 μM RA, either alone or in combination, for 30 min and collected at stage 32–34 for whole-mount in situ hybridization analysis. Expressions of *Hoxb-1* (panels A–D), *XPTB* (liver, panels E–H), *XHex* (panels I–L), *Cyl*18 (intestine, panels M–P), and troponin Ic (heart, panels Q–T) are illustrated. Yellow and white arrowheads in panels A–D indicate *Hoxb-1* expression in posterior pharyngeal endoderm and rhombomere 4, respectively. The posterior pharyngeal endoderm expression of *Hoxb-1* is inhibited under all three conditions (panels B–D). White arrowheads in panels I, J, and L indicate thyroid expression of *XHex*. Red arrowheads in panels G, K, and S indicate that the expression of these markers in liver and heart is inhibited.

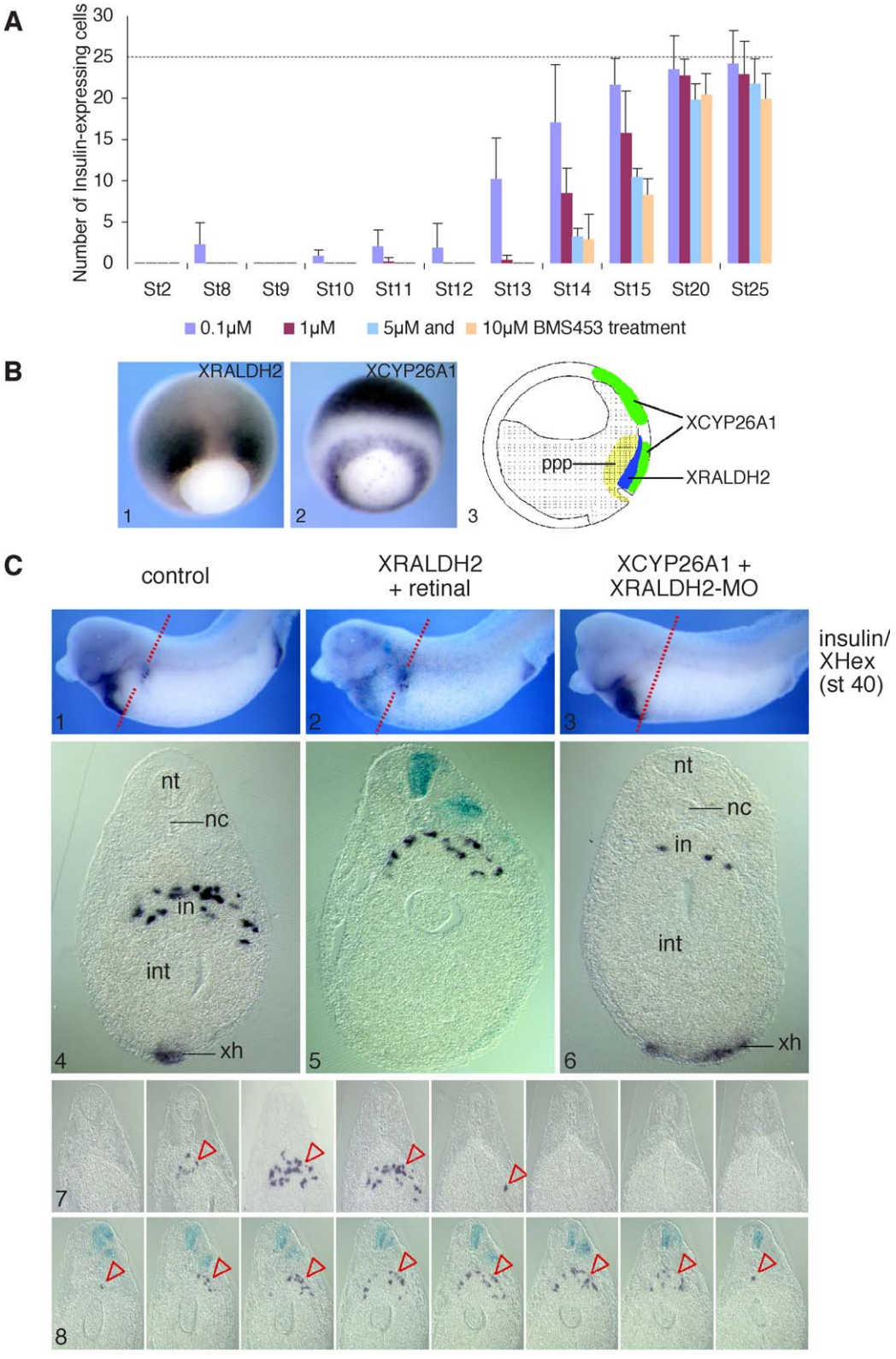
It turns out that RA treatment of *Xenopus* embryos specifically inhibits *XHex* and *XPTB* expression in the liver, as well as *XHex* expression in thyroid gland, while BMS453 has no significant effect (Figs. 3E–L; Table 1). Although RA treatment blocks *Hoxb-1* expression both in pharyngeal endoderm and in rhombomere 4 (Fig. 3C), RA signaling is specifically required for *Hoxb-1* expression only in posterior pharyngeal endoderm, but not for rhombomere 4 expression (Fig. 3B), which is consistent with the data from studies in mouse (Matt et al., 2003; Wendling et al., 2000). Thus, the effects observed on liver marker gene expression are different from those shown for the ventral pancreas and pharyngeal endodermal markers. While increased RA signaling seems to expand the ventral pancreas, it interferes with liver and posterior pharyngeal endoderm development. In contrast to the development of the pancreas and posterior pharynges, liver development in *Xenopus* embryos does not rely on RA signaling.

Since it is well established that signals from the cardiac mesoderm influence both pancreas and liver development (Zaret, 2002), we also analyzed the effect of modulating RA signaling on heart development by use of troponin Ic as a marker (Drysdale et al., 1994). Troponin Ic expression in the developing heart is inhibited in the presence of excessive RA, which is consistent with previous studies (Drysdale et al., 1997), but not affected by the RA inhibitor BMS453 (Figs. 3Q–T). There is a very good correlation in respect to the RA dose dependence that we observe for liver and heart gene expression (Table 1). Thus, it seems likely that the inhibition of liver development may, at least in part, be consequence of the inhibition of heart formation. Finally, expression of the intestinal marker gene *Cyl18* (Chen et al., 2003) is not found to be influenced significantly by increased or decreased RA signaling (Figs. 3M–P).

Fig. 2. RA promotes endocrine development at the expense of exocrine development in the dorsal pancreas. Stage 11 albino embryos were treated with either 0.25 μM BMS453 or 5 μM RA for 30 min and collected at stage 39/40 for whole-mount in situ hybridization analysis. (A) *ESR*10 expression in the dorsal pancreas primordium is downregulated by both RA and BMS453. (Panels 1–3) *ESR*10 expression in whole embryos. (panels 4–6) transversal sections (corresponding to the red dashed lines shown in panels 1–3). Note that *ESR*10 expression in the ventral pancreas is not affected by RA or BMS453 treatment (red arrowheads in panels 1–3). *ESR*10 expression in the dorsal pancreas is blocked by either 0.25 μM BMS453 (100%, $n = 32$, panels 2 and 5) or 5 μM RA (48%, $n = 36$, panels 3 and 6) treatment. (B) NeuroD expression is upregulated by RA and downregulated by BMS453 (panels 1–3, whole embryos; panels 4–9, transversal sections). NeuroD expression is restricted to the dorsal pancreas (panel 1, red arrowhead) in control embryo, covering 3–4 transversal sections (30 μM per section, panel 4 and serial sections in panel 7). 0.25 μM BMS453 treatment completely ablates NeuroD expression (100%, $n = 58$, panels 2 and 5 and serial sections in panel 8). 5 μM RA significantly expands NeuroD expression (panel 3, red arrowheads), spanning 9–12 transversal sections (78%, $n = 56$, panel 6 and serial sections in panel 9). dp indicates dorsal pancreas; int, intestine; nc, notochord; and nt, neural tube.

RA signaling in the context of pancreas development is required before the end of gastrulation

With the aim of defining the exact developmental stages when RA signaling is required for pancreas development, we have subjected *Xenopus* embryos at various early stages of development to a transient exposure of increasing concentrations of BMS453 (Fig. 4A). Intermediate and high doses of BMS453 totally ablate insulin expression if applied before stage 13. A low dose of BMS453 retains



residual insulin expression at most stages tested, with a significant recovery of insulin-positive cells observed from treatment at stage 13 and later. Application of the RA inhibitor after stage 13 never resulted in a complete suppression of insulin-expressing cells, even at the highest dose tested. Thus, we conclude that RA signaling is required for pancreas specification during the process of gastrulation. We also performed a similar series of experiments with RA-treated embryos (data not shown). The timing of RA-mediated effects is similar to what has been observed with BMS453.

We had previously characterized the two key enzymes for RA metabolism in *Xenopus* embryos, namely, XCYP26A1 (Hollemann et al., 1998), mediating RA degradation, and XRALDH2 (Chen et al., 2001), responsible for RA synthesis. Both genes are expressed during gastrulation in a complementary, nonoverlapping pattern. XRALDH2 mRNA is strongly expressed in the internal involuting mesoderm, while XCYP26A1 is predominantly expressed in the prospective anterior neural plate (Fig. 4B). Thus, the internal involuting mesoderm may serve as an RA signaling center for the adjacent endoderm that will give rise to liver and pancreas precursor cells. In an attempt to manipulate the level of RA signaling during gastrulation, we performed mRNA microinjection experiments with either XRALDH2 or XCYP26A1. Expansion of the RA signaling center by overexpression of XRALDH2 resulted in a phenotype comparable to the one observed upon exogenous application of RA, as evident from inhibition of *XHex* and expansion of insulin expression (Fig. 4C). Conversely, suppression of endogenous RA signaling by ectopic expression of XCYP26A1 combined with a knockdown of RALDH2 resulted in effects comparable to those obtained with BMS453, that is, inhibition of insulin expression (Fig. 4C).

RA signaling positively regulates the expression of early pancreatic precursor cell transcription factors

To further explore the effects of RA signaling, we analyzed the expression of transcription factors that are specifically expressed in pancreatic precursor cells before the differentiation of endo- and exocrine lineages. *Pdx1* (referred to as *XlHbox8* in *Xenopus*) and *Hlx*b9 (*xHB9* in *Xenopus*) are early and general markers of pancreatic precursor cells (Edlund, 2002). In tailbud-/tadpole-stage *Xenopus* embryos, these genes are uniformly expressed in both dorsal and ventral pancreas (Saha et al., 1997; Wright et al., 1989). Both are upregulated upon application of excessive RA, with a pronounced expansion of the dorsal pancreas along the anteroposterior axis and a less severe but significant increase of expression in the ventral pancreas. Conversely, inhibiting RA signaling with BMS453 results in complete repression in the dorsal pancreas and a partial inhibition ventrally (Fig. 5).

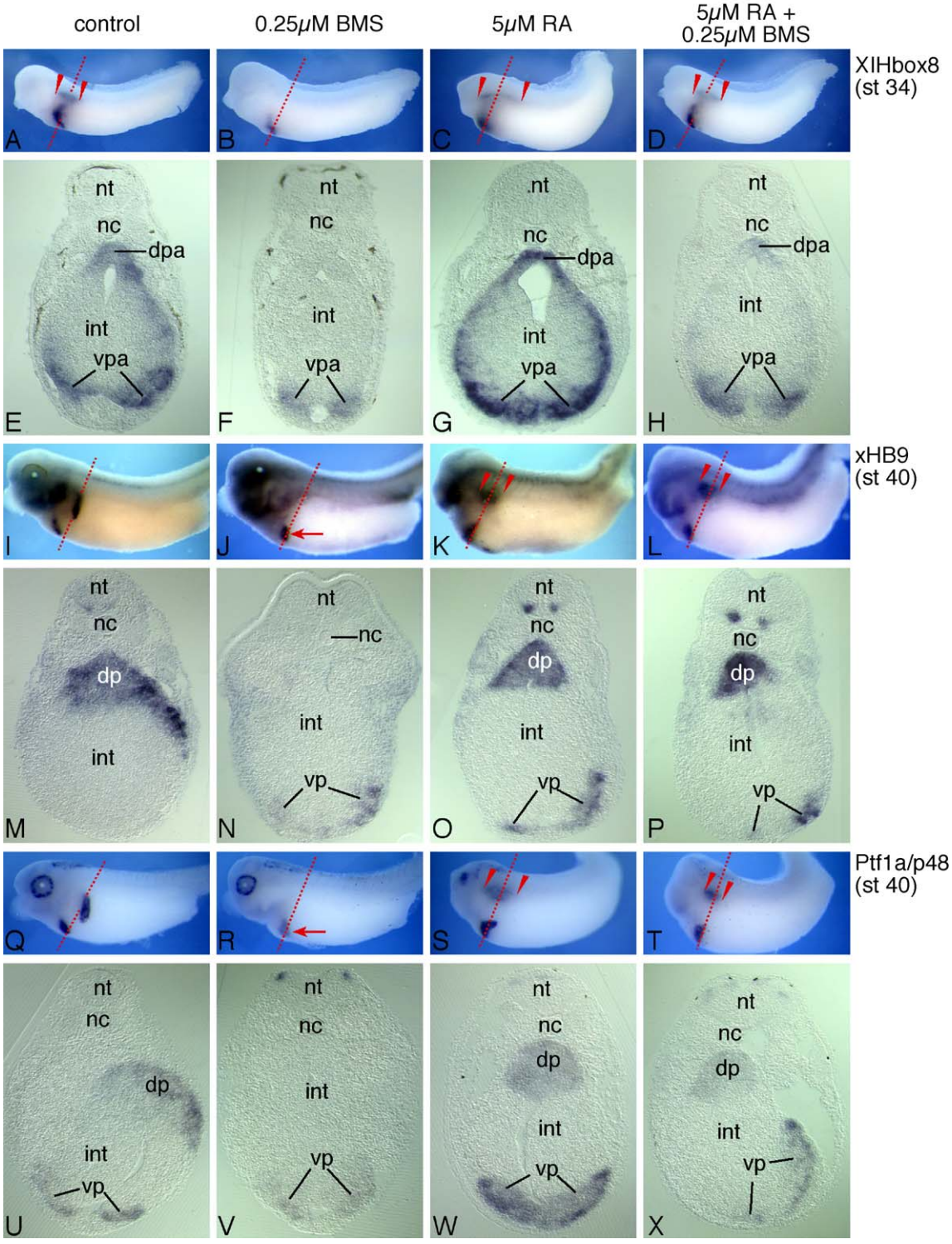
As a third marker, we have used p48, which is specific for pancreatic precursor cells during early embryogenesis and becomes restricted to the exocrine pancreas during later stages of pancreas development in the mouse (Krapp et al., 1998). In *Xenopus*, p48 is expressed in a similar pattern (S.A., Y.C., T.P., unpublished observations and Figs. 5Q and U). Its response to modulating RA signaling is similar to the effects observed for the other two pancreas precursor cell markers *XlHbox8* and *xHB9*. Dorsal expression is expanded upon upregulation of RA signaling and inhibited upon blocking RA signaling (Fig. 5, panels R–T and V–X). Interestingly, in respect to the dorsal pancreas, this situation is in contrast to the RA response of exocrine differentiation genes like *XPD*lp or trypsinogen, which we find to be negatively regulated by RA signaling in the dorsal pancreas (see above), indicating that the RA-dependent expression of p48 reflects its function in pancreatic precursor cells rather than its activity in the context of exocrine differentiation. Thus, RA expands and BMS453 reduces the pancreatic precursor cell population in *Xenopus* embryos.

Inhibition of RA signaling results in ectopic expression of Shh in the dorsal pancreatic anlage

Shh expression is specifically excluded from the dorsal and ventral pancreatic buds in vertebrate embryos, and

Fig. 4. Gastrula stage embryos require RA signaling for pancreas specification. (A) RA signaling before the end of gastrulation is essential for pancreas specification. Embryos were treated with increasing amounts of BMS453 (as indicated) for 30 min at different developmental stages (as indicated) and collected at stage 34. Insulin-expressing cells were counted (5 embryos per treatment) by dissecting the embryos after whole-mount in situ hybridization; the corresponding numbers and standard deviations are displayed in the graph, the P value is less than 0.005 (Student t test). The dashed line indicates the average number of insulin-expressing cells in control stage 34 embryos (standard deviation: 8%). (B) Expression pattern of XRALDH2 and XCYP26A1 during gastrulation suggests that an endogenous RA concentration gradient is created for the specification of putative dorsal pancreas precursor cells. (Panels 1 and 2) Posterior-dorsal view of stage 11 embryos stained for XRALDH2 and XCYP26A1 expression by whole-mount in situ hybridization. (Panel 3) Diagram demarcating the prospective dorsal pancreatic precursor cells relative to the expression domains of the genes analyzed. (C) Modulation of RA signaling via alteration of XRALDH2 and XCYP26A1 activities affects pancreas and liver development. To increase RA signaling, 2 ng of XRALDH2 mRNA and 50 pg of LacZ mRNA were coinjected into one blastomere of 2-cell stage embryos from the marginal zone and treated with 5 μM retinal at stage 12.5 for 1 h (5 μM retinal alone did not result in significant effects, data not shown). Stage 40 embryos were stained with X-gal before whole-mount in situ hybridization (panels 2, 5, and serial sections in panel 8; number of insulin-expressing cells: 80 ± 8, $n = 32$; $P < 0.005$). To decrease RA signaling, 2 ng of XCYP26A1 mRNA and 5 pmol XRALDH2 morpholino antisense oligos were coinjected at the one-cell stage from the animal pole and collected at stage 40. Whole-mount in situ hybridization was simultaneously done with *XHex* and insulin probes (panels 3 and 6; number of insulin-expressing cells, 6 ± 3, $n = 28$; $P < 0.005$). Control embryos stained for *XHex* and insulin expression are shown in panels 1, 4, and 7 (number of insulin-expressing cells, 60 ± 5). in indicates insulin; int, intestine; nc, notochord; nt, neural tube; ppp, putative dorsal pancreatic precursor cells; and xh, *XHex*.

overall inhibition of Hedgehog signaling results in heterotopic pancreas development; conversely, elevated levels of Shh have been found to impair pancreas formation (Apelqvist et al., 1997; Hebrok et al., 1998). We therefore tested if Shh expression is affected in RA- or BMS453-treated *Xenopus* embryos. In control tailbud stage embryos, Shh is expressed in the brain, notochord, and floor plate, as well as in the anteriormost portion of the endoderm that lines the oral and pharyngeal cavities, while there is no detectable signal in the dorsal pancreatic anlage (Ekker et al., 1995 and Fig. 6). Upon BMS453 treatment, Shh expression expands posteriorly into the territory of the dorsal endoderm at tailbud/tadpole stages of development. In RA-treated embryos, Shh expression in oral cavity and forebrain is



reduced, probably due to the typical RA-induced loss of the corresponding anterior structures. Taken together, the data obtained reveal that inhibition of RA signaling by BMS453 treatment during gastrulation leads to ectopic expression of Shh in the presumptive dorsal pancreas region of tailbud stage embryos.

