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Identification of embryonic pancreatic genes using *Xenopus* DNA microarrays

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Abstract

The pancreas is both an exocrine and endocrine endodermal organ involved in digestion and glucose homeostasis. During embryogenesis, the anlagen of the pancreas arise from dorsal and ventral evaginations of the foregut that later fuse to form a single organ. In order to better understand the molecular genetics of early pancreas development, we sought to isolate markers that are uniquely expressed in this tissue. Microarray analysis was performed comparing dissected pancreatic buds, liver buds, and the stomach region of tadpole stage *Xenopus* embryos. A total of 912 genes were found to be differentially expressed between these organs during early stages of organogenesis. K-means clustering analysis predicted 120 of these genes to be specifically enriched in the pancreas. Of these, we report on the novel expression patterns of 24 genes. Our analyses implicate the involvement of previously unsuspected signaling pathways during early pancreas development.

INTRODUCTION

The embryonic pancreas in vertebrates forms from dorsal and ventral protrusions of the primitive gut epithelium. These pancreatic buds grow, fuse, and branch to eventually form the definitive pancreas. Like the mammalian pancreas, the pancreas of the frog *Xenopus* is composed of both endocrine and exocrine tissues and can be readily studied due to easy access to large numbers of externally developing embryos (Blitz et al. 2006). The exocrine tissue of both mammals and amphibians is organized into grape-like clusters of cells called acini and their associated ducts, which produce and secrete enzymes into the duodenum that function in digestion of food. Embedded within this exocrine tissue are five types of endocrine cells, which secrete hormones into the bloodstream to regulate somatic tissue uptake and hepatic release of glucose as metabolic conditions require. Endocrine cells are organized into the so-called Islets of Langerhans that produce insulin (β cells), amylin (β cells), glucagon (α cells), somatostatin (δ cells), ghrelin (ε cells), and pancreatic polypeptide (PP cells). Malfunction of the pancreas is the root cause of several diseases including

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diabetes mellitus, pancreatitis and pancreatic cancers. Diabetes mellitus is a metabolic disorder characterized by hyperglycemia (high blood sugar) and can be considered to break down into two subtypes. Type 1 diabetes is caused by autoimmune destruction of the insulin-producing β cells (reviewed in Devendra et al., 2004), while Type 2 diabetes is characterized by a system-wide resistance to insulin that sometimes progresses to the loss of β cell function. Type 1 diabetes is an incurable chronic condition, which can only be remedied by insulin supplementation. Thus, it has been argued that considerable benefit to patients with type 1 diabetes (and severe forms of type II diabetes) could be derived via an increased understanding of the molecular and developmental underpinning of pancreas differentiation that fosters the development of β cell replacement and/or regeneration therapies.

In recent years rapid progress has been made in understanding the molecular genetic basis of pancreas formation. The pancreas undergoes a series of developmental processes: 1) the formation of the endoderm germ layer, 2) regional specification of the endoderm to form a "pre-pancreatic" domains, 3) the formation/outgrowth of pancreatic buds and subsequent fusion of the dorsal and ventral buds, and 4) the differentiation and expansion of pancreatic tissues with endocrine, exocrine and duct cells (Murtaugh and Melton, 2003). In both zebrafish and *Xenopus* (teleost and tetrapod), there exists a conserved gene regulatory network controlling early endodermal development (Stainier, 2002; Koide et al., 2005; Wardle and Smith, 2006). Coordinated expression of transcription factors such as VEGT and SOX17, and the members of the MIX and GATA family genes are required for the early endoderm formation (Zhang et al., 1998; Weber et al., 2000; Xanthos et al., 2001; Lee et al., 2001; Latinkic et al., 2003; Kofron et al., 2004; Sinner et al., 2004). Signals emanating from the mesoderm control the establishment of anteroposterior and dorsoventral patterning of the endoderm. Retinoic acid, which is secreted from the anterior paraxial mesoderm is required for the proper development of pancreas in *Xenopus*, zebrafish and mice (Stafford and Prince 2002; Stafford et al., 2004; 2006; Martin et al., 2005; Pan et al., 2007). Signals from notochord promote the induction of dorsal pancreas (Kim et al., 1997), and purified activin βB and FGF2 mimic the notochord function (Hebrok et al., 2000). Growth factors including sonic hedgehog, Wnts, and Notch are also implicated in various processes of pancreatic development (Hebrok et al., 2000; reviewed in Kim & MacDonald, 2002).

Pancreatic cell fates become irreversibly determined through the interactions of various intracellular transcription factors. During embryogenesis, endoderm is specified by GATAs and SOX17, and subsequently the foregut endoderm, which gives rise to the liver, duodenum and pancreas, is established by the expression of FOXA2 and HNF6 (Jacquemin et al., 2000; Lee et al., 2005). The homeodomain-containing transcription factors such as HLXB9, PDX1, PTF1A participate in pancreatic bud formation and growth (Li et al., 1999; Harrison et al., 1999; Ahlgren et al., 1996; Offield et al., 1996; Kim et al., 2002, reviewed in Schwitzgebel, 2001, Kawaguchi et al, 2002). Pancreatic progenitors are specified for endocrine and non-endocrine fates by the differential expression of the transcription factor NGN3. NEUROD1 and PAX6 promote the development of endocrine progenitor differentiation. PDX1, NKX2.2 and NKX.6.1 are expressed in cells that are destined to become specific β-endocrine cells (Sander et al., 2000). The goal of the present study is to

further identify signaling components and transcription factors of potential important for pancreas development.