In vitro conversion of presumptive ectodermal cells into a pancreatic fate by a combination of RA and VegT/β-catenin

Finally, we asked whether the endogenous RA signaling function in pancreas specification could be mimicked in an in vitro system with pluripotent embryonic precursor cells. Our previous studies had demonstrated that a combination of VegT and β-catenin can convert prospective ectodermal cells to a liver fate (Chen et al., 2003). As revealed by RT–PCR analysis, application of RA to VegT/β-catenin-injected animal caps at the equivalent of stage 11 does promote expression of various pancreatic marker genes, including *XlHbox8*, insulin, trypsinogen, and *XPD*Ip (Fig. 7A). Expression of liver and intestine marker genes in VegT/β-catenin-injected caps is not altered by RA application, indicating that different types of cells coexist in the explants. Interestingly, coinjection of noggin leads to a significant increase of pancreas gene expression, as revealed by both RT–PCR and whole-mount in situ hybridization (Figs. 7A and B). At the same time, coinjection of noggin also results in the reduction of liver gene expression (Fig. 7A). This latter finding correlates with the observation from murine foregut endoderm explants treated with noggin (Rossi et al., 2001). Thus, these experiments provide a first hint on that it may be possible to direct pluripotent embryonic precursor cells from *Xenopus* to develop into pancreatic cells by reconstructing the embryonic pathway from primitive endodermal cells to differentiated endocrine and exocrine cells.

Discussion

In the present study, we have characterized the effects of either increasing or decreasing RA signaling on pancreas and liver development in *Xenopus* embryos. Inhibition of RA signaling during gastrula stages results in a total loss of dorsal pancreas development, while the ventral pancreas is only moderately affected. The observed loss of the dorsal pancreatic rudiment correlates with a marked expansion of the Shh expression domain into the dorsal endoderm. In contrast, application of excessive RA during gastrulation expands the endocrine at the expense of the exocrine cell population in the dorsal pancreas via inhibition of Notch signaling activities. Conversely, excessive RA stimulates exocrine pancreatic gene expression in the ventral rudiment and simultaneously inhibits liver development. The RA-induced effects are partially recapitulated in pluripotent embryonic precursor cells programmed to develop into primitive dorsal endoderm by a combination of VegT and β-catenin.

RA-mediated patterning of the endoderm during gastrulation

The data presented in this communication suggest that RA plays an important role for endoderm patterning during gastrulation of *Xenopus* embryos. Formally, however, we cannot exclude the possibility that the effects observed reflect an indirect mechanism, in which RA exerts its regulatory activity on the patterning of the mesoderm, which in turn would play a critical role for the patterning of the endoderm. A prepattern of the endoderm has been proposed to exist already at gastrula stages since, for example, dorsal vegetal explants, but not ventral ones, express *XlHbox8* in a cell-autonomous manner (Gamer and Wright, 1995; Henry et al., 1996). The patterning activities of RA that we have described here, as well as the presence of a RA signaling center (RALDH2-positive cells) in close contact with the prospective pancreas precursor cells, provide good support for the idea that RA is critically involved in establishing a prepattern in gastrula stage endoderm. RALDH2 expression during gastrulation is confined to the involuting mesoderm. It has been suggested that the dorsal pancreatic rudiment may be derived from the suprablastoporal endoderm, while the two ventral pancreatic rudiments may originate from the large yolky cells of the subblastoporal endoderm of stage 10.5 *Xenopus* gastrulae (Chalmers and Slack, 2000; Kelly and Melton, 2000). Thus, the close superposition of RA-producing cells is in line with a critical role of RA as a signaling molecule for the pancreatic precursor cells. The

Fig. 5. Excessive RA signaling expands the expression domain of pancreatic precursor cell markers. Stage 11 albino embryos were treated with 0.25 μM BMS453 and 5 μM RA, either alone or in combination, for 30 min and collected at stages 34 and 40 for whole-mount in situ hybridization analysis. (Panels A–D) *XlHbox8* expression in stage 34 control and manipulated embryos. BMS453 treatment completely blocked *XlHbox8* expression in dorsal pancreas anlage and duodenum and partially inhibited the expression in the ventral pancreas anlage (100%, $n = 106$). RA treatment dramatically increased the pancreatic *XlHbox8* expression domain (as demarcated by red arrowheads; 96%, $n = 112$). (Panels E–H) Transversal sections corresponding to the embryos (red dashed lines) shown in panels A–D. (Panels I–L and Q–T) Expression of *xHB9* and *Ptf1a/p48* in stage 40 control and manipulated embryos. BMS453 treatment completely abolished the expression of both genes in dorsal pancreas (panels J, N, R, and V); ventral expression was only partially reduced (red arrows in panels J and R; *xHB9*: 100%, $n = 41$; *Ptf1a/p48*: 100%, $n = 34$). RA treatment positively regulated *xHB9* and *Ptf1a/p48* expression in both dorsal and ventral pancreas (panels K, O, S, and W). The dorsal pancreatic expression domain is expanded (red arrowheads in panels K and S; *xHB9*: 86%, $n = 41$; *Ptf1a/p48*: 82%, $n = 34$). (Panels M–P and U–X) Transversal sections corresponding to the red dashed lines shown in panels I–L and Q–T. The opposing effects of BMS453 and RA were partially neutralized when both chemicals were applied simultaneously (panels D, H, L, P, T, and X). dp indicates dorsal pancreas; dpa, dorsal pancreas anlage; int, intestine; nc, notochord; nt, neural tube; vp, ventral pancreas; and vpa, ventral pancreas anlage.

argument has been raised that early endodermal explants do contain mesodermal cells and that mesoderm-free explants isolated during neurula stages fail to express genes like *XlHbox8*, while they do so if cocultivated with mesoderm, suggesting that the regional specification of the endoderm requires mesodermal signaling (Horb and Slack, 2001). We also noticed RA-induced expression of *FoxF1*, a mesodermal marker that is normally expressed throughout the gut mesoderm, in our VegT/β-catenin-programmed ectodermal explants that are positive for pancreas marker genes (data not shown). In another series of endoderm grafting experiments, Zeynali et al. (2000) observed that the anterior endoderm in *Xenopus* is determined late (between stages 25 and 28) under the influence of mesoderm. Nevertheless, and as pointed out by Horb and Slack (2001), these observations do not rule out the possibility that an early labile specification exists and depends on the continuous interaction with the mesoderm or other neigh-

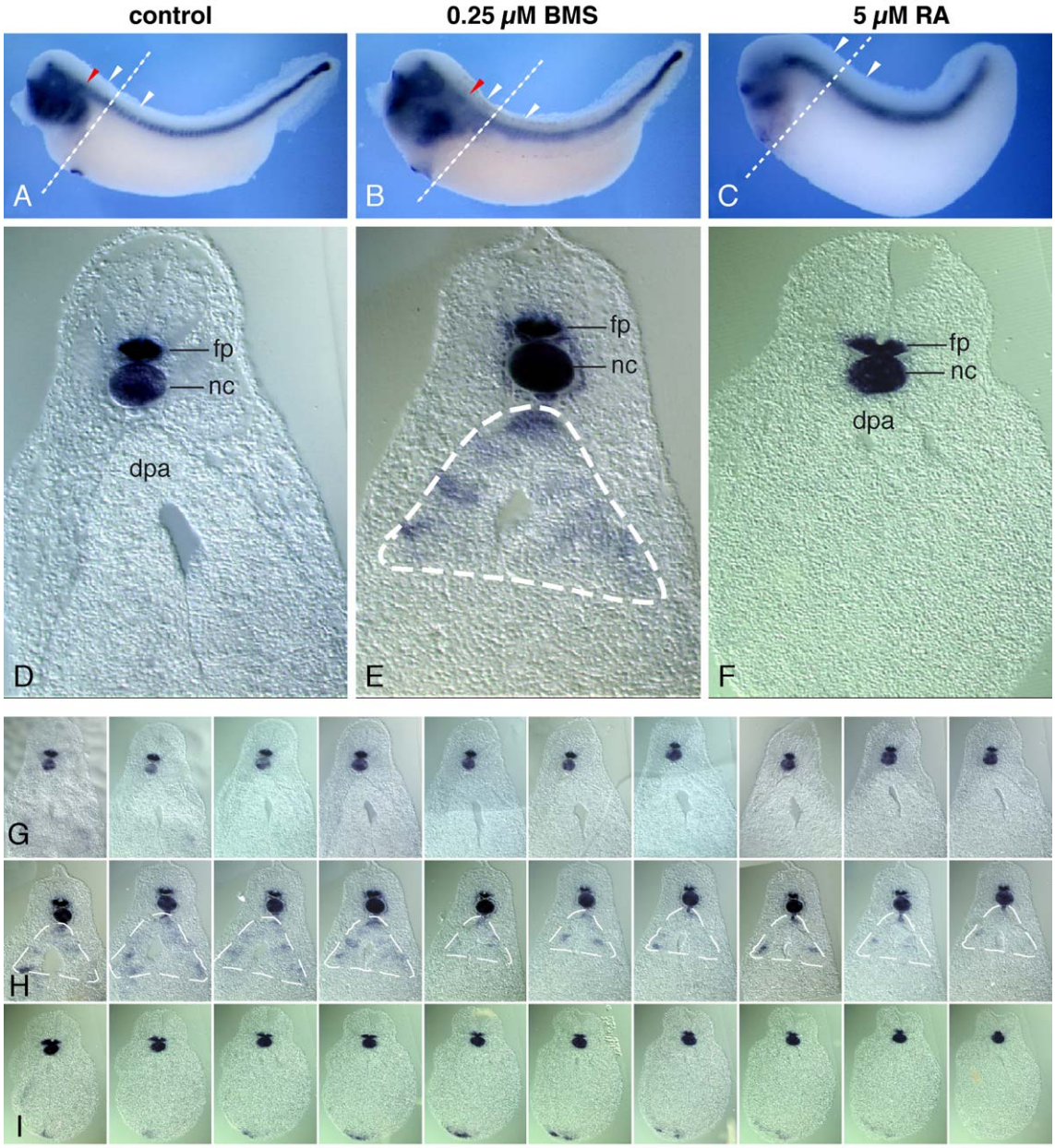


Fig. 6. Inhibition of RA signaling leads to ectopic expression of Shh in the presumptive dorsal pancreas region. Stage 11 albino embryos were treated with either 0.25 μM BMS453 or 5 μM RA for 30 min and collected at stage 36 for whole-mount in situ hybridization analysis. (Panels A–C) Shh expression in stage 36 control and manipulated embryos. (Panels D–F) Transversal sections corresponding to the white dashed lines shown in panels A–C. (Panels G–I) Serial transversal sections span the regions between the two white arrowheads in panels A–C, respectively. Shh is not expressed in the dorsal pancreas anlage of control (panels A, D, and G) and RA-treated embryos (panels C, F, and I, 100%, $n = 62$), but it is expressed in the dorsal endoderm of BMS453-treated embryos (panels B, E, and H, white dashed circles in E and H, 63%, $n = 58$). The red arrowheads in panels A and B indicate the positions of the otic vesicle. dpa indicates dorsal pancreas anlage; fp, floor plate; and nc, notochord.

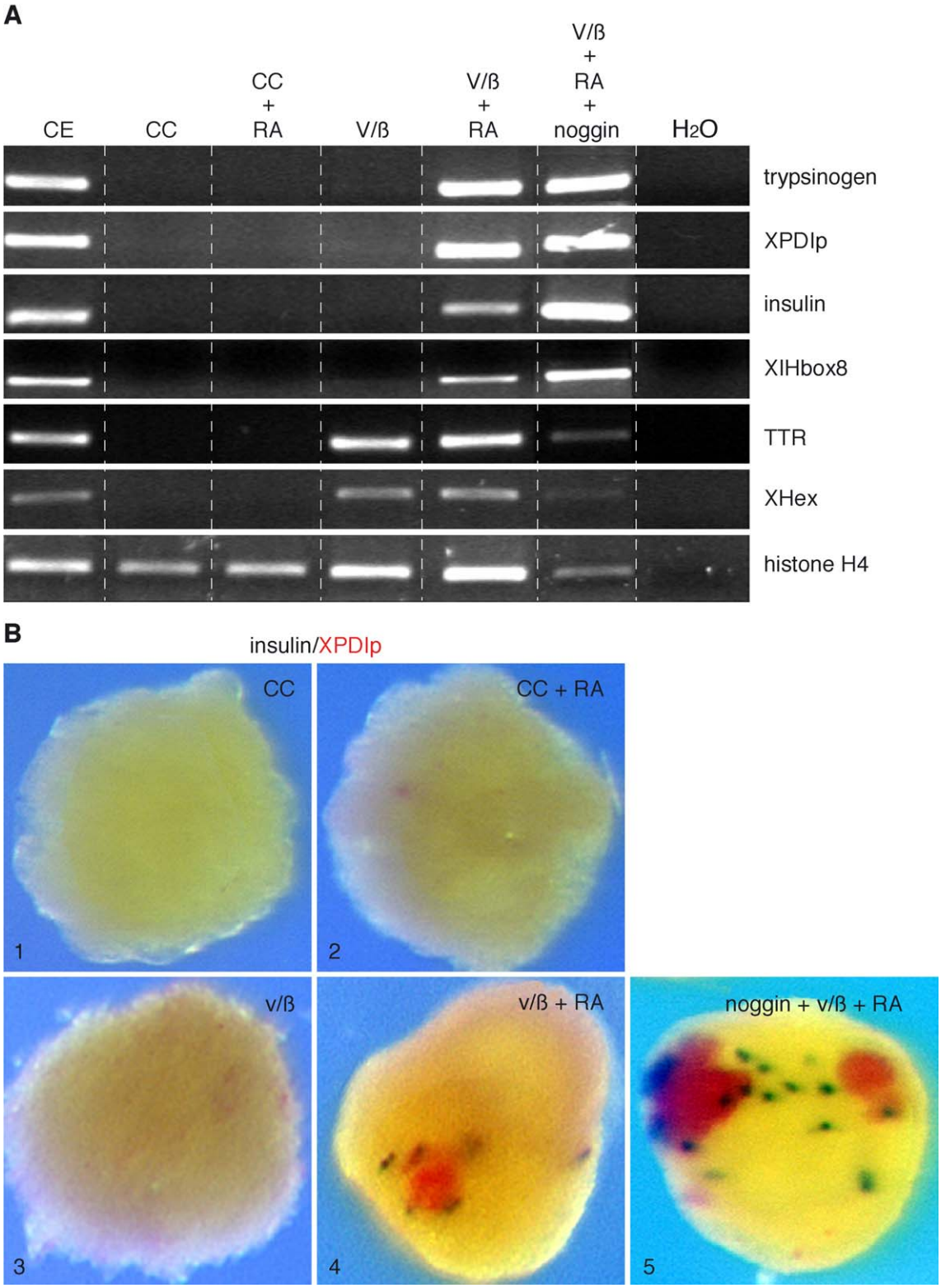


Fig. 7. RA induces pancreas development in VegT/β-catenin-injected pluripotent precursor cells. (A) RT–PCR analysis of liver and pancreas marker gene expression in ectodermal explants (animal caps), which were harvested from stage 9 embryos and cultivated to the equivalent of the embryonic stage 42. (B) Double-staining whole-mount in situ hybridization analysis of insulin (blue) and *XPDI*p (red) expression in stage 42 ectodermal explants treated as indicated. CE, control embryo; CC, control explants (caps); +RA, control or injected caps, were treated with 5 μM RA at the equivalent of stage 11 for 1 h; V/β, VegT/β-catenin-injected caps. The amount of mRNAs injected were 500 pg per embryo of VegT, 200 pg per embryo β-catenin, and 250 pg per embryo noggin.

boring tissues. This latter scenario is in line with the results reported here, with RA serving as such an early patterning activity for the endoderm; the nature of the later acting maintenance signal(s) is not evident, but it (they) is (are) unlikely to be identical to RA itself, since inhibition of RA signaling after gastrulation has no overt effect on pancreas development.

Differential regulation of dorsal versus ventral pancreas development

While RA signaling is required for proper dorsal and ventral pancreas development, excessive RA promotes endocrine at the expense of exocrine development dorsally; conversely, excess RA stimulates exocrine gene expression ventrally. The development of endocrine- and exocrine-specific cells from a group of equipotent precursor cells is known to be regulated by Notch signaling. The Notch-activated bHLH transcriptional repressor *ESR*10 may have a function similar to mammalian *Hes*1 in exocrine differentiation. As *Xenopus ngn3* homologue has not been reported to exist, we employed further downstream gene NeuroD in this study. The loss of exocrine cells upon application of exogenous RA correlates with and is likely to be the result of an inhibition of Notch signaling activity that occurs parallel to an increase in NeuroD expression, which one would expect to be paralleled by enhanced levels of the Notch ligand Delta (not analyzed here). Mechanistically, inhibition of Notch signaling in the presence of increased signal levels may be installed via inhibiting the expression of the Notch receptor, as observed. *ESR*10 is expressed in the ventral pancreas in the absence of NeuroD, and ventral expression of *ESR*10 is not downregulated upon RA application. Thus, ventral expression of this Notch response gene must be regulated via an alternative avenue than in the dorsal pancreas.

Expansion of the Shh-positive endodermal territory upon inhibition of RA signaling as observed dorsally was not observed ventrally. To test for the effect of the ectopic activation of Hedgehog signaling in respect to ventral pancreas development, we have also injected mRNA encoding a constitutively active version of the signal-transducing protein Smo (XSmo-M2, Koebernick et al., 2003), as well as Shh-encoding mRNA. Both treatments resulted in a gut phenotype similar to the one caused by BMS453 treatment. However, pancreatic marker genes were expressed in a pattern similar to control embryos (data not shown). It has previously been reported that misexpression of human SmoM2 in *Xenopus* leads to apancreatic embryos (Zhang et al., 2001). However, in the same study, RT–PCR analysis had already revealed that insulin and *XlHbox8* were expressed in whole digestive tracts isolated from injected embryos. Thus, taken together, these studies seem to suggest that increased Hedgehog signaling activity alone is not sufficient to suppress dorsal or ventral pancreas specification in *Xenopus*. It can, however, not be excluded that the relative or local increase in signaling activity during the relevant stages of embryogenesis achieved in these mRNA injection experiments is insufficient.

RA-mediated effects on endodermal patterning in other vertebrate species

Two recent studies published while the work presented here was already in progress support the idea of a regulatory function for RA signaling in the context of early endodermal patterning. Kumar et al. (2003) have used explants from chicken and quail embryos to demonstrate that lateral plate mesoderm exerts an instructive signaling activity on the adjacent endoderm in respect to the expression of various pancreas marker genes. RA signaling was found to be sufficient to induce *Pdx1* expression in anterior explants that contained both endoderm as well as mesoderm and would normally not develop into pancreatic tissue; RA was not sufficient to induce endodermal *Pdx1* expression in the absence of mesoderm. Thus, RA may work indirectly via the mesoderm rather than directly on the endoderm or it may act directly in concert with mesodermally derived signals. Most relevant for the studies with *Xenopus* embryos presented here, Stafford and Prince (2002) have analyzed the function of RA signaling for pancreas development in the zebrafish. While some of the effects observed in the fish equal those obtained in the frog system, others do not. In both systems, inhibition of RA signaling during gastrulation results in a dramatic reduction of pancreas gene expression. However, application of excess RA resulted in an expansion of both exo- and endocrine cells in the fish, while it resulted in an expansion of the endocrine cell population at the expense of the exocrine cells in the dorsal pancreas of the frog. Furthermore, while inhibition of RA signaling correlated with a loss of liver in the fish, liver development in the frog was unaffected in the same situation. Effects of RA treatment on Notch or Hedgehog signaling were not described by Stafford and Prince (2002). In addition, it had also been reported that, in stark contrast to the situation in mouse and frog, Shh exerts a positive regulatory activity on pancreas development in the fish (diIorio et al., 2002; Roy et al., 2001). The pancreas develops from two separate domains in both *Xenopus* and zebrafish; we note, however, that their response to a modulation of RA signaling is indistinguishable in the fish, while dorsal and ventral pancreas in the frog differ significantly in several aspects of pancreas gene expression (as discussed above). It has been pointed out (Biemar et al., 2001) that the pancreatic primordia in the zebrafish embryo are not truly equivalent to the pancreatic buds, as they develop in other vertebrates including *Xenopus*. Gastrulation in zebrafish results in the formation of a sheet of endodermal cells rather than a proper endodermal tube.

Therefore, the discrepancies of results obtained in a comparison of both systems in respect to pancreas and liver development may, at least in part, reflect the differences in respect to gut tube formation in fish and frog embryos.

Programming pluripotent embryonic precursor cells towards a pancreatic fate

It seems of considerable general interest to establish experimental protocols that allow to generate specific pancreatic cells from pluripotent embryonic precursor cells in vitro. Moriya et al. (2000a,b) have used either dorsal blastopore lip explants or activin-treated animal cap explants to demonstrate that retinoic acid can promote the formation of structures that have a pancreatic morphology and which express *XlHbox8* as well as insulin. We have previously described a related approach that tries to recapitulate the early embryonic events of endoderm formation, also making use of the animal cap system; cultivation of ectodermal explants coinjected with VegT and β-catenin, as a means to generate primitive dorsal endoderm, results in the induction of the liver-specific, but not the pancreas-specific, gene program (Chen et al., 2003). In extension of these earlier studies, we now report on the formation of clusters of exocrine and endocrine pancreatic cells in VegT/β-catenin-injected animal cap explants upon treatment with retinoic acid. Application of noggin to the system further enhances both endocrine (insulin) and exocrine (*XPD*lp) gene expression. In contrast, in an in vitro culture system of dissociated pancreatic cells from the E15.5 mouse fetus, BMPs promoted formation of cystic epithelial colonies containing isletlike structures (Jiang et al., 2002). Based on mutant analysis, it has been shown that BMP signaling has a positive role in zebrafish pancreas specification and development (Tiso et al., 2002). BMPs can also induce *Pdx1* expression in recombinants of endoderm and mesoderm tissues from early chicken embryos (Kumar et al., 2003). The discrepancy between our data and previous studies might be due to the different experimental context in the different systems employed. The function of endogenous BMP signaling in *Xenopus* embryos for pancreas development remains to be investigated.

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Chapter II: Retinoic acid-mediated patterning of dorsal pre-pancreatic endoderm during gastrulation in *Xenopus*

Fong Cheng Pan, Yonglong Chen, Elke Bayha, Anna Grapin-Botton and Tomas Pieler, **Retinoic acid patterning of dorsal pre-pancreatic endoderm during gastrulation involves interaction with BMP signalling,** *to be submitted*

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Retinoic acid patterning of dorsal pre-pancreatic endoderm during gastrulation involves interaction with BMP signalling

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Summary

Early patterning of the endoderm as a prerequisite for pancreas specification involves retinoic acid (RA) as a critical signalling molecule in gastrula stage *Xenopus* embryos. In extension of our previous studies, we made systematic use of early embryonic endodermal and mesodermal explants. We find RA to be sufficient to induce pancreas specification in dorsal but not ventral endoderm. The differential expression of RARs during gastrulation in ventral versus dorsal explants is important for their distinct responsiveness in respect to induction of pancreatic gene expression. Furthermore, BMP signalling, that is overcome dorsally by specific inhibitors such as noggin, prevents the formation of pancreatic precursor cells in the ventral endoderm of gastrula stage *Xenopus* embryos. An additional requirement for mesoderm suggests the production of one or more further pancreas inducing signals by this tissue. Finally, recombination of manipulated early embryonic explants, but also inhibition of RA activity in whole embryos, lead us to conclude that RA signalling as it is relevant for pancreas development operates simultaneously on both, mesodermal and endodermal germ layers.

Introduction

As one of the three primary germ layers, the endoderm will give rise to the epithelial lining of the gastrointestinal tract, which in turn will be the origin for a number of different organs, including the pancreas. In *Xenopus*, the endoderm derives from the cells positioned at the vegetal pole of the embryo (Dale & Slack, 1987). VegT, a vegetally localising maternal transcription factor, serves a key regulatory function in the expression of several downstream endodermal signalling molecules (Xnrs, derriere) and transcription factors (Mix-type homeobox transcription factors, GATA4/5/6, and XSox17). Interaction of these factors in late blastula stage embryos commits the vegetal cells to an endodermal fate (reviewed in Fukuda & Kikuchi, 2005).

Endoderm patterning and pancreas development in chordates define an area of intense research activities. Several signalling pathways have been reported to be involved in these events, and the importance of mesenchymal-epithelial interactions has been revealed. Studies in *Xenopus* using embryonic explants have suggested an important role for TGF-β (activin and Vg1) and FGF signalling in the patterning of the AP axis of the endoderm. Blocking signalling activities using the appropriate dominant negative receptors abolished expression of the anterior endodermal marker *XlHbox8*, while a posterior endodermal marker, IFABP, was not affected (Gamer & Wright, 1995; Henry et al., 1996). Zorn and colleagues (1999) have further shown that the anterior endomesoderm, as defined by the expression of Xhex and cerberus, is specified by a combined action of Wnt/β-catenin and TGF-β signalling.

All these studies were based on the use of vegetal explants isolated from late blastula stage embryos. A more recent investigation performed by Horb and Slack (2001) suggests that the presence of a small number of mesodermal precursor cells in such early vegetal explants might have been overlooked. Endoderm that is completely devoid of mesoderm, as isolated from neurula and tailbud stage embryos, was not found to express any regional marker and it becomes regionally specified only in the presence of adjacent mesodermal tissue.

In the mouse, endoderm receives its initial instructive patterning information from the adjacent mesoderm just after gastrulation. FGF4 can induce the differentiation of endoderm in a concentration-dependent manner and has recently been shown to be required for establishing gut domains along the A-P axis in a paracrine manner (Wells and Melton, 2000; Dessimoz et al., 2005). Later in the development, at somite stage, juxtaposition of endoderm and notochord is an important event for dorsal pancreas specification. FGF2 and activin, which are believed to be delivered by the notochord, suppress Sonic hedgehog (Shh) expression in the dorsal pancreatic endoderm (reviewed in Hebrok, 2003), thus allowing for pancreas development. Additional signalling activities of unknown identity have been attributed to the vascular endothelium (Lammert et al., 2001). In the ventral foregut endoderm, signals from the cardiac mesoderm (FGF2) and septum transversum (BMP), are required to specify a liver fate with bipotential endodermal cells, which would otherwise take the default route to pancreas (reviewed in Zaret, 2002).

Using chick-quail chimeras and in vitro tissue recombination approaches, Kumar and colleagues (2003) have shown that diverse signals, such as BMP, RA or activin, are able to instruct the anterior endoderm to adopt a pancreatic fate, but only in the presence of mesoderm. By use of *swirl* ($BMP2^{-/-}$) and *chordino* ($chordin^{-/-}$) zebrafish mutants, Tiso and colleagues (2002) found that BMP signalling is involved in regulating the A-P pattern of the zebrafish endoderm; anterior endoderm is expanded and the pancreatic domain is reduced in *swirl* mutant embryos and the phenotype is just the opposite for *chordino* mutant embryos.

More recently, we and others were able to define a conserved, essential role for RA signalling in specification of the dorsal pancreas in zebrafish (Stafford & Prince, 2002), *Xenopus* (Chen et al., 2004; Stafford et al., 2004) and mouse (Martin et al., 2005; Motlokov et al., 2005). The rate limiting step in the biosynthesis of RA is carried out by a retinaldehyde dehydrogenase (RALDH); RALDH2 is expressed in the mesoderm during gastrulation, i.e. during the period of development when RA signalling is critically required for pancreas

development in fish and frogs (Stafford and Prince, 2002; Chen et al., 2004). A most recent study by Stafford et al. (2006) was devoted to the question if RA acts directly on endodermal cells to induce a ß-cell fate in the zebrafish; using an elegant cell transplantation approach it was concluded that RA functions as an instructive signal produced in the mesoderm and directly inducing endocrine pancreatic precursors in the endoderm.

In extension of our previous studies on the patterning of the early *Xenopus* endoderm, we now exploit a specific advantage of the *Xenopus* system, as it is defined by the ease of manipulation and cultivation of early embryonic explants. We conclude that RA is sufficient to induce pancreas specification in dorsal but not ventral endoderm, that the differential expression of RARs during gastrulation in both mesoderm and endoderm plays an important role the earliest stages of pancreas specification, and that BMP signalling and absence of RA seem to inhibit the formation of pancreatic precursor cells in the ventral endoderm of gastrula stage *Xenopus* embryos: An additional requirement for mesoderm suggests the production of one or more additional pancreas inducing signals by the mesodermal cells. Furthermore, recombination of early embryonic explants, but also whole embryo manipulation of RA signalling, lead us to conclude that RA signalling, as it is relevant for pancreas development, operates simultaneously on both, mesodermal and endodermal germ layers.

METHODS AND MATERIALS

Embryo cultivation and microinjections

Pigmented or albino *Xenopus laevis* embryos were obtained using standard protocol previously described in Pan et al., (2006) and staged according to Nieuwkoop and Faber (1967). Synthetic capped mRNA for microinjection was in vitro transcribed using mMessage-mMachine^{TM} Ambion kit and purified over an RNeasy column (Qiagen, Germany). Capped mRNA encoding the following genes were injected at the final concentrations as indicated: Noggin (500 pg, Smith et al., 1993); xRARα2 coding region (500 pg, Sharpe & Goldstone, 1997), pCDG-xRARγ2.1 (500 pg, Blumberg et al., 1992); xRXRβ (500 pg, Sharpe & Goldstone, 2000); DNRARα2 (1 ng, Sharpe & Goldstone, 1997).

Animal cap explants, endodermal and mesodermal explants isolation and cultivation

For animal cap assays, two-cell stage embryos were injected bilaterally with appropriate mRNA and animal cap explants were dissected from stage 9 embryos using a gastromaster. The endodermal and mesodermal explants were isolated from stage 11 embryos using forceps. Endodermal explants were isolated by removing the ectoderm and the mesoderm, followed by bisecting in the middle, perpendicular to the dorsal lip to generate dorsal endoderm (DE) and ventral endoderm (VE). Mesodermal explants were prepared by first removing the animal cap followed by careful separation from the adjacent endoderm. For endodermal explants cultured in the presence of mesoderm, whole endoderm plus mesoderm explants were prepared by removing the animal half of mid gastrula embryos; the explants were then bisected perpendicular to the dorsal lip to generate dorsal endoderm plus dorsal mesoderm (DEM) and ventral endoderm plus ventral mesoderm (VEM). All explants were cultured in 1x MBS (17.6 mM NaCl, 480 µM $NaHCO_3$, 200 µM KCl, 2 mM Hepes, 160 µM Mg_2SO_4, 80 µM $CaCl_2$, 60 µM $Ca(NO_3)_2$, pH 7.4). Explants were staged according to the control siblings.

Chemical treatment of embryos and explants

RA (all-trans-RA, Sigma) and BMS453 (a gift from Bristol Myers Squibb) were prepared as 10 mM stock solution and diluted as described previously (Chen et al., 2004), unless otherwise indicated. Embryos and explants were treated with either RA or BMS at stage 10 for 1 hour in 0.1x MBS and 1x MBS, respectively. The chemicals were washed away intensively after treatment using the same buffer.

RT-PCR analysis

Total RNA from whole embryos, animal caps, endodermal and mesodermal explants was isolated using RNeasy kits (Qiagen, Germany) followed by DNaseI treatment to remove genomic DNA. All RNA samples were controlled by 35 cycles of a histone H4-specific PCR reaction for DNA contamination. All the experiments involving RT-PCR analysis were repeated at least two times with ordinary RT-PCR analysis using a protocol described previously (Chen et al., 2004) and performed at least once by use of real time RT-PCR analysis. The results obtained with real time RT-PCR analyses were found to correspond to those obtained with the ordinary RT-PCR analysis. The real time RT-PCR protocol is indicated as follow: cDNA was generated from 500 ng total RNA in 100 µl total volume, 5 µl cDNA was used for real time PCR with iQ Syber Green Supermix (25 µl in total volume) in the iCycler system (BioRad). All samples were normalised to levels of ornithine decarboxylase (ODC), which was used as the loading control. The mRNA concentration was measured by using a standard curve for each analysed gene. All measurements were done in duplicates, and the values in the figures represent the mean value of the corresponding experiment. The sequences and cycles number for primers used in the real time PCR and normal PCR are listed below:

Xenopus histone H4 (25 cycles, Niehrs et al., 1994), forward: 5'-CGGGATAACATTCAGGGTATCACT-3' and reverse: 5'-ATCCATGGCGGTAACTGTCTTCCT-3'; *XPDl*p (30 cycles, Afelik et al., 2004), forward: 5'-GGAGGAAAGAGGGACCAA-3' and reverse: 5'-

GCGCCAGGGCAAAAGTG-3'; *XlHbox8* (32 cycles, Chen et al., 2004), forward: 5'-AATCCACCAAATCCCACACCT-3' and reverse: 5'-GCCTCAGCGACCCAATAGAA-3'; insulin (32 cycles, Henry et al., 1996), forward: 5'-ATGGCTCTATGGATGCAGTG-3' and reverse 5'-AGAGAACATGTGCTGTGGCA-3'; PTF1a-p48 (30 cycles, Afelik et al., 2006), forward: 5'-GAGAAGCGACTGTCCAAG-3' and reverse: 5'-CATCAGTCCATGAGAGAG-3'; transthyretin (32 cycles, Chen et al., 2004), forward: 5'-GGAATCCCCGCTGCCAATC-3' and reverse: 5'-ATGAGAAGGAGTAGGGGGTGA-3'; xtwist (30 cycles,), forward: 5'-AGAACCTGGAGCTGGATC-3' and reverse: 5'-GGCTTGAAAGGCACGACT-3'; RALDH2 (32 cycles, Chen et al., 2001), forward: 5'-CCTGATGGCTTCTCTGCAGCTC-3' and reverse: 5'-GATAGCACCTTGGAGATCCACA-3'; xRARα2.1 5'UTR (30 cycles), forward: 5'-GAAGGAGTCTCCCATGTG-3' and reverse: 5'-GTCCGTAGTGGGGTCCCG-3'; xRARα2.2 5'UTR (30 cycles), forward: 5'-GCGGATGTGTCTGTACAG-3' and reverse: 5'-GTGTTGAGTCCCAAGTGG-3'; xRARγ2.1 (27 cycles), forward: 5'-CCAGAGCCCACACTGCTG-3' and reverse: 5'-ACCATTTCCTCCGAGCTG-3'; xRARγ2." (27 cycles), forward: 5'-GCTATCAGAGCCCACACTG-3' and reverse: 5'-ACCATTTCCTCCGAGCTG-3'; xRXRα (30 cycles), forward: 5'-GAGCTGGCAGGCGTAGGA-3' and reverse: 5'-CAGGAGTCGGTGCAATC-3'; xRXRβ (30 cycles), forward: 5'-CCCAGGGGATGTGGAAG-3' and reverse: 5'-GGGTGTAGCAGCAGTCTG-3'; Ornithine decarboxylase (ODC, 25 cycles), forward: 5'-GCCATTGTGAAGACTCTCTCCATTC-3' and reverse: 5'-TTCGGGTGATTCCTTGCCAC-3'; xBra (30 cycles) forward: 5'-GGATCGTTATCACCTCTG-3' and reverse: 5'-GTGTAGTCTGTAGCAGCATGCTGCTAC-3'; Mixer (25 cycles), forward: 5'-CACCAGCCCAGCATCTAACC-3' and reverse: 5'-CAATCTCACATCAACTGAAG-3'

Whole mount in situ hybridisation

Whole-mount in situ hybridization on whole embryo and animal caps was done in principle as described (Harland, 1991)with modifications as reported in

Hollemann et al. (1999). The probes were prepared as described (Chen et al., 2004)

Vibratome sectioning

Vibratome sections (30 µm) were prepared as described previously using the Leica VT1000S vibratome (Hollemann et al., 1999).

Results

RA is sufficient to induce pancreas specific gene expression in dorsal endodermal, but not in ventral endodermal explants

Our previous studies had revealed that RA signalling is required for pancreas specification in the dorsal endoderm of gastrula stage *Xenopus* embryos, with the RALDH2 expressing adjacent mesodermal tissue functioning as the corresponding signalling centre (Chen et al., 2004). In further pursuit of these observations we addressed the question if, firstly, RA is sufficient for pancreas specification of the dorsal endoderm, and if, secondly, it is also capable of inducing pancreas specific gene expression in the ventral endoderm of gastrula stage embryos.

For this purpose, different types of explants were dissected from stage 11 *Xenopus* embryos, containing dorsal or ventral endoderm (DE or VE), dorsal or ventral mesoderm (DM or VM), or different combinations hereof (DEM, VEM, DE/VM, VE/DM); RA treatment was performed transiently for one hour after isolation. Explants were cultivated up to the equivalent of stage 39, i.e. when endocrine as well as exocrine pancreatic differentiation markers start to be expressed in the corresponding control embryos.