One approach to uncover the processes of pancreas development is to establish a comprehensive transcription profile using “genomics” methodologies. Using DNA microarrays, novel regulators of β cell development and signature marker genes representing specific pancreatic cells were identified in mice (Gu et al., 2004; Chiang and Melton, 2003). In *Xenopus*, we have previously reported on the isolation and characterization of various organ specific endodermal markers using our homemade *Xenopus* neurula stage (stage 15) and tailbud stage (stage 25) cDNA microarrays (Shin et al., 2005; Park et al., 2007). In this current study, we instead utilized *Xenopus* Affymetrix GeneChip arrays representing over 14,400 transcripts and compared the gene expression profiles of the developing pancreas, liver, and stomach of tadpole stage (stage 42) embryos. The stage was chosen for the analyses because the pancreatic and liver buds are easily identifiable by visual inspection and thus can be isolated with minimum contamination of other nearby organs. Additionally, the pancreas of stage 42 embryos expresses genes such as Pdx1, Ptf1a, Sox9 that are critical for pancreas development, suggesting that other genes involved in pancreas development can be still isolated. Here we report on the identification and expression analysis of 24 novel *Xenopus* genes that are enriched in the pancreas. Our analyses implicate the involvement of previously unsuspected signaling pathways during early pancreas development.

RESULTS AND DISCUSSION

Identification of genes expressed in embryonic pancreatic buds using microarrays

We wished to identify genes that are specifically expressed in the developing pancreas of early *Xenopus* embryos to better understand the process of endodermal organ differentiation. During embryonic gut formation, the liver and pancreatic rudiments bud out from the epithelium of the developing digestive tube posterior to the future stomach (Kelly and Melton 2000; Chalmers and Slack, 1998). Like the situation in mammals, the dorsal pancreatic bud originates from the gut tube underneath a portion of the notochord and the ventral pancreatic buds derive from the ventral gut near the heart and liver. These pancreatic primordia are independently induced and later fuse to form a single pancreas. We isolated the pancreas, liver, and stomach buds from stage 42 embryos (swimming tadpole stage shortly after the dorsal and ventral pancreatic buds fuse), and performed Affymetrix gene expression analyses using *Xenopus laevis* genome arrays. These organ buds mostly consist of the endoderm component, but also include the associated mesoderm. Experiments were performed twice, using two independently dissected tissue samples. Expressionist software (GeneData) was used to refine microarray data, including gene expression profiling analyses (see Materials and Methods). To identify genes that are differentially expressed between the pancreas, liver, and stomach, we first selected genes that passed the analysis of variance (ANOVA) with a criterion of p values of less than 0.05. Four thousand two hundred two genes satisfied this standard. Next, the expression levels of each gene in the pancreas, liver and stomach, were compared. Genes with average expression difference ratios of greater than three-fold were selected for further analyses. After manually removing genes that are redundantly represented in the Affymetrix chips, 912 genes were subjected to subsequent

analyses (see Supplementary Table 1). These genes were separated into six expression groups based on K-means clustering analysis (Fig. 1A). Group P contains 120 genes that are specifically enriched in the pancreas. Group L contains 237 genes preferentially expressed in the liver, and 303 genes in Group S are enriched in the stomach. Groups PL, PS and LS contain the genes that are enriched in both the pancreas and liver but not stomach (90 genes), pancreas and stomach but not liver (88 genes), and liver and stomach but not pancreas (74 genes), respectively (Fig. 1A and B). To identify the most closely related human orthologs we performed BLAST searches, followed by reciprocal BLAST analyses. The homologous genes thus represent the best bidirectional BLAST hit between *Xenopus laevis* and human and were then assigned official gene symbols and full names of their human counterparts (Supplementary Table 1). Whenever a *Xenopus* gene encodes a putative protein but fails to identify a human ortholog, “not available” (“N/A”) was assigned. Other remaining *Xenopus* genes were simply listed by EST identification numbers.

We examined the biological properties of the six clustering groups by examining Gene Ontology (GO) terms associated with genes in each cluster (Suppl Table 1, Fig. 1C). Among the 912 genes, the most frequently observed GO term is linked to metabolism (21%), followed by signal transduction (11%), transport (10%), cytoskeletal organization and biogenesis (7%), transcription (7%), cell adhesion (3%), cell proliferation (2%), proteolysis and peptidolysis (2%), blood coagulation (2%), complement activation (2%), and digestion (1%). A GO term of “digestion” was represented at 9% in Group P, in contrast to 1% among the 912 pancreas/liver/stomach genes. Since the exocrine pancreas secretes numerous digestive enzymes into the duodenum, this finding is consistent with a known role of the pancreas. In the embryonic liver (Group L), “blood coagulation” and “complement activation” represented 6% and 5%, respectively. This is consistent with the liver’s important role in hematopoiesis and as the major producer of complement activation proteins to regulate inflammatory processes. Group S is enriched in genes involved in “cytoskeleton organization and biogenesis” (13%) including actin and myosin, in contrast to the other two groups (2% for Group P and 3% for Group L), which may be linked to the contractile action of the stomach, requiring enhanced musculature.

Based on this annotation, we first compared the gene lists in each clustered subgroup against the published literature to corroborate that both our organ bud dissections did indeed enrich for organ-specific mRNAs, and our K-means clustering analysis correctly assigned these into appropriate subgroups (see Table 1). Group P genes included AMY2A, CEL, CLDN6, CPA1, DNASE1, ELA1, ELA2A, HLXB9, INS, PDIA2, and PRSS2, which are known to be expressed in the pancreas (Afelik et al., 2004; Sogame et al., 2003, Horb and Slack, 2002; Kelly and Melton, 2000; Chen et al., 2004; Park et al., 2007). Similarly in Group L, we found genes associated with liver development, which are A2M, ALB, AMBP, BF, FGG, HHEX, KNG1, TBX2 and TF (Zorn and Mason, 2001, Park et al., 2007). Group S included ATP4B, CTSE, SOX2, and STARD10, which are expressed in the stomach (Park et al, 2007). XBP1 is expressed in both the liver and pancreas (Park et al., 2007) and belongs to Group PL. PAX6 is expressed in both the pancreas and intestine of stage 45 embryos (Kelly and Melton, 2000) and is assigned to Group PS. Based on these results, we concluded that

our dissections/microarray data are of high quality and that the clustering analysis successfully separated the 912 genes into appropriate expression groups.

Overview of our findings

Our current microarray analyses show that many of the *Xenopus* genes found to be expressed in the stomach, liver, and pancreas have clear orthologs in human and other species, although their specific roles in each organ have not been recognized previously. We also wished to confirm the findings of the microarray data by whole mount *in situ* hybridization. Among the 120 pancreatic (group P) genes**,** 24 previously uncharacterized candidate genes were selected based on the following criteria. Eighteen genes were selected based on three GO terms, “transcription”, “signal transduction”, and “cell adhesion”. In addition, based on the array results 6 genes expressing at high levels in the pancreas were also chosen from the PL and PS groups because they may serve as useful marker genes in the future. The results of whole mount *in situ* hybridizations performed on dissected guts of tadpole stage embryos are shown in Figures 2 through 4, and information on genes previously not reported to be expressed in the pancreas is listed in Table 2. We also report on the expression patterns of these genes in whole embryos (Suppl Figures 1–5). We note that while most of the in situ hybridization patterns are consistent with the predicted patterns based on the K-means clustering analysis, in some cases *in situ* patterns do not match with the clustering classification. This discrepancy may be attributed to the fact that while we isolated RNA from dissections performed at a single stage (stage 42), we examined gene expression patterns by whole mount *in situ* hybridization at several different stages.

The *in situ* data are presented in Figures 2–4 according to the timing of earliest onset of each genes expression. Among the 24 genes selected based on the above criteria**,** we find that ten genes (BTG1, CLDN4, FGFR4, GJA7, HES3, KIRREL2, NR5A2, REEF5, STXBP6, and TRIM66) are initially expressed in stage 35 embryos in both dorsal and ventral pancreatic buds, with the exception of RND1, which is first detected in the dorsal pancreas and later becomes expressed in the ventral pancreas (Figure 2). We originally identified FGFR4 to be expressed in both the pancreas and the liver based on microarray data. However whole mount *in situ* hybridization of stage 35 embryos revealed that this gene is initially detected in the dorsal and ventral pancreatic buds, but later is expressed in other tissues. During subsequent stages, morphogenetic movements of the endoderm rotate the dorsal and ventral pancreatic buds toward one another on the left side of the trunk (compare stage 35 to stages 40–43). The pancreatic buds fuse with one another medial to the stomach by stage 40 (Kelly and Melton, 2000). Figure 3 shows expression of nine other genes (CBLB, FZD10, KIAA0133, KIAA0152, PCTK2, PDCD4, PLXNB1, PLXND1, and RBPJ) that are initially detected in the dorsal and ventral pancreas of stage 40 embryos. Additionally, several genes belonging to the PL group (CITED2, FAM46C) and the PS group (ICA1, MDK) are detected subsequently (st 42, Figure 4).

Genes expressed in the pancreas

RND1- and PLXNB1-mediated signaling—We detected specific expression of RND1 and PLXNB1 in the developing pancreas of stage 35 and stage 40 embryos, respectively (Figures 2 and 3). Since both PLXNB1 and RND1 physically interact to regulate cell

contraction in cell culture assays (Oinuma et al., 2003), it is tempting to propose the involvement of a RND1-PLXNB1 signaling pathway in pancreas morphogenesis. Recently, RND1 was also shown to interact with FLRT3 (Fibronectin Leucine-rich Repeat Transmembrane 3), a type I transmembrane protein containing extracellular leucine-rich repeats (Ogata et al., 2007). FLRT3 and RND1 physically interact to modulate cell adhesion during early embryogenesis by controlling the cell surface levels of cadherin through regulated endocytosis. RND1- and PLXNB1-mediated signaling may play roles in coordinating pancreatic morphogenesis.

KIRREL2 in pancreas development—The KIRREL gene family encodes transmembrane proteins with 5 Immunoglobulin (Ig) -like domains (Sun et al., 2003), suggesting possible roles as receptors or cell adhesion molecules. At stage 35 and afterward, KIRREL2 is expressed both in the ventral and dorsal pancreas (Figure 2 and suppl Figure 3) but not before. At stage 25 KIRREL expression is also detected in the pronephros, the brain, eyes, and the posterior tip of the notochord of early tailbud stage embryos (suppl Figure 3) and the expression persists until stage 40. In human, there are three KIRREL genes (KIRREL, KIRREL2, and KIRREL3), and KIRREL2 is expressed specifically in β cells of human adult pancreas (Sun et al., 2003). While the *Xenopus* KIRREL family member discussed here is the ortholog to human KIRREL2, it is yet to be determined whether *Xenopus* KIRREL2 is similarly expressed in the β cells of adult *Xenopus* pancreas.