As depicted in Figure 1, DE alone does not activate pancreatic gene expression if cultivated in the absence of RA, while low but significant levels of such activities are detected upon treatment with RA. Consistent with the results reported by Asashima and colleagues (Moriya et al., 2000a), dorsal mesoderm,

encompassing the dorsal lip structure, can also be induced by RA to form pancreatic structures. Co-explantation of dorsal endoderm/mesoderm (DEM) results in the induction of pancreatic gene expression even in the absence of exogenous RA, probably due to the endogenous RA, as produced by the DM and signalling to the adjacent DE. However, the existence of additional signalling activities mediating the communication between dorsal mesoderm and endoderm cannot be excluded at this point. The liver specific gene transthyretin was also activated under these conditions. Treatment of the DEM with exogenous RA results in significantly enhanced levels of all pancreatic marker genes tested.

Conversely, ventral endodermal and mesodermal explants (VE, VM and VEM) fail to activate pancreatic gene expression, neither in the presence or in the absence of RA. Interestingly, a combination of ventral endoderm with dorsal mesoderm is found to result in robust levels of pancreatic gene expression that can be enhanced by addition of exogenous RA. The reciprocal combination, DE plus VM gave similar results to those observed with DE alone. These findings strongly suggest that one or more signals in addition to RA, which by itself cannot induce pancreas specific gene expression in the ventral endoderm, may be involved in the signalling between meso- and endoderm that eventually results in pancreas specification.

Thus, in summary of this first set of experimental observations, we conclude that RA is sufficient to induce pancreas specification in dorsal but not ventral endoderm, which may have been exposed to additional signal(s) originating from the mesoderm prior to or during gastrulation.

Spatially restricted expression of RA-receptors may contribute to the differential competence of dorsal and ventral endoderm to respond to RA

The differential RA responsiveness of dorsal versus ventral endoderm explants, as observed in the experiments described above, might reflect spatially restricted expression of the appropriate RA receptor molecules. Quantitative RT-PCR analysis of a panel of six such RAR- and RXR-type receptors in micro-

dissected gastrula stage embryos reveals a differential distribution for two of these: RARα2.1 is higher in dorsal versus ventral endoderm, and RARγ2.1 is high in dorsal and low in ventral mesoderm (Fig. 2A).

To further explore the role of these two receptors in respect to pancreas specification, we tested if they can confer competence to RA mediated induction of a pancreatic fate when ectopically expressed in endodermal explants isolated from microinjected gastrula stage embryos. XRARα2 alone or in combination with its co-receptor xRXRβ is not able to confer RA induced expression of pancreatic marker genes in VE or VEM isolates (data not shown). Interestingly, ectopic expression of xRARγ2.1, either alone or in combination with XRARα2, results in the induction of pancreas gene activity in response to RA and in the presence of increased levels of the co-receptor xRXRβ in VEM explants (Fig. 2B). In this experimental situation, ectopic RARs will be expressed in both mesodermal and endodermal germ layers, such that indirect effects as generated by RA mediated events in the mesoderm cannot be excluded.

Taken together, these results suggest that the differential expression of RARs during gastrulation in both mesoderm and endoderm contribute to the earliest stages of pancreas specification, as they occur during gastrulation.

Noggin allows the ventral endoderm to adopt a pancreatic fate in response to RA and mesoderm

We have previously reported that animal cap explants, which would normally develop into atypical epidermis, can be programmed for pancreatic gene expression by a combination of VegT, ß-catenin and RA; co-expression of noggin in this system was found to further enhance the level of pancreatic gene activity, while reducing liver-specific gene expression (Chen et al., 2004). The proportion of cells in the animal cap explants that are positive for endo- and exocrine pancreatic marker genes can even be further increased by increasing the dose of RA (Fig. 3C).

In extension of these studies we report here that a combination of VegT, ß-catenin and noggin is sufficient to result in pancreas gene activity, even in the absence of exogenously added RA (Fig. 3A). However, addition of the competitive RA inhibitor BMS453 to the system ablates pancreas gene transcription, suggesting a requirement for endogenous RA (Fig. 3A). Indeed, noggin also promotes a robust increase in the level of the mRNA for the key enzyme in the biosynthesis of RA, RALDH2, in animal cap explants (Fig. 3B), that may be sufficient to provide a critical amount of RA for pancreas specification.

Since levels of BMP signalling in the endoderm of gastrula stage embryos are known to be high ventrally but low dorsally, also due to the secretion of BMP inhibitors like noggin from the dorsal lip (reviewed in De Robertis and Kuroda, 2004), we sought to investigate if ectopic expression of noggin in the ventral endoderm/mesoderm explants (VE and VEM) could confer competence to activate pancreatic marker genes in response to RA. While the VE explant alone remained silent in the presence of a combination of noggin and RA, addition of ventral mesoderm was sufficient to induce low but significant levels of pancreas gene activity in VEM explants (Fig. 3D).

Thus, BMP signalling and absence of RA seem to inhibit the formation of pancreatic precursor cells in the ventral endoderm of gastrula stage *Xenopus* embryos; the additional requirement for mesoderm suggests the production of one or more pancreas inducing signals by the mesodermal cells.

RA responsiveness is required in both mesoderm and endoderm in the context of early embryonic pancreas specification

The requirement for mesoderm to result in pancreas specific gene activity in the different endoderm explant assays described above raises the question if RA signalling molecules are directly received by the endodermal cells, or if they are relayed via the mesoderm, or if a combination of both modes of signalling may exist. In order to address this question, we made use of a dominant negative

variant of RARα2 (Sharpe and Goldstone, 1997), the competitive RA inhibitor BMS453 and two different assay systems.

Firstly, endogenous RA signalling was selectively inhibited in dorsal mesoderm (DM) and dorsal endoderm (DE) explants respectively, before co-cultivation of both specimens and analysis of pancreas specific gene activity (Fig. 4A). Microinjection of mRNA encoding DN-RARα2 into, or BMS 453 incubation of both, DE and DM, results in a significant reduction of pancreatic gene activity in DE+DM explants, with most severe effects obtained for the inhibition of insulin expression. Selective inhibition of RA signalling in either DM or DE alone, prior to co-culture with wild type DE or DM, respectively, similarly reduces pancreas gene transcription, even though to a lesser extent.

Secondly, the DN-RARα2 was preferentially directed to either the mesodermal or endodermal germ layer by means of mRNA injection into four-cell or eight-cell stage, embryos, respectively; whole embryos were cultivated to the equivalent of embryonic stage 36 and 39, and analysed for pancreas and liver specific gene activities (Fig. 4B). It turns out that both selective inhibition of RA signalling in the mesoderm or in the endoderm exerts similar effects; while the development of the liver remains largely unaffected, pancreas development is clearly inhibited. Furthermore, ventral pancreas is severely affected when endodermal RA signalling is blocked.

Thus, results obtained in both the endoderm/mesoderm explants and whole embryo microinjection experiments equivocally argue that RA signalling as it is relevant for pancreas development operates simultaneously in both mesodermal and endodermal germ layers.

Discussion

In the present study we report that RA is sufficient to induce pancreas specification in dorsal but not ventral endoderm; the differential expression of RARs during gastrulation in both mesoderm and endoderm plays an important role in this regionally restricted responsiveness. In addition, BMP signalling together with the absence of RA prevent the formation of pancreatic precursor cells in the ventral endoderm of gastrula stage *Xenopus* embryos; the further requirement for the presence of mesoderm suggests production of one or more additional pancreas inducing signals by the mesodermal cells. Furthermore, results obtained via recombination of early embryonic explants, but also whole embryo manipulation of RA signalling, lead us to conclude that RA signalling as it is relevant for pancreas development operates simultaneously on both, mesodermal and endodermal germ layers (Fig. 5).

Differential responsiveness of dorsal versus ventral endoderm toward RA

Fate mapping experiments have revealed that the pancreas originates from dorsal endoderm (Chalmers and Slack, 2000; Kelly and Melton, 2000). In isolation, we did not find dorsal endoderm (DE) explants, dissected from stage 11 *Xenopus* gastrulae, do not express pancreatic marker genes, including *XlHbox8.* Previous studies reporting on autonomous expression of endodermal markers such as *XlHbox8*, had employed whole or dorsal/ventral vegetal explants from stage 8/9 blastulae (Gamer and Wright, 1995; Henry et al., 1996). A more recent study by Horb and Slack (2001) had arrived at the conclusion that such early explants form some mesodermal cells, suggesting that the endoderm is not specified in the absence of mesoderm. In contrast to culture in isolation, our DE explants adopt a pancreatic fate upon treatment with exogenous RA, or when co-cultured with the adjacent mesoderm, which is likely to serve as a source for endogenous RA. These findings suggest that RA is sufficient to direct the dorsal endoderm toward a pancreatic fate. However, in order to achieve maximal levels of pancreas gene expression, RA and dorsal mesoderm are required to be present at the same time, indicating that additional signalling activity from the mesoderm may also be involved.

Conversely, VE explants were not able to adopt a pancreatic fate, even in the presence of RA and ventral mesoderm. The differential distribution of RARs and their isoforms defines complex patterns during embryogenesis (reviewed in Mark et al., 2006). We found that xRARα2.1 and xRARγ2.1 are predominantly expressed in the dorsal endoderm and dorsal mesoderm, respectively. When these two xRARs are ectopically introduced into VEM explants, pancreatic gene expression can be induced upon application of RA.

RA-mediated patterning of the dorsal prepancreatic endoderm operates both directly and relayed via the mesoderm

In the present study, we used recombination of endodermal and mesodermal explants, as well as modulation of RA signalling in whole embryos, to address the question, if RA acts directly or/and indirectly via the mesoderm to specify the dorsal endoderm towards a pancreatic fate. The data obtained suggest that RA, acts both directly and indirectly. The observed preferential expression of at least two different RARs in the dorsal endoderm (namely xRARα2.1) and in the dorsal mesoderm (namely xRARγ2.1) is in line with this notion.

The importance of the mesoderm in RA mediated pancreas specification has also been revealed in other systems. Using chick explants, Kumar et al. (2003) have shown that RA is able to induce ectopic *Pdx1* expression in anterior endoderm only in the presence of mesoderm. In the mouse, RA-responsive cells were detected in both the mesenchyme and endoderm of the pancreatic anlage in RALDH2 -/- mice upon treatment with RA (Martin et al., 2005 & Motlokov et al., 2005). Conversely, a most recent study by Stafford et al. (2006) using the zebrafish system arrives at a different conclusion. Using cell transplantation techniques, it is demonstrated that RA receptor function is required in the foregut endoderm but not in the mesoderm for the development of insulin-expressing ß-cells; it was not reported if the development of other endocrine and exocrine pancreatic cells has similar signalling requirements. Pancreas development in the zebrafish has also been reported to differ in

comparison with other vertebrate systems in respect to the role of hedgehog mediated signalling (diIorio et al., 2002; Roy et al., 2001).

Interaction of BMP and RA signalling in the context of pancreas specification

Our present findings also support the notion that down-regulation of BMP signalling in conjunction to RA signalling is required for pancreas specification during gastrulation. Ventral endodermal explants were able to adopt a pancreas fate in the presence of RA and mesoderm when BMP signalling is down regulated by noggin. A similar situation was observed in the animal caps assay, in which the pancreatic cell population was enlarged in the presence of noggin and increasing concentrations of RA. Early studies by Gamer and Wright (1995) had shown that vegetal explants, either injected with BMP4 or isolated from UV treated embryos, do not express *XlHbox8*; induction of *XlHbox8* expression in the noggin-injected ventral vegetal explants was not observed. Our study confirms and extends these finding by demonstrating that, in order to induce a pancreatic fate in ventral endoderm, three different elements are required: down-regulation of BMP signalling, RA and the presence of mesoderm.

Sasai and colleagues (1996) have reported that organiser factors, such as chordin and noggin, can induce dorsal endoderm formation independent of mesoderm in the animal caps assay. They also pointed out that these factors are needed to pattern the endoderm during gastrulation, which is in line with data reported here. Moreover, we also demonstrate that noggin can induces *xRALDH2* expression in the animal cap assay, revealing a further interdependence of noggin and RA signalling in the context of dorsal endoderm patterning.

While our data support the notion of a negative function for BMP signalling in the context of pancreas specification, several other reports have suggested that pancreas development requires BMP signalling. BMPs promote formation of cystic epithelial colonies containing islet-like structures in an *in vitro* culture system of dissociated pancreatic cells from E15.5 mouse embryos (Jiang et al.,

2002). Furthermore, BMPs have been found to play a positive role in zebrafish pancreas development using mutants that are homozygous for BMP (*swirl*) and chordin (*chordino*). Explants studies in the chick have revealed that, in the presence of mesoderm, somite-stage anterior endoderm explants treated with BMPs activate expression of *Pdx1* (Kumar et al., 2003).

These discrepancies might reflect the time point at which BMP signalling is needed for pancreas development. In *Xenopus*, down regulation of BMP signalling must occur during gastrulation. We cannot exclude the possibility for a requirement of BMP signalling in later phases of pancreas development in the frog, as observed in other systems. This aspect remains to be investigated.

Pancreas specification reprogrammed in vitro

One major obstacle in strategies to cure Type I diabetes is to obtain a sufficient supply of β-cells for transplantation. Many attempts have been made to establish a protocol for generating β-cells from murine ES cell. Such protocols have gained induction of pancreatic and β-cell differentiation by transfecting the master regulatory genes Pdx1 or/and Pax4 into the nestin-positive neural progenitor cells (reviewed in Kania, 2004). β-cells generated from neural progenitor cells, though expressing insulin and being responsive to glucose stimulation, have been found to undergo apoptosis right after differentiation (Hansson et al., 2004). More recent studies have focused on attempts to generate definitive endoderm from either murine or human ES cells, which can then differentiate into β-cells (Kubo et al., 2004, D'Amour et al., 2005). Our protocol for the generation of different pancreatic cells from pluripotent embryonic precursor cells, as defined by the *Xenopus* animal cap explant system, using VegT, β-catenin, noggin and RA is designed to mimic the normal embryonic situation for the dorsal mesendoderm and could be useful for those studies aiming at the generation of definitive endoderm and β-cells from mouse or human ES cells.

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Figure legends

Figure 1

RA induces pancreatic gene expression in the dorsal, but not in the ventral, gastrula endoderm explants during gastrulation.

Stage 11 pigmented embryos were dissected to generate dorsal endoderm (DE); dorsal mesoderm (DM); dorsal endoderm plus dorsal mesoderm (DEM); ventral endoderm (VE); ventral mesoderm (VM); ventral endoderm plus ventral mesoderm (VEM); dorsal endoderm plus ventral mesoderm (DE+VM); and ventral endoderm plus dorsal mesodem (VE+DM). The explants were treated with or without 5 μM RA right after isolation for 1 hour and cultured until the control siblings reach stage 39. The explants were subjected to semi-quantitative and quantitative RT-PCR analysis for pancreas marker expression. The markers used are indicated on the right side of the graph and the same markers will be used throughout the study: XlHbox8 and Ptf1a/p48 are pancreatic precursor cell markers; insulin is an endocrine-specific marker; XPDIp is an exocrine-specific marker; TTR is a liver-specific marker; Darmin is an intestine-specific marker; xtwist is a mesodermal marker for controlling the purity of the isolated endodermal explants, and Histone H4 is a loading control. For all analyses involving quantitative RT-PCR, expression levels of each probe tested were normalised to ODC and were compared to the expression levels of the control embryo (CE) at that corresponding stage.

Figure 2

Differential expression of RARs contributes to the compentence of endoderm to activate pancreas gene expression in response to RA.

(A) Stage 11 embryos were dissected to generate dorsal endoderm (DE), dorsal mesoderm (DM), ventral endoderm (VE) and ventral mesoderm (VM). The isolated explants were collected immediately after isolation and subjected to semi-quantitative and quantitative RT-PCR analysis for expression of various xRARs and xRXRs. xRARα2.1 and xRARγ2.1 were found to be predominantly

expressed in the dorsal portion of the endoderm and mesoderm, respectively. xBra and Mixer are markers for controlling the purity of the isolated mesodermal and endodermal explants. (B) xRARγ2.1, either alone or in combination with xRARα2, is able to induce a pancreatic fate in the VEM explants in the presence of its co-receptor xRXRβ. xRARγ2.1 (500 pg/embryo), either alone or in combination with xRARα2 (500 pg/ embryo) or/and xRXRβ (500 pg/embryo) was injected into 4 vegetal cells of 8-cell stage embryos. Ventral endoderm/mesoderm (VEM) explants were isolated from the injected embryos at stage 11. The isolated VEM was treated with or without 5 μM RA for one hour right after isolation and cultured until control embryos had reached stage 39. These explants were subjected to semi-quantitative and quantitative RT-PCR analysis for pancreas marker expression.

Figure 3

Down-regulation of BMP signalling confers competence for pancreas specification in ventral endoderm in response to RA only in the presence of mesoderm.

(A) Noggin is able to induce pancreas specification in VegT/β-catenin co-injected animal cap explants in the absence of exogenous RA. Animal caps were isolated at stage 8 from VegT (500 pg/embryo) and β-catenin (200 pg/embryo), as well as VegT/β-catenin and noggin (500 pg) injected embryos. The isolated animal caps were divided into 3 groups, one group without treatment, one group treated with 5 μM RA and the last group treated with 0.25 μM BMS453 at stage 11 for 1 hour. These animal caps were then cultured until control siblings had reached stage 42, and analysed for pancreas gene expression using semi-quantitative and quantitative RT-PCR. (B) Noggin is able to induce xRALDH2 expression in animal cap explants. Animal caps were isolated from noggin or VegT/β-catenin (concentrations of injected RNA are as indicated above) injected embryos at stage 8. The isolated animal caps were treated with or without 5 μM RA at stage 10.5 for 1 hour, collected at stage 11 and examined for xRALDH2 expression using RT-PCR analysis. (C) Pancreas specification can be efficiently recapitulated in animal cap explants by a

combination of VegT, β-catenin, noggin and RA. Animal caps were isolated from VegT (V) / β-catenin (β) / noggin injected albino embryos at stage 8. The isolated animal cap were subjected to RA treatment at stage 11 for 1 hour, cultured until control siblings had reached stage 40, and analysed for XPDIp (red) and insulin (dark blue) expression using in situ hybridisation. (Panels 1– 3) VegT/β-catenin/noggin co-injected animal caps treated with 8 µM, 16 µM and 32 µM RA, respectively. (D) Down regulation of BMP signalling by noggin results in a competence of VEM to adopt a pancreas fate in response to RA. Noggin was injected into 4 vegetal cells of stage 4 embryos. Ventral endoderm (VE) and ventral endoderm plus mesoderm (VEM) explants were isolated from the noggin-injected embryos at stage 11. These explants were treated with or without 5 µM RA for one hour after isolation, cultured until control siblings reached stage 39 and analysed for pancreas gene expression using RT-PCR analysis. In all the analyses involving quantitative RT-PCR, expression levels of each probe tested were normalised to ODC and compared to the expression levels of control embryos (CE) at that corresponding stage.