FGF signaling in pancreas development—FGF signaling has been implicated in mediating epithelial-mesenchymal interactions in the developing pancreas (Miralles et al., 1999) and promotes the expansion of pancreatic progenitor cells (Papadopoulou and Edlund, 2005). We identified FGFR4 expression in both the liver and pancreas of developing *Xenopus* (Figure 2). This finding is consistent with a previous report that FGFR4 is expressed in the liver and the endodermal epithelium of the E12–14 mouse pancreas (Stark et al., 1991). In mice, FGF10 has been implicated in the normal growth of early pancreas buds (Bhushan et al., 2001), and the maintenance of PTF1A expression in the dorsal pancreatic bud (Jacquemin et al., 2006). Misexpression of FGF4 in the early pancreas using the PDX1 promoter was shown to disrupt pancreas development (Dichmann et al., 2003). In zebrafish, FGF10 and FGF24 are shown to display redundant activity in patterning the pancreatic lateral plate mesoderm (Manfroid et al., 2007).

FZD10 and WNT signaling in pancreas development—While numerous studies have demonstrated roles for Wnt signaling in pancreas development, the precise functions of this pathway are yet to be determined. Gene targeting of β-catenin in the pancreas using PDX1-Cre recombinase suggests that s-catenin is required for development of the exocrine pancreas but not the endocrine cells (Dessimoz et al., 2005; Murtaugh et al., 2005; Wells et al., 2007). In a more recent study, Wnt signaling has been implicated in islet β cell proliferation (Rulifson et al., 2007). Our clustering analysis (Suppl Table 1) shows that FZD10 belongs to Group P. However, whole mount *in situ* hybridization revealed that FZD10 is weakly expressed in the liver as well (Figure 3). FZD10 was previously shown to mediate canonical Wnt signaling in the presence of Wnt7b and LRP5 (Wang et al., 2005).

Hedgehog signaling in pancreas development—Hedgehog (Hh) signaling likely plays multiple roles in pancreas development. Inhibition of sonic hedgehog (Shn) signaling in the endoderm is crucial for specification of the dorsal pancreas domain (Hebrok et al., 1998; Kim and Melton, 1998). Furthermore, appropriate levels of Hh signaling also appear to regulate cell proliferation/organ size, morphogenesis, and the function of pancreatic tissue at the later stages of development (Hebrok et al., 2000). Our clustering analysis (Suppl Table 1) has grouped the Hh ligands (Shh and Dhh) into Group S and grouped the Hh receptors (PTCH2, and SMO) into Group LS, and therefore these signaling components are lacking in the early pancreas, consistent with the observation that the inhibition of Hh signaling is important for early pancreas development.

RBPJ and HES3: Notch signaling—Loss-of-function experiments have shown that the Notch signaling pathway promotes the self-renewal of pancreatic progenitor cells and/orexocrine cell lineage commitment (Apelqvist et al., 1999, Jensen et al., 2000). We find that notch pathway genes such as HES3 (a *Xenopus* gene closely related to human HES5; Figure 2) and RBPJ (a nuclear component of the Notch signaling pathway; Figure 3), are expressed in the *Xenopus* pancreas, whereas DLL1 (a Notch/Delta ligand) is expressed in the stomach (Suppl Table 1). ESR10, another enhancer of split related gene closely related to HES3 and HES5, was previously shown to be expressed in the *Xenopus* pancreas (Chen et al., 2004). These data are consistent with the notion that Notch/Delta signaling and its downstream components such as HES3/5 and ESR10 play important roles in pancreas development.

Other novel genes expressed in developing pancreas

CITED2: CBP/p300-interacting transactivator has a Glu/Asp-rich carboxy-terminal domain 2 and encodes a transcriptional modulator. Knockout of the CITED2 gene results in embryonic lethality with heart and neural tube defects (Yin et al., 2002; Weninger et al., 2005; Chen et al., 2007). CITED2 is also required for mouse fetal liver development (Qu et al., 2007) and our microarray data suggest that it is expressed in the developing embryonic liver. While expression of CITED2 has been reported in the heart and the kidney of mouse and chick embryos (Dunwoodie et al., 1998; Schlange et al., 2000; Boyle et al., 2007), our finding of its expression in the pancreas (Figure 4) is novel.

PCTK2: PCTAIRE protein kinase 2 is a Cdc2-related protein kinase and is known to be expressed in terminally-differentiated neurons (Hirose et al., 1997). However, its expression in the pancreas (Figure 3) has not yet been reported. Since a related protein, PCTK1, was shown to play a role in membrane trafficking from endoplasmic reticulum to Golgi (Palmer et al., 2005), we speculate that PCTK2 might be involved in the similar secretory pathway during pancreas development.

REEP5: Receptor accessory protein 5 is a member of the Receptor Expression Enhancing Protein (REEP) family. REEP1 is involved in cell surface expression of odorant receptors that belong to the G-protein coupled receptor (GPCR) superfamily (Saito et al., 2004). Specific expression of REEP5 in the pancreas (Figure 2) has not been reported previously.

TRIM66: Tripartite motif containing 66, also known as transcriptional intermediary factor 1 delta (TIF1delta), has been implicated in heterochromatin-mediated gene silencing (Khetchoumian et al., 2004). TRIM66 was reported to be expressed in testes (Khetchoumian et al., 2004), but its expression in the pancreas (Figure 2) has not been described.

STXBP6: Syntaxin binding protein 6, also known as amisyn, was isolated as a syntaxin-binding protein enriched in brain (Scales et al., 2002) and is thought to act as an inhibitor of exocytosis (Constable et al., 2005). STXBP6 is also expressed in the cement gland, an amphibian-specific transient structure that is notable for being highly secretory. STXBP6 is also expressed in cranial ganglia before mid-tailbud stage (Suppl Figure 5). At the mid-tailbud stage (stage 30–31), STXBP6 expression is first detected in the ventral pancreas (Suppl Figure 5), and later (stage 35) in both pancreatic buds (Figures 2 and Suppl Figure 5). At stage 40, STXBP6 expression is maintained in the cement gland, cranial ganglia, and pancreas (Figure 2 and Suppl Figure 5).

BTG1: B-cell translocation gene 1 is a member of the family of antiproliferative genes. BTG1 expression is maximal at the G0/G1 phases of the cell cycle and decreases when cells progress through G1 (Rouault et al., 1992). It interacts with a variety of transcription factors including retinoic acid and estrogen receptors, MyoD, and CAF1 (CCR4 associated factor 1), to regulate cellular differentiation (Busson et al., 2005; Prevot et al., 2001). While BTG1 is expressed in the adult pancreas, and weakly in the stomach and the liver (Prevot et al., 2001), the function of BTG1 in the embryonic pancreas (Figure 2) is unknown.

CBLB: Cas-Br-M (murine) ecotropic retroviral transforming sequence b is an E3 ubiquitin ligase and is expressed in the embryonic pancreas (Figure 3). CBLB is involved in the negative regulation of autoimmunity (Bachmaier et al., 2000; Chiang et al., 2000) and has been suggested as a susceptibility gene for Type 1 diabetes based on its association with autoimmunity in animal models and its role in T-cell co-stimulatory signaling (Yokoi et al., 2002). However, no naturally occurring mutations of CBLB have so far been linked to type 1 diabetes in human (Kosoy et al., 2004).

GJA7 and PLXND1: Gap junction protein alpha 7, also known as connexin 45, is a component of gap junctions. Its expression has been described in vascular cells of pancreatic islets (Theis et al., 2004) and is expressed in the embryonic *Xenopus* pancreas (Figure 2). GJA7-deficient mice have vascular defects and die between E9.5 and E10.5 (Kruger et al., 2000). Plexin D1 (PLXND1) is also involved in vascular patterning (Torres-Vazquez et al., 2004), and we find that it is expressed in both the developing liver and pancreas (Figure 3). GJA7 and PLXND1 may have their roles in vascular patterning during pancreas development.

ICA1: Islet Cell Autoantigen 1 is an arfaptin-related protein associated with membrane trafficking at the Golgi complex and immature secretory granules in neurosecretory cells (Spitzenberger et al., 2003). ICA1 is also known as ICA69, and is a candidate gene in autoimmunity associated with type I diabetes (Pietropaolo et al., 1993). The expression is particularly strong in adult pancreatic tissues (Mally et al., 1996), but we also find that 1CA1 is expressed during embryonic pancreas development (Figure 4).

MDK: Midkine is a heparin-binding growth factor involved in many biological processes including angiogenesis, cancer, and neural development (reviewed in Kadomatsu and Muramatsu, 2004). MDK has also been implicated in diabeticnephropathy by accelerating the intracellular signaling network evoked by hyperglycemia (Kosugi et al., 2006). We find that MDK is expressed in the pancreas and the stomach but not in the liver of *Xenopus* (Figure 4), consistent with the previous observations in mouse embryos (Kadomatsu et al., 1990). While the expression of MDK is up-regulated in pancreatic cancer (Aridome et al., 1995), its role during pancreas development is unclear.

NR5A2: Nuclear Receptor subfamily 5, group A, member 2 is a Ftz-F1-related nuclear orphan receptor B and expressed in the pancreas and the liver (Figure 2), and the similar expression patterns were observed in mice (Annicotte et al. 2003; Fayard et al., 2003). PDX1 controls the expression of NR5A2, which in turn controls the expression of CEL (Carboxyl Ester Lipase) (Fayard et al., 2003). In *Xenopus*, NR5A2 has been suggested to be a transcriptional activator (Ellinger-Ziegelbauer et al., 1994), but its exact function and spatiotemporal expression studies during organogenesis have not been examined. While NR5A2 deficient mice have been generated, its *in vivo* role during pancreas development has not been reported (Pare et al., 2004).

PDCD4: Programmed Cell Death protein 4 is a recently discovered tumorsuppressor protein that inhibits protein synthesis by interfering with the assembly of the cap-dependent translation initiation complex. (Cmarik et al., 1999). In connection with the pancreas, PDCD4 was shown to suppress the expression of carbonic anhydrase type II. Since tumor cells require a high bicarbonate flux for their growth, carbonic anhydrase suppression results in growth inhibition of endocrine tumors such as insulinomas and glucagonomas (Lankat-Buttgereit et al., 2004). PDCD4 expression during pancreas development (Figure 3) has not been reported in other animals, though PDCD4 seems to be expressed in a wide variety of tissues.

Other genes with unknown function: FAM46C (Family with sequence similarity 46, member C), KIAA0133, and KIAA0152 (Figures 3 and 4) are evolutionarily conserved genes, although their functions are yet to be determined. KIAA0152 is expressed in human pancreatic islets (Ylipaasto et al., 2005).

Why were some other known pancreas markers not in the list?—In *Xenopus*, transcription factors such as SOX9, PTF1A/P48, PDX1 (XLHBOX8) are all expressed in the developing pancreas, but these are not included in our gene lists. These genes were inaccessible to our microarray analysis as PTF1A and PDX1 were not included in the *Xenopus* Affymetrix version 1.0 microarray chip design. In the case of SOX9, the expression level in the pancreas was high, however its expression values in the liver and the stomach varied significantly between replicate experiments. Therefore, these data did not pass the standards we set for our ANOVA analysis (our cutoff was a p value ≤ 0.05).