Figure 4

RA acts both directly and indirectly on the dorsal endoderm in the context of pancreas specification.

(A) RA signalling is required both in endoderm and mesoderm to specify the pancreas. Dorsal mesoderm (DM) and dorsal endoderm (DE) explants were dissected from stage 11 embryos injected with DNRARα2 (1 ng) at the 4 cell-stage, or treated with 0.25 µM BMS at stage 10. These manipulated DM and DE explants were recombined with each others or with the wild type DE and DM, respectively. The recombined explants were cultured until stage 39 and analysed for pancreas-specific gene expression using both semi-quantitative and quantitative RT-PCR. (B) Blocking RA signalling specifically in either the endoderm or the mesoderm of whole embryos further supports the notion that RA acts both directly on the endoderm and indirectly via the mesoderm in the context of pancreas specification. To block RA signalling specifically in the

endoderm, DNRARα2, together with the lineage tracer β-gal, was injected into the vegetal pole of 8-cell stage embryos. In order to block RA signalling in the mesoderm, DNRARα2 and β-gal were injected into the marginal zone of 4-cells stage embryos. The injected embryos were cultivated until control siblings had reached stage 36 or 39, and subjected to β-gal staining before whole mount *in situ* hybridization was carried out. (Panels 1 –3) Lateral view of stage 36 embryos stained for *XlHbox8* expression (anterior towards the left). (Panels 4 – 6) Lateral view of stage 36 embryos stained for *Ptf1a/p48* expression. (Panels 7 – 9) Lateral view of stage 39 embryos stained for *XPDIp* expression. (Panels 10 – 12) Lateral view of stage 36 embryos stained for Xhex expression. (Panels 13 –15) Serial transversal sections (each 30 µM thick) of stage 36 embryos stained for insulin expression. Insulin expression is lost in the dorsal pancreas when RA signalling is specifically block in the mesoderm (panel 14), or in the endoderm (panel 15). Red arrowheads demarcate the ventral pancreas, white arrowheads demarcate the dorsal pancreas. The statistics obtained from this experiment are indicated at the right bottom corner of each panel.

Figure 5

A scheme representing the RA-mediated early endodermal patterning events in the context of pancreas specification. During gastrulation, RA, generated in the dorsal mesoderm, signals both directly and indirectly to the dorsal endoderm to specify dorsal pancreas. Inhibition of BMP signalling by noggin or chordin is also important for dorsal pancreas specification. Additional mesodermal factor(s), is (are) required to act in conjunction with noggin and RA to pattern the dorsal endoderm towards pancreas fate.

Figure 1

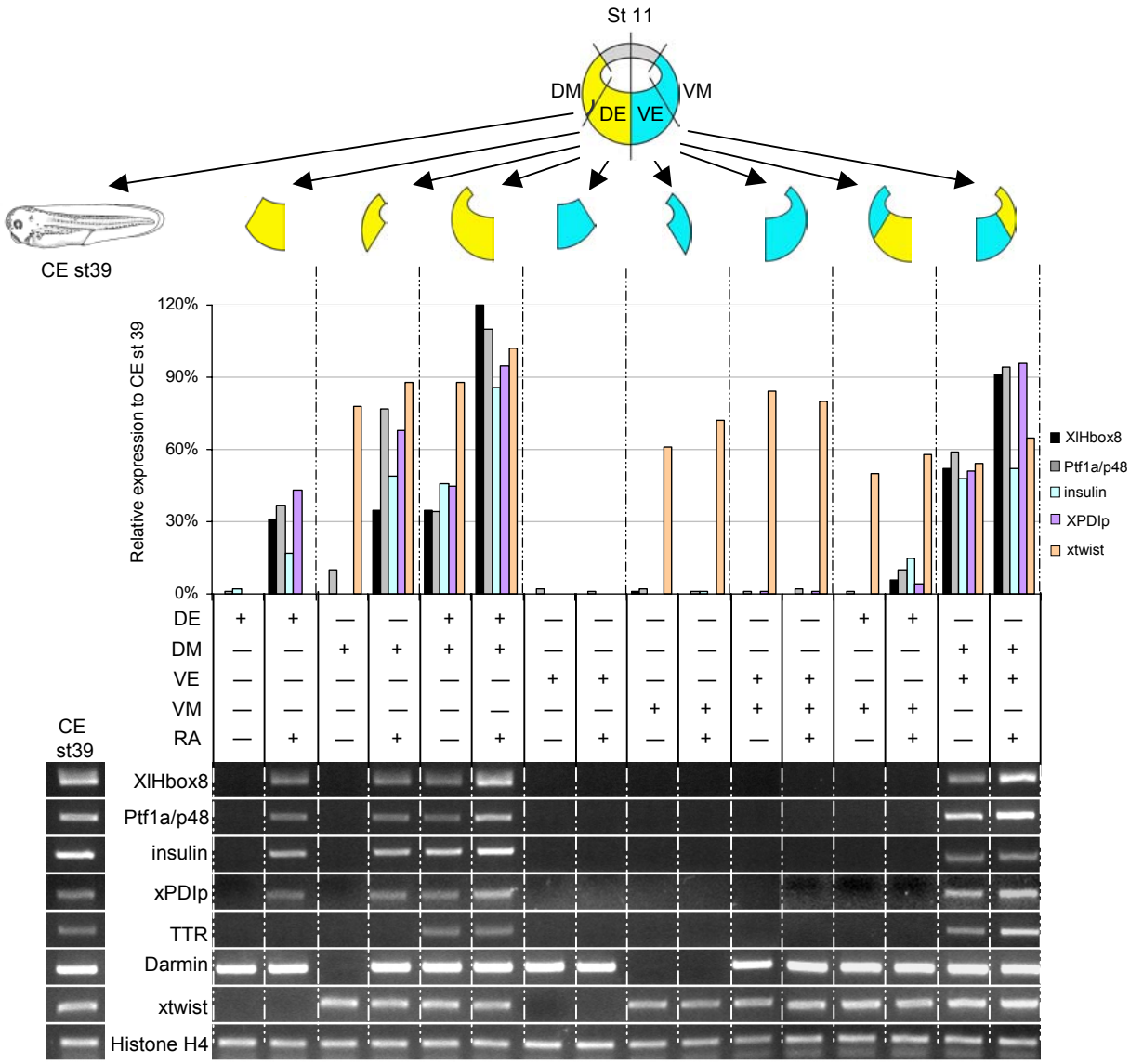
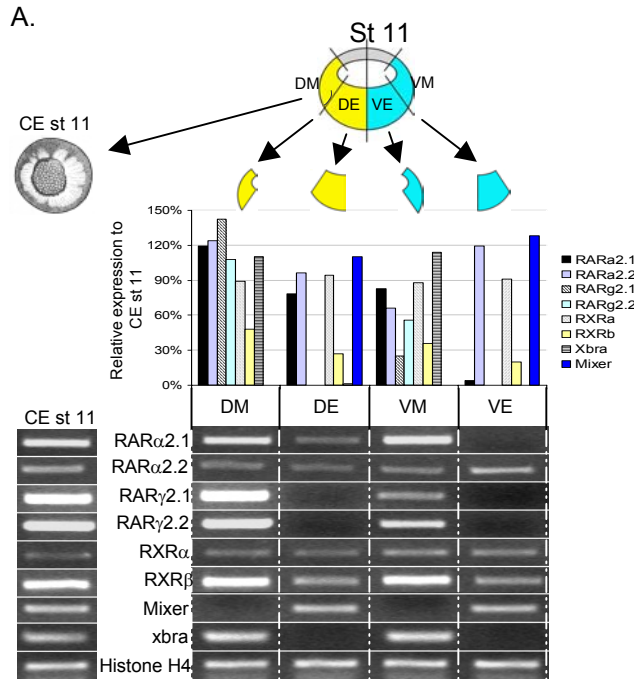

St 11
DM
VM
DE
VE
CE st39
120%
90%
60%
30%
0%
Relative expression to CE st 39
XlHbox8
Ptf1a/p48
insulin
XPDlp
xtwist
DE
DM
VE
VM
RA
CE st39
XlHbox8
Ptf1a/p48
insulin
xPDlp
TTR
Darmin
xtwist
Histone H4

Figure 2


A.
St 11
DM
VM
DE
VE
CE st 11
Relative expression to CE st 11
150%
120%
90%
60%
30%
0%
RARa2.1
RARa2.2
RARg2.1
RARg2.2
RXRa
RXRb
Xbra
Mixer
DM
DE
VM
VE
CE st 11
RARα2.1
RARα2.2
RARγ2.1
RARγ2.2
RXRα
RXRβ
Mixer
xbra
Histone H4

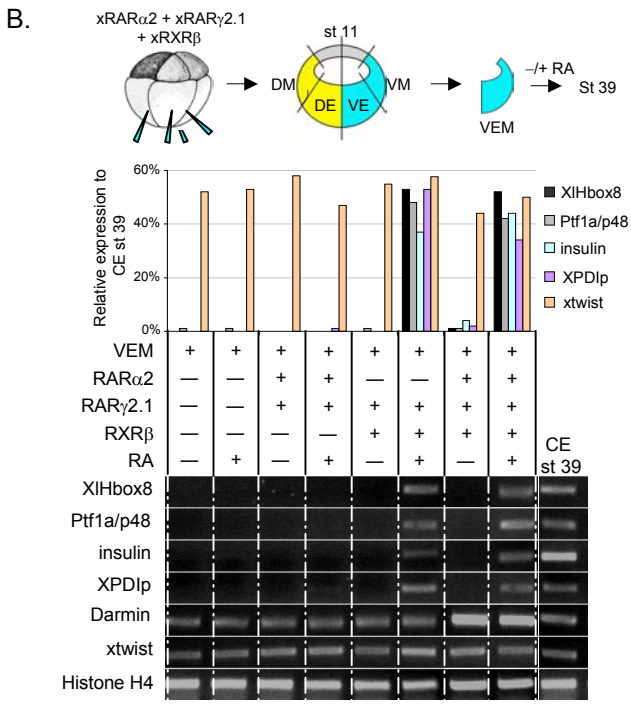

B.
xRARα2 + xRARγ2.1
+ xRXRβ
st 11
DM
VM
DE
VE
–/+ RA
St 39
VEM
Relative expression to CE st 39
60%
40%
20%
0%
XlHbox8
Ptf1a/p48
insulin
XPDIp
xtwist
VEM
RARα2
RARγ2.1
RXRβ
RA
CE st 39
XlHbox8
Ptf1a/p48
insulin
XPDIp
Darmin
xtwist
Histone H4

Figure 3

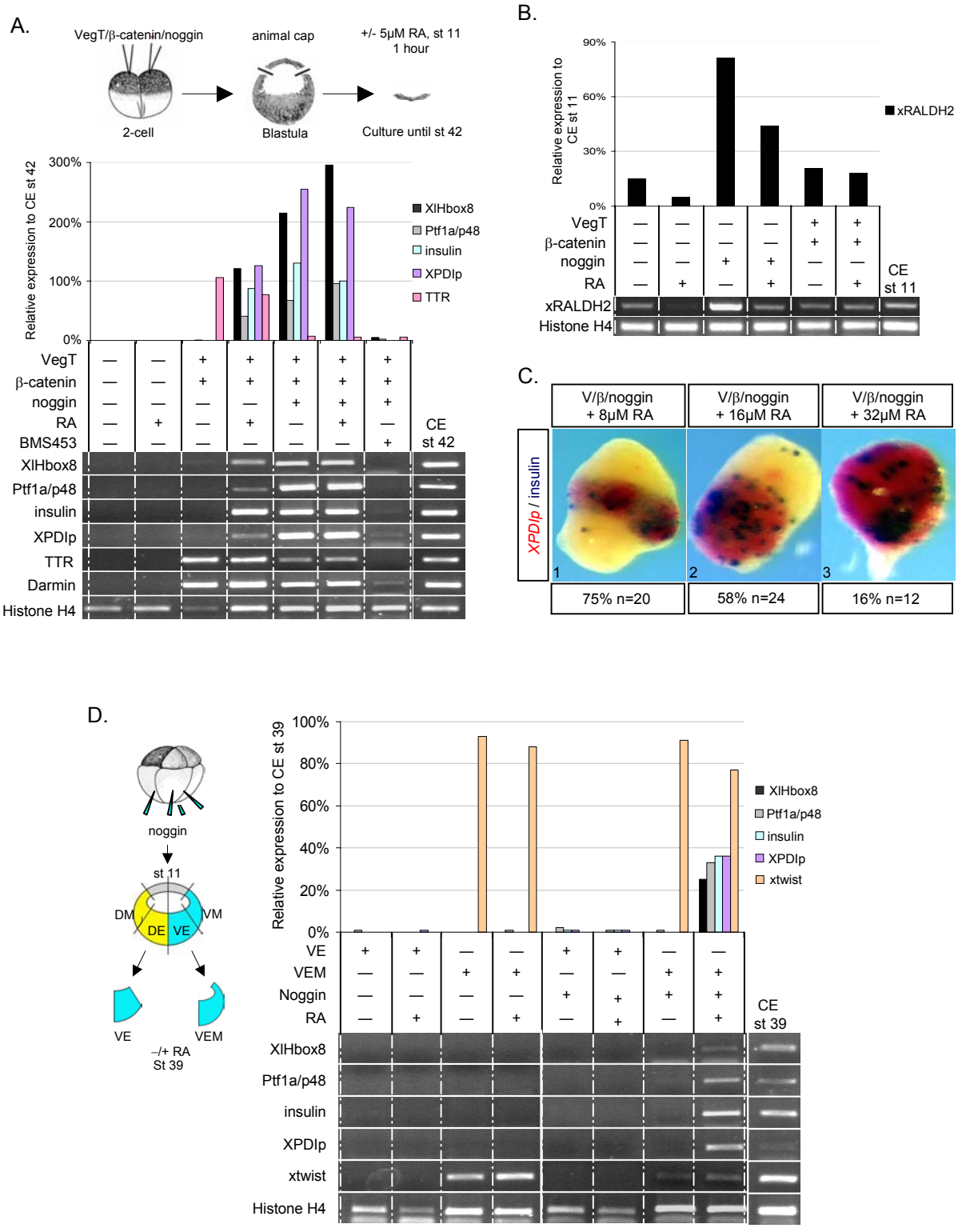

A.
VegT/β-catenin/noggin
animal cap
+/- 5μM RA, st 11
1 hour
2-cell
Blastula
Culture until st 42
Relative expression to CE st 42
300%
200%
100%
0%
XlHbox8
Ptf1a/p48
insulin
XPDIp
TTR
VegT
β-catenin
noggin
RA
BMS453
CE
st 42
XlHbox8
Ptf1a/p48
insulin
XPDIp
TTR
Darmin
Histone H4
B.
Relative expression to
CE st 11
90%
60%
30%
0%
xRALDH2
VegT
β-catenin
noggin
RA
CE
st 11
xRALDH2
Histone H4
C.
V/β/noggin
+ 8μM RA
V/β/noggin
+ 16μM RA
V/β/noggin
+ 32μM RA
XPDIp / insulin
1
2
3
75% n=20
58% n=24
16% n=12
D.
noggin
st 11
DM
DE
VE
VM
VE
VEM
–/+ RA
St 39
Relative expression to CE st 39
100%
80%
60%
40%
20%
0%
XlHbox8
Ptf1a/p48
insulin
XPDIp
xtwist
VE
VEM
Noggin
RA
CE
st 39
XlHbox8
Ptf1a/p48
insulin
XPDIp
xtwist
Histone H4

Figure 4

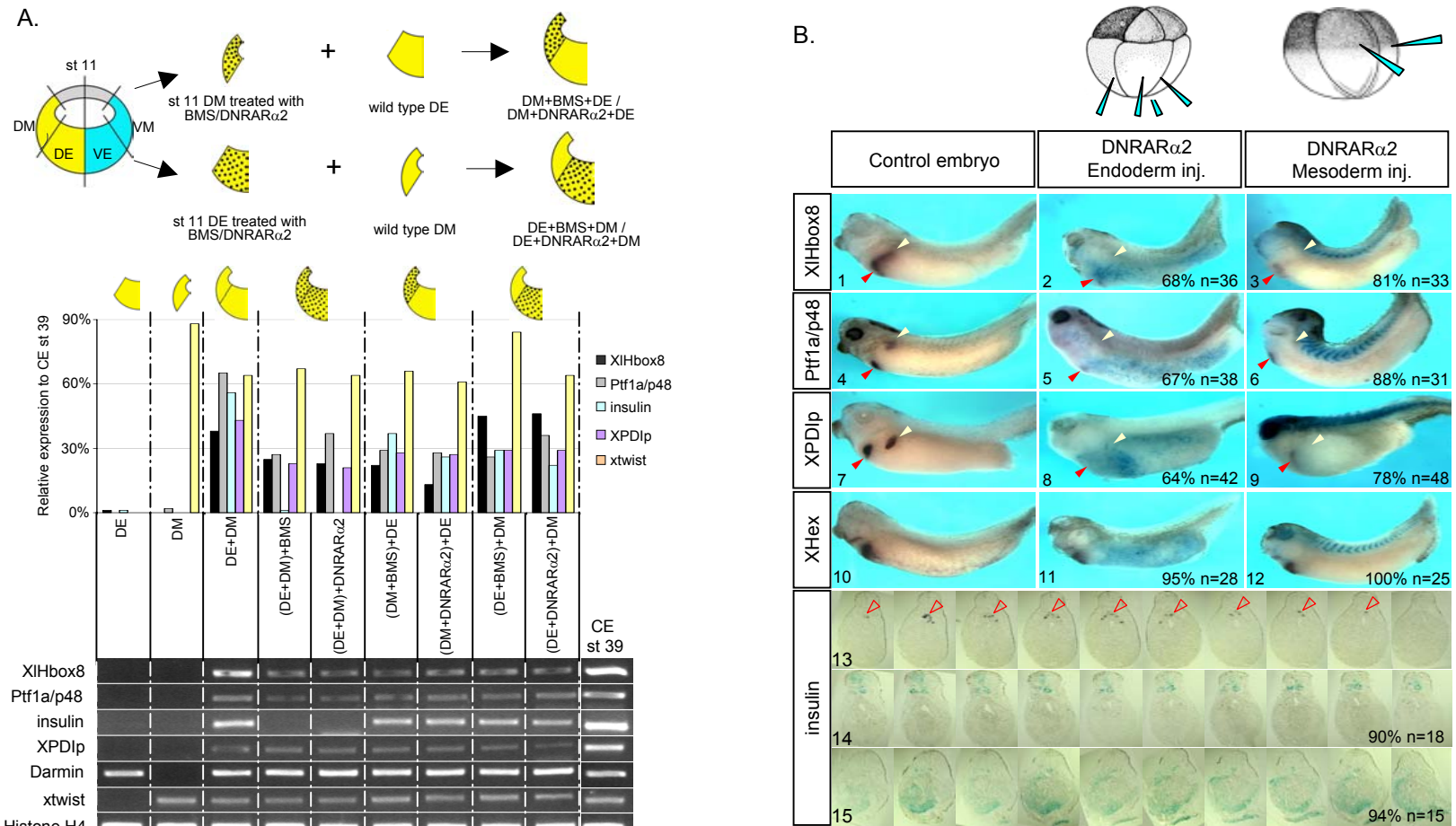
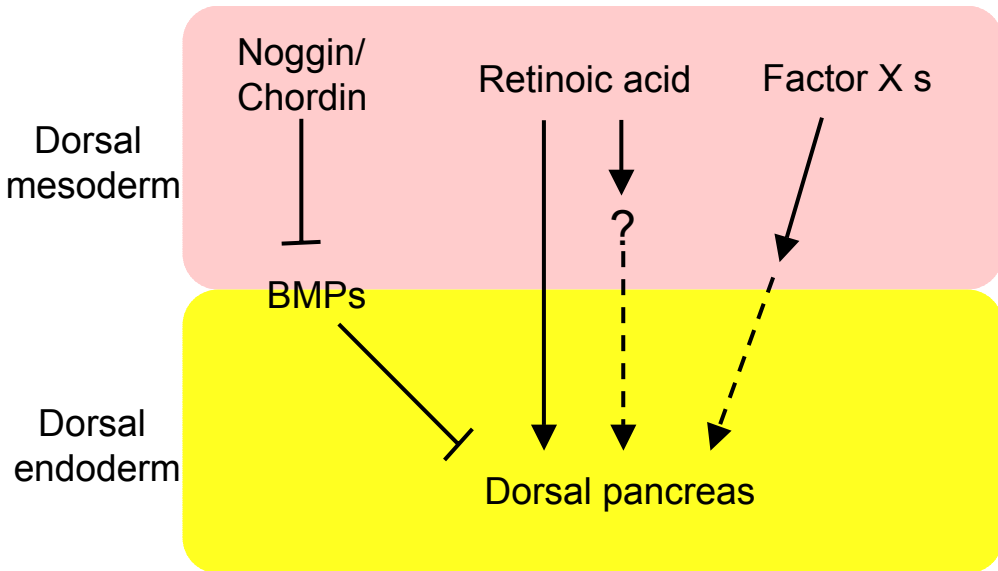