Synexpression group genes—The term “synexpression groups” was coined to describe functionally related genes that share similar spatial expression patterns (Niehrs and Pollet, 1999). Thus, it was proposed that tissue specific patterns of coexpression of genes may be a

predictor for genes that work together in a common pathway or toward a common goal. Interestingly, many genes involved in steps of early neural patterning (e.g., NKX2.2, NKX6.1, NGN3, NEUROD, PAX6, PAX4) are also expressed in the pancreas, and this observation has led to the notion that neural and pancreatic genes/tissues may share a functional similarity, perhaps in regulating a group of genes controlling secretory function. Our current *Xenopus* situ hybridization study agrees with this view as a majority of the genes expressed in the *Xenopus* pancreas are also expressed in the brain (71 %, n=24). Along this theme, we also report a synexpression group of pancreas genes (BTG1, CLDN4, FZD10, GJA7, KIRREL2, RBPJ, and RND1) that are coexpressed at the tip of growing tail buds (Suppl Figures 1–3, and Suppl Figure 5).

Usefulness of the *Xenopus* system to study organ development—*Xenopus* has so far been under-utilized as a means of studying the processes of organogenesis (Blitz et al., 2006). However, among the advantages offered by *Xenopus* that could contribute significantly to a better understanding of the development of organs such as the pancreas is the provision of a fresh set of molecular markers. Availability of such markers would be useful to better define the developmental processes of differentiation, in this case, leading to the formation of a functional pancreas during embryogenesis. More significantly, identification of such markers could aid in the development of novel diagnostic tools for the detection of pancreatic diseases in humans and in manipulating the growth and differentiation of human and mouse stem cells to develop cures for pancreatic disorders such as diabetes mellitus. Since this strategy was successful in identifying genes expressed in the pancreas, we predict that *in situ* hybridizations for genes belonging to Groups L and S will similarly reveal new markers for liver and stomach development.

EXPERIMENTAL PROCEDURES

Isolation of endodermal organ buds

Xenopus laevis embryos were fertilized *in vitro*, dejellied in 2% cysteine hydrochloride (pH 7.8) and maintained in 0.1X Modified Barth's Saline (MBS) (pH 7.4). At stage 42, embryos were anesthetized in 0.01% ethyl 3-aminobenzoate methanesulfonate (Sigma, A5040) in 0.1X MBS. Then, the embryos were transferred to 1X MBS containing 0.01% ethyl 3-aminobenzoate methanesulfonate. Using watchmakers forceps the gut was removed from the rest of the tadpole tissues, and the pancreas, liver, and stomach buds were isolated and subjected to RNA isolation using TRIZOL reagent (Invitrogen).

GeneChip analyses

Total RNAs isolated from the endodermal organs were subjected to microarray analyses using *Xenopus laevis* Genome Arrays (Affymetrix, version 1.0). Two biologically independent experiments were carried out. Microarray data were refined and analyzed using Expressionist software (GeneData, Inc.). Analysis of variance (ANOVA) was performed to identify statistically significant genes (with p values less than 0.05) that are expressed differentially between the pancreas, liver, and stomach. Six gene groups representing enrichment in pancreas (P), liver (L), stomach (S), pancreas and liver (PL), pancreas and stomach (PS), liver and stomach (LS) were generated by k-means clustering.

Whole mount *In situ* hybridization

Whole mount *in situ* hybridization was carried out essentially as described previously (Harland, 1991). Embryos at various stages were fixed in MEMFA, washed in methanol, and stored in ethanol at −20°C. For the preparation of gut tissue, the anterior and posterior regions of embryos were removed by cutting transversely at the levels of heart and proctodeum, from which the ectodermal layer, notochord and somites were removed. cDNA clones were obtained from a *Xenopus* EST cDNA library (kind gift of Dr. Naoto Ueno, NIBB). To generate anti-sense *in situ* hybridization probes, cDNA inserts were PCR-amplified using M13 forward and reverse primers, and DIG-labeled RNA probes were directly generated using T7 RNA polymerase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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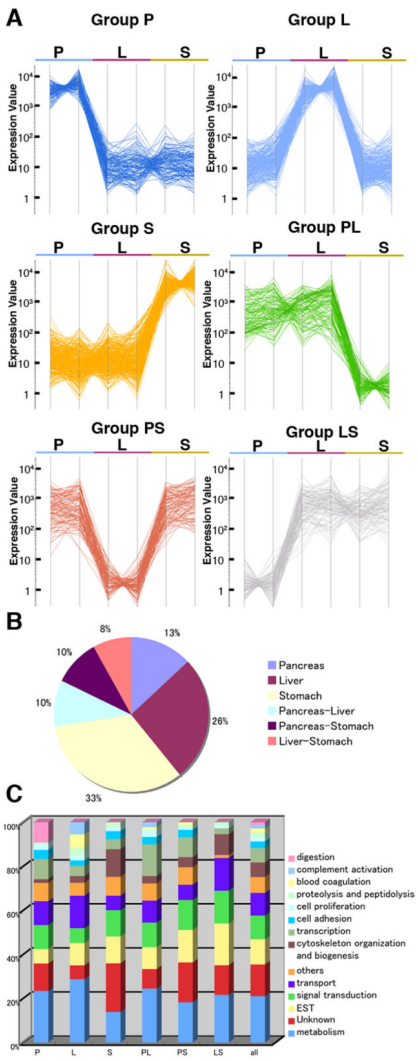

Fig. 1.