A.
st 11
DM
VM
DE
VE
st 11 DM treated with BMS/DNRARα2
wild type DE
DM+BMS+DE / DM+DNRARα2+DE
st 11 DE treated with BMS/DNRARα2
wild type DM
DE+BMS+DM / DE+DNRARα2+DM
Relative expression to CE st 39
90%
60%
30%
0%
XIHbox8
Ptf1a/p48
insulin
XPDIp
xtwist
DE
DM
DE+DM
(DE+DM)+BMS
(DE+DM)+DNRARα2
(DM+BMS)+DE
(DM+DNRARα2)+DE
(DE+BMS)+DM
(DE+DNRARα2)+DM
CE st 39
XIHbox8
Ptf1a/p48
insulin
XPDIp
Darmin
xtwist
Histone H4
B.
Control embryo
DNRARα2 Endoderm inj.
DNRARα2 Mesoderm inj.
XIHbox8
Ptf1a/p48
XPDIp
XHex
insulin
1
2
68% n=36
3
81% n=33
4
5
67% n=38
6
88% n=31
7
8
64% n=42
9
78% n=48
10
11
95% n=28
12
100% n=25
13
14
90% n=18
15
94% n=15

Figure 5


Noggin/
Chordin
Retinoic acid
Factor X s
Dorsal
mesoderm
?
BMPs
Dorsal
endoderm
Dorsal pancreas

Chapter III: *I-SceI* meganuclease mediated transgenesis in *Xenopus laevis*

Fong Cheng Pan, Yonglong Chen, Jana Löber, Kristine Henningfeld and Tomas Pieler, ***I-SceI* meganuclease mediated transgenesis in Xenopus**, *Developmental Dynamics.,* 2006, **235**,247–252

Statement of contribution:

Subcloning, microinjection, raising transgenic frog embryos, genomic DNA isolation, southern blot analysis, data collection and manuscript preparation.

TECHNIQUES

I-SceI Meganuclease-Mediated Transgenesis in *Xenopus*

Fong Cheng Pan, Yonglong Chen, Jana Loeber, Kristine Henningfeld, and Tomas Pieler*

Several experimental approaches have been described to generate transgenic frogs. Here, we report on the application of a novel method in *Xenopus*, making use of *I-SceI* meganuclease. The characteristic feature of this endonuclease is that it has an extended recognition site of 18 bp, which is expected to exist only once in 7×10^{10} bp of random DNA sequences. Various reporter constructs flanked by two *I-SceI* recognition sites were injected together with the *I-SceI* meganuclease into one-cell stage *Xenopus* embryos. We observed an overall transgenesis frequency of 10% or more under optimized condition. The injected genes were integrated into the genome and transmitted to F1 offspring. Southern blot analysis showed that between one and eight copies of the transgene were integrated. Meganuclease-aided transgenesis, thus, provides a simple and highly efficient tool for transgenesis in *Xenopus*. *Developmental Dynamics 235:247–252, 2006.*

Key words: *I-SceI* meganuclease; transgenesis; *Xenopus*

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INTRODUCTION

Xenopus laevis is one of the major model systems for the analysis of vertebrate development. In the past, transgenic frogs have been used for various purposes such as (1) to dissect regulatory regions of genes (Knox et al., 1998; Casey et al., 1999), (2) to misexpress genes during development under specific spatial and temporal control (Hartley et al., 2001, 2002), and (3) to generate mutations in genes through gene trap approaches (Bronchain et al., 1999).

First attempts to create transgenic frogs had been reported in pioneering studies by Etkin et al. (1984) more than 2 decades ago. Linearized plasmid DNA was injected into fertilized *Xenopus* eggs and was found to be maintained in the form of long extrachromosomal concatemers during early stages of development, eventual integration into the genome occurring at later stages. Although this method is fast and easy, mosaic expression of the transgene in F0 is predominantly observed; uneven distribution of the episomal DNA before integration also results in low germ line transmission rates. Furthermore, the injected genes showed incorrect spatial and temporal regulation.

Approximately 10 years later, Kroll and Amaya (1996) developed a more efficient method for generating transgenic frogs. It involves restriction endonuclease-mediated integration (REMI) of DNA into demembranated sperm nuclei, followed by transplantation of the nuclei into unfertilized eggs. One of the major advantages of this method is that the transgene is integrated into the male genome before fertilization, resulting in high germ line transmission rates. However, the concentration and amount of the restriction enzyme used need to be well-controlled to avoid the risk of genome fragmentation. Inappropriate transplantation of the nuclei also leads to low survival rates of the embryos.

In principle, other transgenic methods reported for different model systems, such as fish, are also applicable to *Xenopus* embryos. These strategies include the use of DNA-NLS complexes (Liang et al., 2000) to achieve more efficient integration of transgenes, as well as the use of transposable elements, such as Sleeping Beauty (Davidson et al., 2003) and

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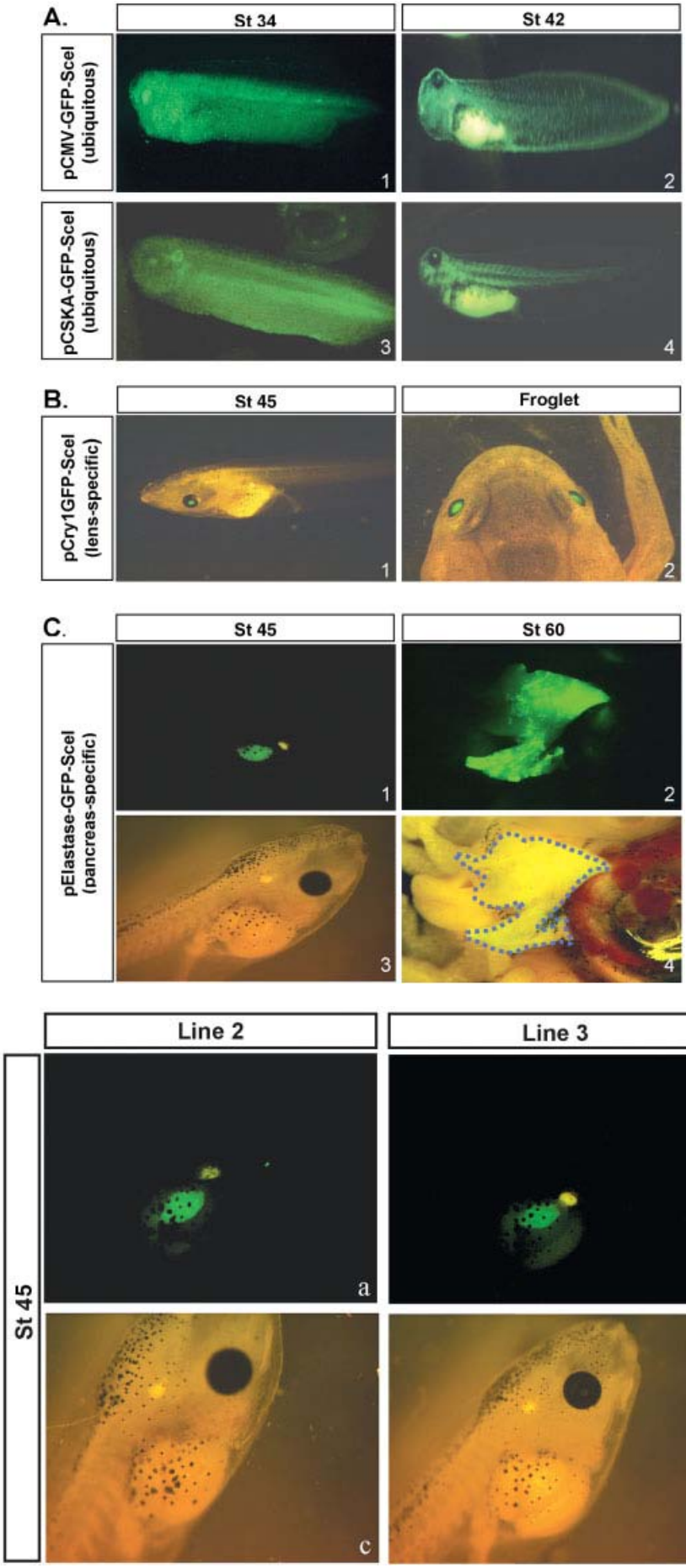

Fig. 1. Generation of transgenic frog embryos with various promoters using I-SceI meganuclease. **A–C:** Transgenic embryos were generated with the ubiquitous promoters pCMV-GFP-SceI (1 and 2) and pCSKA-GFP-SceI (3 and 4; A); the lens-specific promoter pCry1GFP-SceI (B); and the pancreas-specific promoter pElastase-GFP-SceI (C).

Fig. 2. a–d: F1 offspring obtained from F0 founder frog line 2 darkfield (a) and brightfield (c), and line 3 darkfield (b) and brightfield (d), showing strong and specific green fluorescent protein expression in the pancreas.

Frog Prince (Miskey et al., 2003), along with the transposase to obtain site-specific integration.

Use of the rare cutting meganuclease *I-SceI* to generate transgenic medaka fish has been reported recently (Thermes et al., 2002). *I-SceI* meganuclease is an endonuclease encoded by the mobile group I intron found in *Saccharomyces cerevisiae* mitochondria. The characteristic feature of this endonuclease is that it has an extended recognition site of 18 bp, which is expected to exist only once in 7×10^{10} bp of random DNA sequences. Several groups have also reported the successful use of this endonuclease for gene targeting in mammalian cells (Choulika et al., 1994; Cohen-Tannoudji et al., 1998; Richardson et al., 1999).

Here, we report the use of *I-SceI* meganuclease for the creation of transgenic *Xenopus laevis* embryos. Stable transgenic lines were established using various ubiquitous and tissues-specific promoters coupled to the green fluorescent protein (GFP) reporter. We observed high-frequency transgenesis and efficient germ line transmission.

RESULTS AND DISCUSSION

Generation of Transgenic Frog Embryos With Different Promoter Constructs Using *I-SceI* Meganuclease

We have applied an experimental approach to generate transgenic *Xenopus laevis* that was originally described for medaka fish by Thermes et al. (2002). The promoter of interest coupled to a reporter gene was subcloned into a vector containing two *I-SceI* recognition sites. Plasmid DNA was cleaved by and injected together with the *I-SceI* meganuclease into one-cell stage embryos within 1 hr after fertilization (see the Experimental Procedures section for details).

We have tested four different promoter constructs that drive the ubiquitous or tissue-specific expression of GFP: pCMV-GFP-SceI, which contains the CMV promoter (Kroll and Amaya, 1996), and pCSKA-GFP-SceI, which contains the cytoskeletal actin promoter from *Xenopus borealis* (Thermes et al., 2002) both driving ubiquitous expression of the GFP reporter, as well as pCry1GFP-SceI, containing the 490-bp fragment of γ-crystallin promoter (Offield et al., 2000), and pElastase-GFP-SceI, which contains the pancreatic-specific elastase promoter (Beck and Slack, 1999), driving tissue-specific expression in lens and pancreas, respectively. Transgene expression of embryos injected with pCMV-GFP-SceI and pCSKA-GFP-SceI were first detected after gastrulation and yielded highest levels at tail bud stage (stage 34). Strong GFP expression continued through tadpole stage (stage 42) and was still detectable after metamorphosis (Fig. 1A and data not shown). Transgenic embryos generated by injecting pCry1GFP-SceI started to express the transgene specifically in the lens of tail bud stage embryos (Fig. 1B), correlating with the activation of the endogenous promoter (Offield et al., 2000). Lens-specific expression was still detectable in the froglet stage. Similarly, transgenic embryos injected with pElastase-GFP-SceI showed pancreas-specific GFP expression at stage 45 (Fig. 1C). This corresponds to the endogenous expression of elastase in *Xenopus laevis* embryos (Beck and Slack, 1999). We were able to detect a strong and specific GFP signal during metamorphosis at stage 60 (Fig. 1C) and in the adult (data not shown).

Coinjection of DNA and *I-SceI* Meganuclease Increases the Frequency of Nonmosaic Transgene Expression

To optimize the experimental protocol used for the generation of transgenic *Xenopus* embryos, DNA and enzyme concentration were varied systematically. Plasmid pCMV-GFP-SceI was injected with or without meganuclease. In the absence of meganuclease, almost all of the GFP-positive embryos showed mosaic expression of the transgene (Table 1). When the same plasmid was injected together with meganuclease, the number of nonmosaic, GFP-expressing embryos increased significantly. Mosaic expression of GFP might be due to replication and persistence of injected plasmids as extrachromosomal episomes, which are inherited by only a subset of cells (Etkin and Pearman, 1987; Etkin et al., 1987). Mosaic expression of the transgene is gradually lost at later stages of development. Similar results were obtained when pCSKA-GFP-SceI (40 pg/embryo) and pCry1GFP-SceI were injected with or without meganuclease (Table 1).

We have also injected different amounts of pElastase-GFP-SceI with different concentrations of *I-SceI* meganuclease (Table 1). Best results were obtained with the highest concentration of DNA used, further increase in plasmid concentration leads to high mortality of the embryos due to gastrulation defects. Variation of the enzyme concentration from 1×10^{-3} U/embryo to 8×10^{-3} U/embryo had no significant effect on the transgenic efficiency (Table 1). Further increase in meganuclease concentration leads to high mortality of the embryos due to gastrulation defects.

In all cases, injected embryos showed high survival rates of more than 50% and almost all of these embryos survived until after metamorphosis. This finding is an advantage over the REMI method in which injected embryos showed low survival rates upon transplantation of nuclei and most of the transgenic embryos generated using this method have difficulties surviving until the frog stage due to the generation of aneuploid embryos (Sparrow et al., 2000).

Germ-Line Transmission of Transgenes and Southern Blot Analysis of Transgenic Lines

Transgenic embryos injected with pElastase-GFP-SceI were scored for GFP expression in the pancreas. F0 transgenic embryos that were positive for GFP in the pancreas were raised to sexual maturity and mated with wild-type frogs. To check for germ-line transmission, F1 tadpoles were scored for GFP-expression in the pancreas. Six mature F0 founder frogs were raised to maturity. A significant degree of germ line transmission, which ranged from 1–76%, was observed for most of these lines (Table 2). A germline transmission rate of higher than 50% suggests that the F0 founder frog

TABLE 1. Transgenesis Efficiency for Different Promoter Constructs as Well as for Different DNA and Enzyme Concentrations

Constructs	DNA (pg/embryo)	*I-SceI* (U/embryo)	Injected N	Survival rate N	Survival rate %	GFP-expressing from survival N	GFP-expressing from survival %	Nonmosaic expression (%) N	Nonmosaic expression (%) %
pCMV-GFP-SceI	40	4×10^{-3}	377	361	96	343	95	42	12
	40	—	464	364	78	345	95	2	0.5
pCSKA-GFP-SceI	40	4×10^{-3}	442	362	82	355	98	34	10
	40	—	453	429	95	355	83	1	0.2
pCry1GFP-SceI	40	4×10^{-3}	479	406	85	336	83	58	14
	40	—	378	295	78	199	68	8	3
pElastase-GFP-SceI	4	4×10^{-3}	320	296	93	6	2	5	2
	20	4×10^{-3}	324	224	69	16	7	14	6
	40	4×10^{-3}	776	660	86	54	8	45	7
	60	4×10^{-3}	663	524	79	65	13	59	11
	40	1×10^{-3}	527	307	58	22	7	18	6
	40	4×10^{-3}	776	660	85	54	8	45	7
	40	8×10^{-3}	984	600	61	46	8	41	7
	40	—	583	407	70	5	1	2	0.5

TABLE 2. Germline Transmission of Transgenic Carrying pElastase-GFP-SceI

F0 founder frog	Sex	Gene transmission rates: Total embryos	GFP-positive embryos	%
Line 1	M	~200	2	~1
Line 2	M	559	428	76
Line 3	M	34	16	47
Line 4	M	292	68	23
Line 5	M	302	30	10
Line 6	F	127	38	30

had multiple transgene-integration loci, whereas germline transmission rates that are lower than approximately 50% indicate that there is mosaicism in the F0 founder germ line. The rates close to 25% are likely to reflect that the founders were "half transgenics," which is sometimes also seen with the REMI transgenesis method (Marsh-Armstrong et al., 1999; Hartley et al., 2002), suggesting a late integration event at the two-cell stage.