Clustering analysis of genes expressed in the embryonic pancreas, liver, and stomach. (A) Six expression groups generated by K-means clustering analysis. Individual line represents the expression level of a given gene in the pancreas (P), liver (L) or stomach (S). Abbreviations: PL, Pancreas and liver; PS, pancreas and stomach; LS, liver and stomach. (B) A chart indicating the percentages of six groups, which are represented by a total of 912**.** **(**C**)** Percentages of highly represented GO terms for each cluster.

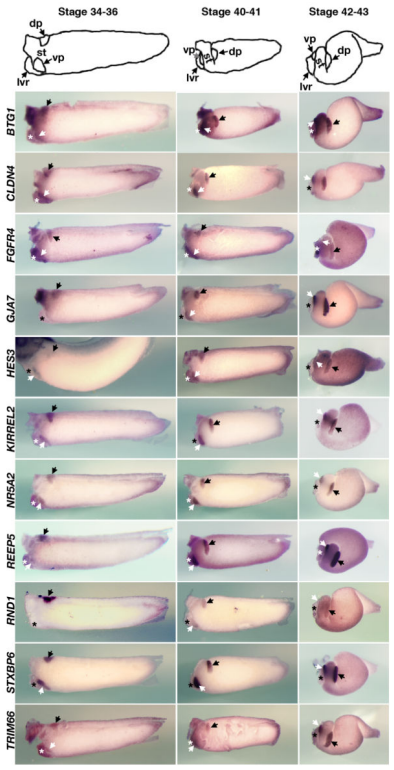

Fig. 2.

Expression of novel pancreatic genes in dissected guts of tadpole stage embryos. Gene expression patterns were revealed by whole mount *in situ* hybridization at three representative stages, stage 34–36, stage 40–41, and stage 42–43. These genes are expressed in the pancreatic rudiments of stage 34–36 embryos. The dorsal and ventral pancreatic buds are indicated with the black and white arrows, respectively and the liver bud is marked by an asterisk. A whole embryo was shown for HES3. While the *Xenopus laevis* gene designated here as HES3 (also known as *Xenopus* ESR2) has a best blastP match to human HES5, blastN to *X. tropicalis* and synteny relations identifies it as a gene distinct from *tropicalis* hes5 called hes3. Anterior to the left, dorsal to the top.

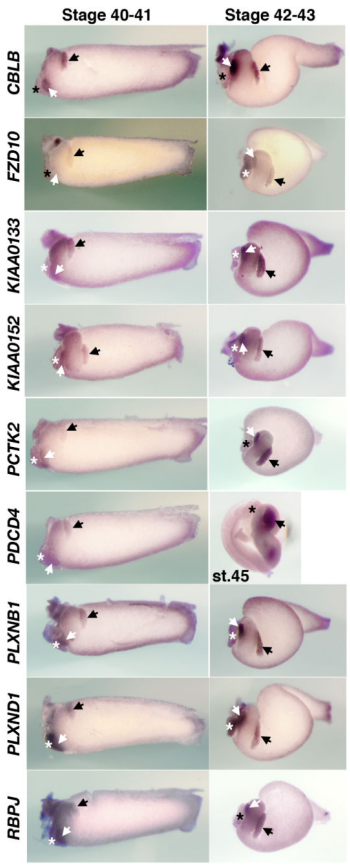

Fig. 3.

Expression of novel pancreatic genes in the gut of stage 40–43 embryos. The dorsal and ventral pancreatic buds are indicated with the black and white arrows, respectively and the liver bud is marked by an asterisk. PDCD4 expression shown is a stage 45 embryo,