The pattern of transgene expression for the founder frogs during tadpole stages was not recorded. However, the tissue specificity and levels of transgene expression were maintained and comparable to those observed in typical F0 founder frog (Fig. 2).

The pioneering study by Etkin and Pearman (1987) had shown that the plasmid DNA-injected embryos were able to transmit the transgene only through the male germline. Here, we show that the transgene is also transmitted through the female germline using the meganuclease approach. We obtained a mature F0 female founder frog that transmitted the transgene to 30% of its F1 offspring.

To determine the nature of DNA integration, such as the number of insertion loci and the length of concatemers, we performed Southern blot analysis on genomic DNA isolated from independent F1 offspring of four different lines carrying pElastase-GFP-SceI. Isolated genomic DNA was digested with *Bam*HI that liberates the insert from the plasmid pElastase-GFP-SceI (Fig. 3A). Analysis of four independent transgenic lines identified a 1.4-kb fragment that is common to all individuals from all lines, confirming integration of the GFP encoding portion. The restriction pattern of the offspring from line 2 suggests that the plasmid was incompletely digested (either at *I-SceI* cleavage site 1 or site 2) such that embryos contain insertion of the entire plasmid in tandem repeat as indicated by the presence of the corresponding characteristic fragments (3.2 kb, 1.4 kb, and 0.5 kb; Fig. 3B, lanes 1 and 2). In line 3, the absence of a correspondingly intense 3.2-kb signal indicates formation of tail to tail concatemers (Fig. 3B, lanes 3 and 4). The restriction patterns of lines 2, thus, suggests that the *I-SceI* cleavage sites were maintained in the genome of these transgenic lines, similar to what has been reported by Thermes et al. (2002) for medaka fish. In line 4, the absence of the 3.2-kb and 0.5-kb fragments indicates integration of the insert alone (Fig. 3B, lane 5). The presence of other fragments in all lines tested is likely to reflect different chromosomal integration sites and, thereby, generation of different junctional fragments. A GFP-negative control embryo showed no hybridization to the probe (Fig. 3B, lane 8).

The copy number of integrated concatemers was estimated using a dilution series of plasmid DNA. Assuming a DNA content of 3.1×10^9 bp per

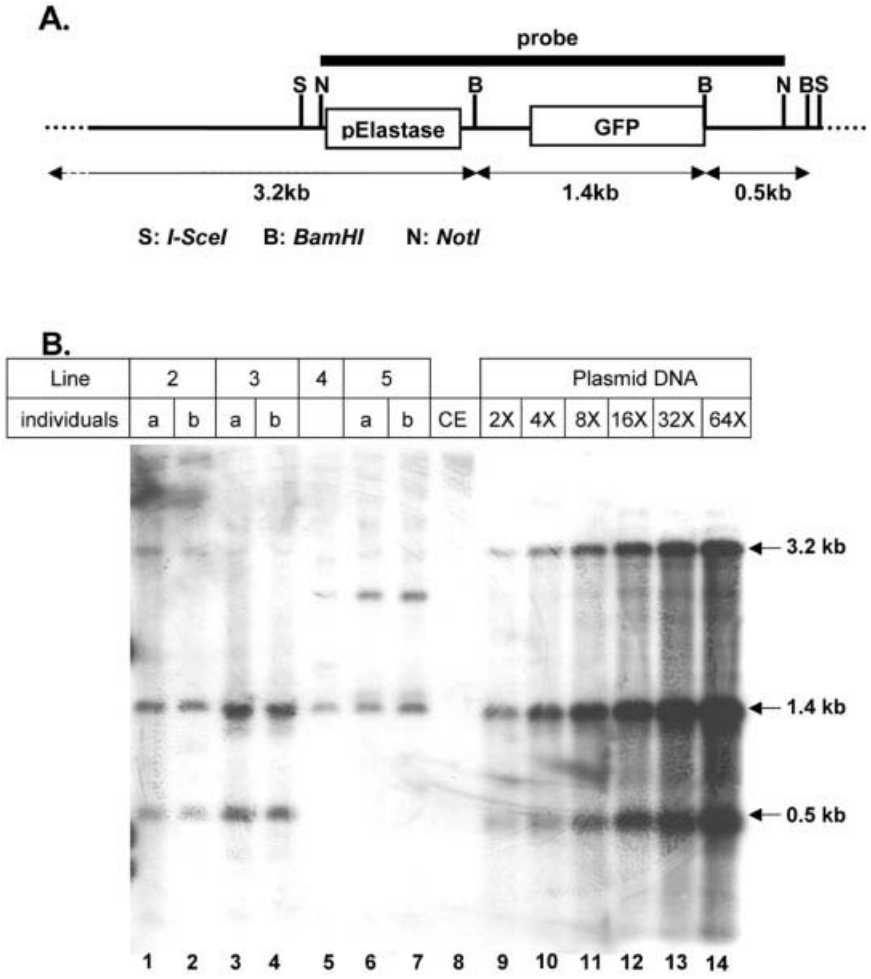

Fig. 3. Southern blot analysis of transgenic lines. **A:** Schematic diagram of pElastase-GFP-SceI containing a pancreas-specific promoter and a green fluorescent protein (GFP) coding region. **B:** Southern blot analysis was performed with genomic DNA isolated from F1 offspring of four F0 founder frogs (lines 2–5) injected with pElastase-GFP-SceI and scored positive for GFP in the pancreas. The a and b indicate two different individuals from the same line. Genomic DNA was digested with *Bam*HI and hybridized with insert probes resulting from *Not*I digestion. The copy number of integrated DNA was estimated using a dilution series (as indicated) of plasmid DNA digested with *Bam*HI. 2X-64X, plasmid standards. Quantities indicated on top of each lane correspond to 2 to 64 copies of the plasmid integrated into the genome.

haploid genome (Tymowska, 1973), 15 pg of *Bam*HI-digested plasmid pElastase-GFP-SceI were loaded to estimate the intensity of one copy plasmid DNA integrated into the *Xenopus laevis* genome. Copy numbers ranging from one or two to a maximum of approximately eight copies were observed (Fig. 3B). These numbers are significantly lower than those observed in the transgenics obtained by the REMI method, in which the linearized plasmid tends to form concatemers of more than 10 copies that also resulted in variable transgene expression levels and phenotypes (Hartley et al., 2002).

In conclusion, we have applied a novel approach to generate transgenic frogs that may have several advantages. First, the meganuclease approach is simple such that large numbers of transgenic embryos can be generated in a single experiment. Second, the transgenesis efficiency is higher than those reported for other methods. The transgenes show faithful temporal and spatial expression and high survival rates. We have also demonstrated germline transmission of the transgenes. Meganuclease coinjection, thus, provides a simple and highly efficient tool for transgenesis in *Xenopus*.

EXPERIMENTAL PROCEDURES

Plasmid Construction

pCMV-GFP-SceI was generated by inserting a 2.0-kb *Sal*I–*Not*I fragment (containing the CMV promoter coupled to GFP reporter sequence and a SV40 polyA tail) from pCSGFP3 into the ISceI-pBSII SK+ vector containing two I-SceI recognition sites (Thermes et al., 2002). pCSKA-GFP-SceI was as described (Thermes et al., 2002). pElastase-GFP-SceI was generated from pElastase-GFP (Beck and Slack, 1999) restricted with *Not*I (New England Biolabs, Ipswich, MA). The 2.1-kb *Not*I fragment, containing the 205-bp, pancreas-specific elastase promoter coupled to GFP and a β-globin poly A tail, was subcloned into the ISceI-pBSII SK+ vector. A 1.5-kb *Nhe*I–*Not*I fragment, containing 490 bp of γ-crystallin1 promoter coupled to GFP and a SV40 polyA tail, was generated from pCry1GFP3 (a gift from Robert Grainger) and subcloned into the *Spe*I and *Not*I sites of the ISceI-pBSII SK+ vector to create pCry-GFP-SceI.

Preparation of *Xenopus* Eggs and Embryos

Pigmented *Xenopus laevis* eggs were obtained by injecting the dorsal lymph sacs of females with 50 IU of human chorionic gonadotrophin (hCG) in the evening followed by 1,000 IU hCG the next morning before egg collection. Eggs were fertilized in vitro with minced testes in 0.1× MBS (1.76 mM NaCl, 48 μM $NaHCO_3$, 20 μM KCl, 200 μM Hepes, 16 μM Mg_2SO_4, 8 μM $CaCl_2$, 6 μM $Ca(NO_3)_2$, pH 7.4), dejellied with 2% cysteine hydrochloride (pH 7.8–8.0) and cultured in 0.1× MBS buffer. Embryos were staged according to Nieuwkoop and Faber (1967).

Microinjection of Plasmid DNA With Meganuclease

Plasmid DNA was digested with *I-SceI* meganuclease (Roche Diagnostics, Germany, or New England Biolabs) before injection [1–15 ng/μl plasmid DNA; 0.5 × commercial meganuclease buffer (Roche Diagnostics, Germany, or New England Bio-

labs); 1–10 U/μl *I-SceI* meganuclease]. The reaction mixture was incubated for 40 min at 37°C, and 4 nl of the reaction mixture was injected into one-cell stage embryos within 1 hr after fertilization, using a microinjector (Eppendorf, Germany). The reaction mixture was injected in between the sperm entry site and the center of the animal pole, the region where the pronuclei of sperm and egg will fuse, to bring the injected DNA in close proximity to the forming nucleus. Injected embryos were cultured at 12.5°C until two-cell stage and to allow integration of the transgene before first cleavage, and then transferred to 18°C for normal culturing.

Fluorescence Microscopy

Embryos were scored for GFP expression using a MZFLIII Leica dissecting microscope with a 370- to 420-nm excitation filter and a 455-nm LP emission filter. The GFP-positive embryos were sorted and bred to maturity. Mature GFP-positive frogs were mated with wild-type frogs to analyze for germline transmission of the transgene. F1 embryos were sorted according to GFP expression, and the germline transmission rates were determined.

Genomic DNA Extraction and Southern Blot Analysis

Individual 2-month-old tadpoles (F1 pElastase-GFP) were ground to a fine powder in liquid nitrogen using a prechilled mortar and pestle. Genomic DNA was extracted using the QIAGEN Blood and Cell Culture DNA kit (Qiagen, Germany). Isolated genomic DNA was digested to completion with *Bam*HI. DNA standards were prepared by digesting pElastase-GFP-SceI used to generate the transgenic frog by *Bam*HI. Assuming an estimated DNA content of 3.1×10^9 bp per haploid genome (Thermes et al., 2002), 15 pg of digested plasmid were loaded to estimate the signal intensity corresponding to a single gene copy. DNA samples (10 μg/lane) were separated in 0.8% agarose gels in 1× TBE and subjected to Southern blot analysis according to standard procedures (Sambrook and Russell, 2001).

DNA probes for Southern blot analysis were generated by restricting the pElastase-GFP-SceI with *Not*I and gel purification with Qiagen Gel Extraction and Purification Kit (Qiagen, Germany). The 2.1-kb *Not*I fragment was then labeled with the nonradioactive ECL Direct nucleic acid labeling and detection system (Amersham Bio-Science, UK).

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5. General discussion

5.1 RA-mediated patterning of the endoderm during gastrulation

RA has been shown to play an important role in the A-P patterning of the mesoderm and ectoderm during embryogenesis (reviewed in Maden 2001). Studies on its role in endodermal patterning have been just initiated recently. In this study, we investigated the role of RA in patterning the early endoderm in the context of pancreas and liver development.

Results obtained from time-course-studies have indicated that RA signalling is crucial for pancreas specification during gastrulation. *RALDH2*, the main endogenous RA synthesizing enzyme, is strongly expressed in the dorso-lateral involuting mesoderm during gastrulation, i.e. during the period of development when RA signalling is critically required for pancreas development in fish and frogs (Stafford and Prince, 2002; Chen et al., 2004). Fate mapping analyses in *Xenopus* have revealed that the pancreas originates from the dorsal endoderm (Chalmers and Slack, 2000; Kelly and Melton, 2000). The presence of a RA signalling centre (*RALDH2* expressing cells) in close contact with the prospective pancreatic precursor cells, supports the idea that RA is involved in prepatterning of the dorsal endoderm during gastrulation.

Based on this hypothesis, we subsequently proved that dorsal endodermal explants isolated during gastrulation, which by themselves do not express any pancreatic genes, can adopt a pancreas fate upon RA treatment or when co-cultured with the dorsal mesoderm (the endogenous RA source). These findings suggest that RA is an important signal to direct the dorsal endoderm towards a pancreatic fate. It was proposed that a prepatterning of the endoderm already exists at gastrula stages, since vegetal explants isolated from early gastrula embryo, express region-specific markers in a cell-autonomous manner (Gamer and Wright, 1995; Henry et al, 1996). However, an argument has been raised that these early vegetal explants may contain mesodermal cells and that mesoderm-free explants, isolated during neurula stage, fail to express region-specific marker, suggesting that regional specification of the endoderm occurs

at later stages and requires mesodermal signalling (Horb and Slack, 2001). Nevertheless, it was not ruled out that an early labile specification exists and is sustained through continuous interaction with the mesoderm. Our findings support the idea that early labile regional specification of the endoderm by RA does occur. This notion is further evidenced by the study by Chen et al. (2003) who showed that dissociated endodermal cells isolated at the end of gastrulation express liver and intestine-specific markers cell-autonomously. The ability of these endodermal cells to express region specific markers cell-autonomously, in the absence of cell-cell interaction, suggests that specification may have occurred early by the end of gastrulation.

We have also shown that both RA and dorsal mesoderm are required to achieve maximal levels of pancreatic gene expression in the dorsal endoderm, indicating that additional signalling activities from the mesoderm may be involved. Conversely, ventral endodermal explants are not competent to adopt a pancreatic fate even in the presence of RA and mesoderm, indicating that certain signalling molecule(s), absent in the ventral part of the gastrula embryo, is(are) required together with RA for pancreas specification. Our findings reveal that down-regulation of BMP signalling by *noggin,* that is expressed dorsally, is important for inducing a pancreatic fate in the ventral endoderm in conjunction with RA, suggesting that *noggin* might be one of the mesodermal factor(s) that is (are) required for pancreas development.

In addition to down regulation of BMP signalling, the differential responsiveness of dorsal versus ventral endoderm to RA may, also, be due to the differential spatial expression of RARs. We found that xRARα2.1 and xRARγ2.1 are predominantly expressed in the dorsal endoderm and dorsal mesoderm, respectively. Ectopic expression of these two receptors induced a pancreatic fate in the ventral endodermal explants in the presence of RA and mesoderm, suggesting that differential spatial expression of these two receptors is also important for the RA-mediated patterning of gastrula endoderm towards the pancreatic fate. Differential distribution of RARs and their isoforms has been shown to play an important role during embryogenesis (reviewed in Mark, 2006). Pfeffer & De Robertis (1994) have shown that in *Xenopus*, expression of

xRARγ2, but not xRARγ1, in the chordoneural hinge is important for tail development. Furthermore, Crawford and colleagues (1995) have shown that xRARγ2 isoforms, i.e. xRARγ2.1 and xRARγ2.2, are differentially distributed in *Xenopus* embryos during early embryogenesis and that they differentially respond to RA and UV treatment, suggesting that these receptors play different roles during morphogenesis. These findings further support our notion that the dorsally restricted expression of these RARs plays an important role for RA-mediated early patterning of dorsal prepancreatic endoderm.

5.2 RA signals both directly and indirectly to pattern the dorsal prepancreatic endoderm

We are particularly interested in knowing whether RA signals are received directly or indirectly by the dorsal endoderm for pancreas specification. Using manipulated endodermal and mesodermal recombinant explants, we have shown that the RA mediated patterning of the dorsal prepancreatic endoderm involves both direct and indirect mechanisms. Similar results were obtained when we specifically blocked RA signalling by injection of DN-RARα2 in a particular germ layer in the whole embryo. The differential spatial expression of RARs in the dorsal endoderm and mesoderm mentioned above also supports the notion that RA might act both, directly and indirectly via the mesoderm to pattern the gastrula endoderm towards a pancreatic fate.

Studies in other model systems have also revealed the importance of the mesoderm in RA-mediated pancreas specification. Using quail-chick explants, Kumar et al. (2003) have shown that RA is able to induce ectopic *Pdx1* expression in the anterior endoderm but only in the presence of mesoderm. In the mouse, RA-responsive cells have been detected in both the mesenchyme and endoderm of the pancreatic anlage in *RALDH2* $^{-/-}$ mice upon treatment with RA. Furthermore, dorsal pancreatic mesenchyme formation was impaired, as marked by the loss of Isl1 expression in these *RALDH2* $^{-/-}$ mice, suggesting that RA signals might be relayed via mesoderm (Martin et al., 2005). These findings are in line with our proposed indirect mechanism of RA signalling in pancreas specification in *Xenopus*.

However, a most recent study by Stafford et al. (2006) using the zebrafish system gave a different conclusion. Using cell transplantation techniques, it was demonstrated that RAR function is required in the foregut endoderm, but not in the mesoderm, for the development of insulin-expressing β-cells; however, it was not reported whether the development of other endocrine and exocrine pancreatic cells has similar signalling requirements. Furthermore, they did not exclude the possibility that RA signals from the lateral plate mesoderm, which they did not examine in their study, might also play a role in pancreas specification in zebrafish, as it has been suggested in the chick studies (Kumar et al., 2003). Our findings indicate that both direct and indirect mechanism are important for pancreas specification in frogs, which is in agreement with the studies in mouse and chick systems.

5.3 A conserved role of RA signalling in dorsal pancreas specification

In addition to the early endodermal patterning events mediated by RA, we also examined the effect of modulating RA signalling on pancreas and liver development in *Xenopus*. Blocking RA signalling during gastrulation, with the RA antagonist BMS453, resulted in dorsal pancreas agenesis with a loss of pancreatic precursor and differentiation marker expression. However, ventral pancreas primordia was only partially inhibited. Exogenous RA treatment caused expansion of the endocrine pancreas at the expense of exocrine cell population in the dorsal pancreas via inhibition of Notch signalling. Studies in the mouse, avian, and zebrafish, have provided evidence for a similar role of RA in pancreas development.

In zebrafish, inhibition of RA signalling during gastrulation resulted in a reduction of pancreatic gene expression. However, in contrast to the frog, exogenous RA leads to expansion of both exocrine and endocrine cell populations in the zebrafish (Stafford and Prince, 2002).

In the mouse, RA produced by *RALDH2* in the dorsal pancreatic mesenchymal cells was crucial for dorsal pancreas development (Motlokov et al., 2005; Martin et al., 2005). *RALDH2* null mutant mice do not form dorsal pancreas, as marked

by the loss of Pdx1 expression, whereas ventral pancreas was not affected, which is in agreement with the findings in the frog. Maternal supplementation of RA to the *RALDH2*$^{-/-}$ mutant mice between E7.5 and E9.5 rescued dorsal pancreas development, indicating that RA is specifically required for the normal development of the dorsal pancreatic endoderm at a stage preceding Pdx1 function. Similar results were also obtained in experiments with quail embryo derived from Vitamin A Deficient (VAD) quail (Stafford et al., 2004). Thus, taken together, results obtained in mouse, avian, frog and fish suggest a conserved role for RA signalling in dorsal pancreas development.

5.4 RA signalling in the context of liver and ventral pancreas development

In *Xenopus,* formation of the ventral pancreas was partially inhibited when RA signalling was blocked, while liver development was not affected. This suggests that liver development does not rely on RA signalling. On the other hand, exogenous RA treatment caused expansion of the exocrine cell population in the ventral pancreas, and liver development was inhibited in a concentration dependent manner. The differential response of the dorsal and ventral pancreas indicates differential developmental regulation of these two anlagen by RA. Indeed revious studies have shown that dorsal and ventral pancreas receive different stimuli from the adjacent mesodermal derivatives in the course of development (reviewed in Kumar and Melton, 2003).

It is well established that signals from the cardiac mesoderm suppress ventral pancreas development and promote a liver fate (Zaret, 2002). Inhibition of liver development, with increasing concentration of RA, may be a consequence of inhibition of heart formation, since excess exogenous RA blocks heart development (Drysdale et al., 1997, Chen et al., 2004). This effect of excess RA on liver development is in line with the observation that the ventral pancreas is expanded.

In contrast to the situation in the frog, blocking RA signalling inhibits both pancreas and liver development in zebrafish, whereas the liver and pancreas

anlagen were expanded upon exogenous RA treatment (Stafford and Prince, 2002). The discrepancies in the results obtained in the fish and frogs with respect to the effect of RA in pancreas and liver development may, at least in part, be due to the differences in endoderm and gut tube formation in the fish and frog embryos. In zebrafish, endoderm progenitors are found at the lateral margin of the blastoderm intermingled with mesoderm progenitors (the RA source). Incomplete segregation of mesodermal and endodermal progenitor cells during gastrulation might reflect the requirement for RA signalling in both pancreas and liver development (reviewed in Tam et al., 2003). In contrast, the endodermal progenitors in *Xenopus* exist as a more discrete population in the vegetal mass and in the suprablastoporal region of the late blastula. During gastrulation, juxtaposition of the dorsal suprablastoporal cells (which give rise to dorsal pancreas), but not the dorsal subblastoporal cells (which give rise to ventral pancreas and liver), to the RA synthesizing cells might be responsible for the different requirement for RA in the development of these two organs (reviewed in Tam, 2003; Kelly and Melton, 2000; Chen et al., 2004).

It has also been pointed out that the pancreatic primordia in the zebrafish embryo are not truly equivalent to the pancreatic buds, as they develop in other vertebrates including *Xenopus*. Moreover, gastrulation in zebrafish results in the formation of a sheet of endodermal cells rather than a proper endodermal tube (Biemar et al., 2001).

Our findings in the frog are in agreement with the results obtained in other studies in mouse and avian. Liver development is not affected in *RALDH2*$^{-/-}$ mutant mice and the quail embryos derived from Vitamin A deficient (VAD) quail, which is contrary to the results obtained in fish but in line with the findings in frogs. However, the effect of excess RA on the liver development in these models were not examined since maternal supplement of exogenous RA can hardly increase the endogenous physiological RA levels in these mutant mice or quail embryos (Martin et al., 2005; Motlokov et al., 2005; Stafford et al., 2004).

5.5 Interaction of BMP and RA signallings in the context of early patterning of the dorsal prepancreatic endoderm

Our findings support the notion that down-regulation of the BMP signalling by *noggin* is required for pancreas specification in conjunction with RA signalling during gastrulation. VE explants were able to adopt a pancreatic fate in the presence of RA and mesoderm, but only when BMP signalling was down regulated by *noggin*. Similar effects were observed in the animal cap assays, in which the pancreatic cell population was increased in the presence of *noggin* and the increasing RA concentration. A previous study by Gamer & Wright (1995) has shown that vegetal explants, either injected with BMP4 or isolated from UV-treated embryos, do not express *XlHbox8*. However, they did not observe an induction of *XlHbox8* expression in noggin-injected ventral vegetal explants. Our study now extend this finding and shows that in order to induce a pancreatic fate in the ventral endoderm, three components are required: (i) down regulation of BMP, (ii) presence of RA and, (iii) mesoderm factor(s) present in both the dorsal and ventral mesoderm.

Sasai and colleagues (1996) also showed that organiser factors, such as *chordin* and *noggin*, could induce endoderm formation independent of mesoderm in the animal cap assay. The endoderm formed in these animal caps are of dorsal endodermal type. They also pointed out that these factors are required to pattern the dorsal endoderm during gastrulation, which also supports our own data. Moreover, we also demonstrated that *noggin* can induce *xRALDH2* expression in the animal cap assay, suggesting that noggin and RA might interact in the process of specifying the dorsal endoderm towards a pancreatic fate during gastrulation.

While our findings support the notion that blocking BMP signalling is required for pancreas specification, several other reports have suggested that pancreas development requires BMP signalling. Jiang and colleagues (2002) showed that BMPs promote formation of cystic epithelial colonies containing islet-like structures in an *in vitro* culture system of dissociated pancreatic cells from E15.5 mouse fetus. It was also shown that BMPs have a positive effect on zebrafish

pancreas development using BMP (*swirl*) and chordin (*chordino*) mutant fish (Tiso et al., 2003). Explant studies in chick (Kumar et al., 2003) have shown that somite-stage anterior endoderm explants treated with BMPs are able to express *Pdx1* in the presence of mesoderm. Discrepancies in the results obtained by us and others could be explained by consideration of the time point when BMP signalling is required for pancreas development. In our case, down regulation of BMP signalling is needed during gastrulation for patterning of the dorsal endoderm towards the pancreatic fate. We do not excluded the possibility that BMP signalling has a positive effect at a later time point of pancreas development in *Xenopus*, for example to induce somite-stage endoderm to express pancreatic markers as it was reported in the chick. However, this possibility still remains to be investigated.

5.6 Interaction of Shh and RA signalling in the context of pancreas specification

In the mouse and chick, *Sonic hedgehog* (*Shh*) is expressed at high levels in the stomach and duodenum epithelium, but is excluded from the dorsal pancreatic domain. During pancreas induction, permissive signals from the notochord are required to suppress *Shh* expression in the dorsal pancreatic domain (Kim et al., 1997; Hebrok et al., 1998). Ectopic expression of *Shh* in the dorsal pancreas results in dorsal pancreas agenesis, whereas overall inhibition of *Shh* leads to heterotopic pancreas formation (Apelqvist et al., 1997; Hebrok et al., 1998). In this study, we found that inhibition of RA signalling results in expansion of *Shh* expression into the prospective dorsal pancreatic endoderm, correlating with the loss of pancreatic gene expression in this region. This observation suggests a negative regulation of *Shh* signalling by RA in dorsal pancreas development in *Xenopus*. It will be interesting to know how and when this interaction occurs. We do not know yet whether RA is able to induce the notochord signals, *activin-βB* and *FGF2* (Hebrok et al., 1998) to suppress the *Shh* expression in the dorsal pancreas. This issue is a subject of further studies.

In contrast to the situation observed in the dorsal pancreas, inhibition of RA signalling does not result in ectopic expression of *Shh* in the ventral pancreas

indicating that dorsal and ventral pancreas development are differentially regulated by RA as it was mentioned above.

In the *RALDH2*$^{-/-}$ mouse, ectopic expression of *Shh* in the dorsal pancreas was not observed, suggesting that the dorsal pancreas agenesis in these mice was not due to the ectopic expression of *Shh* (Martin et al., 2005), which is contrary to the finding in the frog. An effect of RA on *Shh* signalling has also not been reported in zebrafish. Curiously, *Shh* exerts a positive regulatory effect on pancreas development in the fish, which is in stark contrast to the situation in mouse, avian and frog (dilorio et al., 2002; Roy et al., 2001). Furthermore, Chang et al. (1997) have shown that a RA response element (RARE) is present in the upstream regulatory sequence of the *Shh* promoter, suggesting a positive effect of RA on *Shh* signalling in the context of pancreas specification in the fish.

5.7 Interaction of Notch and RA signalling during pancreas development

Endocrine and exocrine fate determination is mediated by Notch signalling via lateral inhibition. Our study shows that an excess of exogenous RA expands the endocrine pancreas at the expense of exocrine cells population by negatively regulating Notch signalling. In RA treated *Xenopus* embryos, *ESR10*, a downstream target of Notch receptor (which has similar function to *Hes1*), is downregulated in the dorsal pancreas, whereas its expression in the ventral pancreas is not altered. *NeuroD*, a downstream target of Ngn3, is upregulated in the dorsal pancreas. *NeuroD* is not expressed in the ventral pancreas even in control embryos. The loss of the exocrine portion of the dorsal pancreas upon application of exogenous RA correlates with and is likely to be a result of the inhibition of Notch signalling activity, as marked by the loss of *ESR10* expression, which is in parallel with an increasing expression of *NeuroD* (Chen et al., 2004). The same phenotype was observed in mice overexpressing Ngn3 under the control of Pdx1 promoter in the pancreatic epithelium. These mice displayed an increase in the endocrine pancreas and decrease of the exocrine compartment due to the premature differentiation of precursor cells into endocrine cells. However, since RA-treated *Xenopus* embryos do not survive

beyond stage 44, we were not able to analyse whether there is only endocrine hyperplasia or if the dorsal pancreas could also have a hypoplastic phenotype, as has been shown in the Ngn3 overexpressing mice (Apelqvist et al., 1999; Schwitzgebel et al., 2000).

Differential expression of the Notch signalling components in the dorsal and ventral pancreas also suggests that Notch signalling is regulated by a different mechanism in these two rudiments, as it is the case for Shh, and RA signalling.

In zebrafish and quail, no link has been reported between Notch and RA signalling (Stafford and Prince, 2002; Stafford et al., 2004). In the mouse, the *RALDH2*$^{-/-}$ mutant mice do not survive beyond E10.5 and maternal supplement of exogenous RA can hardly increase the endogenous physiological RA level in these mutant mice, which makes it difficult to analyse the expression of Notch signalling components in this case (Martin et al., 2005; Motlokov et al., 2005).

5.8 Pancreas specification reprogrammed *in vitro*

Type I diabetes is the result of an autoimmune attack against the insulin producing β-cells. The major obstacle in treating this disease is to obtain enough β-cells for transplantation. Generation of β-cells from human ES cells will provide new hope for curing this disease. Many attempts have been made to establish a protocol for generating β-cells from ES cell, especially of mouse origin. These protocols mostly involve induction of pancreas and β-cell differentiation by transfecting the master regulatory genes, such as Pdx1 or/and Pax4, into nestin-positive neural progenitor cells (reviewed in Kania, 2004). β-cells generated from neural progenitor cells undergo apoptosis right after differentiation (Hansson et al., 2004). In order to generate β-cells that can be used for transplantation, β-cells should be differentiated from the ES cells of endodermal origin. Recent studies have focused on generating definitive endoderm from either mouse or human ES cells, which can later differentiate into β-cells (Kubo et al., 2004, D'Amour et al., 2005).

In *Xenopus*, *in vitro* generation of pancreatic cells using pluripotent precursor cells, the animal cap cells, was initiated by Moriya et al. (2000a,b). Using dorsal lip explants and activin-treated animal cap explants, they have demonstrated that RA can promote the formation of pancreas-like structures that express *XlHbox8* and insulin. In our initial studies, using VegT and β-catenin in combination with RA, we were also able to turn on exocrine and endocrine gene expression in these animal cap explants. Application of *noggin* and increasing concentrations of RA further enhanced the pancreatic cell population while at the same time suppressing the liver fate. These protocols with VegT, β-catenin, *noggin* and RA, in fact, mimic the normal embryonic induction of the formation of the dorsal mesendoderm and could be useful for those studies aiming at the generation of the definitive endoderm and β-cells from mouse or human ES cells.

5.9 *I-SceI* mediated-transgenesis in *Xenopus* and its use for the study of the later phases of pancreas development.

In this study, we have established a new method for generating transgenic frogs using *I-SceI* meganuclease, which we adapted from the fish system (Thermes et al., 2002). We were able to obtain a transgenesis frequency of 10% or more under optimal conditions as well as germ-line transmission of the transgenes. *I-SceI*-mediated transgenesis may provide a simple and efficient tool to study later development in *Xenopus laevis*, for example later phase of pancreas development. Similar results have been obtained recently with *Xenopus tropicalis* that further support the success in the application of this approach in the frog system (Ogino et al.,2006).

By driving GFP under the promoter of a gene that is specifically expressed either in the ventral or the dorsal foregut endoderm, cells from these two regions can be isolated by FACS. Differentially expressed genes involved in cell fate specification can be obtained and the differential molecular regulation between the dorsal and the ventral pancreas can be examined.

A previous study by Horb et al. (2003) showed that transgenic frog embryos driving the Pdx1-VP16 expression under the control of a liver specific promoter, transthyretin, transdifferentiate liver cells into pancreas. They used the elastase promoter-GFP construct, which was also used in our study, as a real time marker of the transdifferentiation process. Various transcription factors, eg, Ptf1a/p48 or ESR10, can now be expressed ectopically under the control of a tissue-specific promoter in an effort to examine the potential of these transcription factors to induce ectopic pancreas (Blitz et al., 2005).

To apply more specifically the transgenic approach to the RA study reported here, transgenic frogs carrying the RARE-GFP transgene can be generated. The dorsal endoderm or dorsal mesoderm of gastrula-stage transgenic embryos expressing the RARE-GFP construct can be isolated and treated with or without RA, before subjecting it to microarray analysis for screening of RA target genes involved in dorsal endoderm patterning during gastrulation.

Conclusion

In this study, we investigated the role of RA signalling in liver and pancreas development, as well as the early endodermal patterning events mediated by RA during gastrulation. Our study shows that RA is essential for pancreas but not liver specification during gastrulation. Blocking RA signalling results in an expansion of *Shh* expression into the dorsal pancreatic domain and a subsequent loss of pancreatic gene expression in the respective region. However, ventral pancreas development is only partially inhibited. Application of exogenous RA leads to the expansion of endocrine pancreas at the expense of the exocrine cell population in the dorsal pancreas via inhibition of Notch signalling. Conversely, in the ventral foregut endoderm, increased RA concentration leads to a gradual inhibition of the liver development and expansion of the ventral pancreas.

Based on the observation that the early patterning of the gastrula endoderm by RA as a prerequisite for the pancreas specification, we also investigated these early patterning events by RA. Using gastrula stage embryonic explants, we show that RA is sufficient to induce pancreas specification in dorsal but not ventral endoderm. This differential responsiveness of the dorsal versus ventral endoderm is, partly, mediated by the differential spatially restricted expression of RARs. Furthermore, down-regulation of BMP signalling by *noggin* is required to pattern the dorsal endoderm in conjunction with RA and additional signal(s) in the mesoderm. Results obtained from embryonic explants and whole embryo experiments allow us to conclude that RA acts both directly on the endoderm and indirectly via the mesoderm for pancreas specification during gastrulation.

In addition, we have established a new method for generating transgenic frogs using *I-SceI* meganuclease. We observed a transgenesis frequency of 10% or more under optimal conditions as well as a high germ-line transmission rate in the generated transgenic frogs. Thus, *I-SceI* meganuclease-mediated transgenesis provide a simple and highly efficient tool for generating transgenic frogs.

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CURRICULUM VITAE

PERSONAL DATA

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ACADEMIC QUALIFICATIONS

2002 – present	PhD student in Developmental Biology Department of Developmental Biochemistry (Prof. T. Pieler) Goettingen Center for Molecular Biosciences (GZMB) Project title: " *Regulation of Xenopus pancreas development: Retinoic acid (RA) signaling in liver versus pancreas specification in Xenopus*".
2001 – 2002	MSc. In Molecular Biology International Master/Ph.D Molecular Biology Program, Goettingen, Germany. Master thesis title: " *Retinoic acid (RA) signaling in Xenopus pancreas development*".
1998 – 2000	BSc (Hons) in Molecular Biology (Microbiology) National University of Malaysia (University Kebangsaan Malaysia, UKM), Selangor, Malaysia. BSc thesis title: "*Subcloning of Gen cry1B Bacillus thuringiensis subspecies entomocidus HD9*".
1995-1998	Diploma in Medical Laboratory Technology University Science of Malaysia (University Sains Malaysia, USM), medical campus Kelantan, Malaysia. Diploma annual projects: "*Genetic Transformation of Vibrio cholerae (1997)"* and "*The Use of PCR for the detection and Identification of Helicobacter pylor (1996)i*".

PUBLICATIONS

1. Chen, Y.*, Pan, F. C.*, Brandes, N., Afelik, S., Soelter, M., and Pieler, T. (2004). Retinoic acid is essential for pancreas development and promotes endocrine at the expense of exocrine cell differentiation in Xenopus. ***Developmental Biology*** 271: 144-160. (* equal contribution)

2. Pan, F.C., Chen, Y., Loeber, J., Henningfeld, K., and Pieler, T. (2005) *I-SceI* mediated transgenesis in *Xenopus*. . *Dev. Dyn.* **235**, 247-252

3. Pieler, T., Pan, F.C., Afelik, S. and Chen, Y. 2006. Molecular Genetics of Liver and Pancreas Development. *In Cell Signaling and Growth Factors in Development: From Molecules to Organogenesis (ed. K. Unsicker and K. Krieglstein),* **Vol. 2**. pp. 823-840. Wiley-vch, Weinheim.

4. Pan, F.C., Chen, Y., Bayha, E., Grapin-Botton, A., and Pieler, T. Retinoic acid patterning of dorsal pre-pancreatic endoderm during gastrulation involves interaction with BMP signalling. (to be submitted)

ATTENDED CONFERENCES

1. 4th German-Italian Xenopus Meeting, October 2003, Menaggio, Italy (poster)

2. 1st Horizons in Molecular Biology, December 2003, Goettingen, Germany (poster)

3. 5th Gfe Developmental Biology Summer school, September 2004, Guenzburg, Germany. (poster)

4. 16th Gfe Scientific Meeting of the Society of Developmental Biology, April 2005, Muenster, Germany. (poster)

5. SFB meeting on Inflammation, Tissue Damage, Regeneration and Tumor Development in Gastrointestinal Tract. 15/04-16/04/05. Goettingen, Germany

6. GROWBETA. 19/11-20/11/04. Goettingen, Germany

7. SFBD meeting, Strassbourg (Obernai). Oct 2005.(poster)

8. X-Omics Satelite meeting, Oct 2005. Obernai (France). (talk)