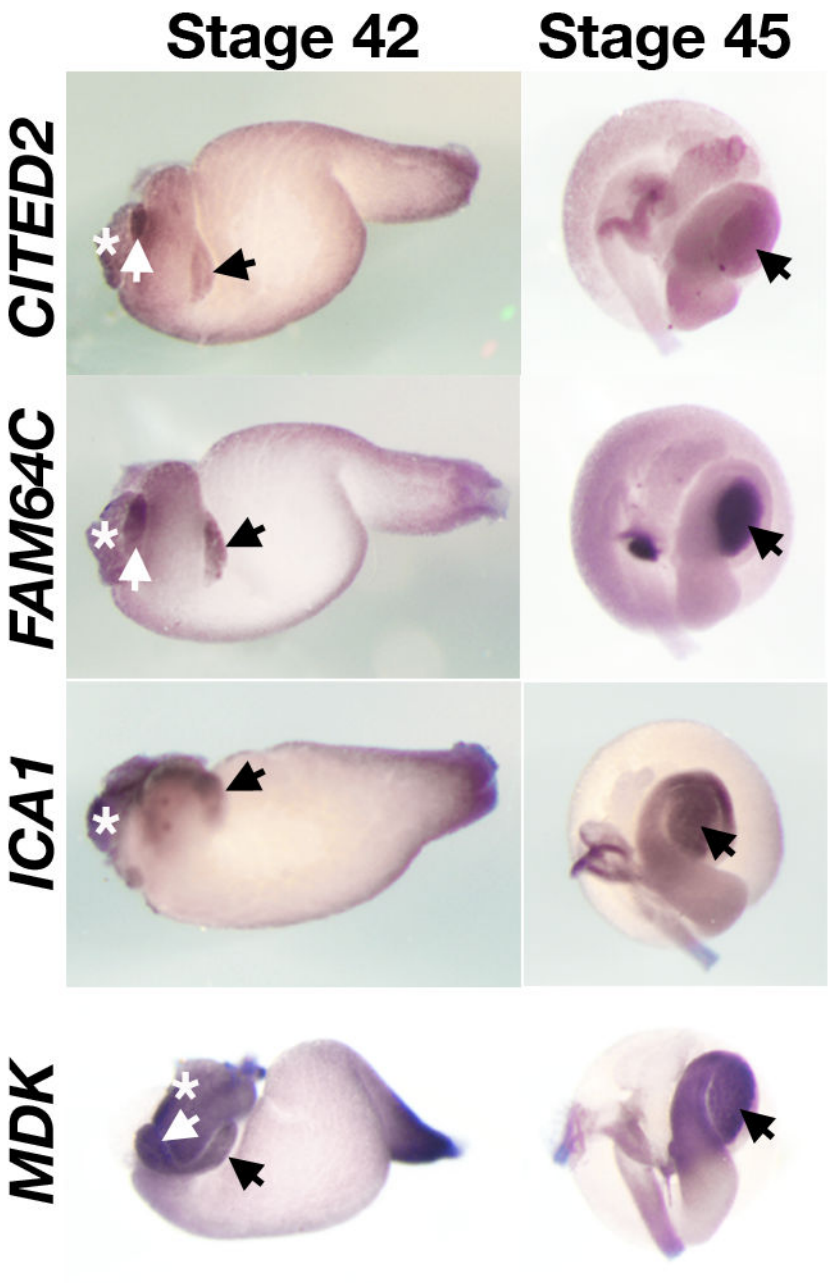

Fig. 4.

Expression of pancreatic genes in the gut of stage 42 and 45 embryos. The black arrows at stage 45 indicate the pancreas after the fusion of dorsal and ventral pancreas and the liver bud is marked by an asterisk.

Table 1

Partial list of known marker genes identified in this study.

Gene symbol	Gene description	Biological process	Group	Xenopus Unigene ID	References
AMY2A	amylase, alpha 2A; pancreatic	digestion	P	Xl.21603	Horb et al., 2002
CEL	carboxyl ester lipase (bile salt-stimulated lipase)	digestion	P	Xl.24591	Sogame et al., 2003
CLDN6	claudin 6	cell adhesion	P	Xl.4725	Park et al., 2007
CPA1	carboxypeptidase A1 (pancreatic)	digestion	P	Xl.26019	Kelly and
DNASE1	deoxyribonuclease I	digestion	P	Xl.932	Sogame et al., 2003
ELA1	elastase 1, pancreatic	digestion	P	Xl.24478	Horb et al., 2002
ELA2A	elastase 2A	digestion	P	Xl.23779	Sogame et al., 2003
HLXB9	homeo box HB9	transcription	P	Xl.286	Chen et al., 2004
INS	insulin	signal transduction	P	Xl.817	Kelly and Melton, 2000;
PDIA2	protein disulfide isomerase-associated 2		P	Xl.24424	Sogame et a
PRSS2	protease, serine, 2 (trypsin 2)	digestion	P	Xl.119	Horb et al., 2002
A2M	alpha-2-macroglobulin	transport	L	Xl.24285	Park et al., 2007
ALB	albumin	transport	L	Xl.395	Zorn and Mason, 2001
AMBP	alpha-1-microglobulin/bikunin precursor	anti-inflammatory response	L	Xl.26215	Zorn and Mason, 2001
BF	B-factor, properdin	complement activation	L	Xl.5152	Park et al., 2007
FGG	fibrinogen, gamma polypeptide	blood coagulation	L	Xl.5168	Zorn and Mason, 2001
HHEX	hematopoietically expressed homeobox	transcription	L	Xl.225	Zorn and Mason, 2001
KNG1	kininogen 1	blood coagulation	L	Xl.10766	Park et al., 2007
TBX2	T-box 2	transcription	L	Xl.931	Park et al., 2007
TF	transferrin	transport	L	Xl.534	Zorn and Mason, 2001
ATP4B	ATPase, H+/K+ exchanging, beta polypeptide	transport	S	Xl.241	unpublished
CTSE	cathepsin E	proteolysis and peptidolysis	S	Xl.35410	unpublished
STARD10	START domain containing 10	transport	S	Xl.2843	Park et al., 2007
SOX2	SRY (sex determining region Y)-box 2	transcription	S	Xl.188	Park et al., 2007
XBP1	X-box binding protein 1	transcription	PL	Xl.4624	Park et al., 2007
PAX6	paired box gene 6 (aniridia, keratitis)	transcription	PS	Xl.647	Kelly and Melton, 2000

Table 2

List of novel *Xenopus* pancreatic genes identified in this study

Gene symbol	Gene description	Biological process	Group	Xenopus Unigene ID	Clone
BTG1	B-cell translocation gene 1, anti-proliferative	transcription	P	Xl.25750	XL005n04
CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	protein ubiquitination	P	Xl.17445	XL013i01
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	transcription	PL	Xl.25598	XL019i09
CLDN4	claudin 4	cell adhesion	P	Xl.23306	XL001g02
FAM46C	family with sequence similarity 46, member C	unknown	PL	Xl.4393	XL197e22
FGFR4	fibroblast growth factor receptor 4	signal transduction	PL	Xl.1016	XL070o16
FZD10	frizzled homolog 10 (Drosophila)	signal transduction	P	Xl.559	XL048i11
GJA7	gap junction protein, alpha 7, 45kDa (connexin 45)	transporter	P	Xl.3337	XL038c12
HES3	hairy and enhancer of split 3 (Drosophila)	transcription	P	Xl.12067	XL031a05
ICA1	islet cell autoantigen 1, 69kDa	transporter	PS	Xl.70385	XL183c20
KIAA0133	KIAA0133	unknown	P	Xl.13330	XL045a04
KIAA0152	KIAA0152	unknown	P	Xl.2971	XL075f01
KIRREL2	kin of IRRE like 2 (Drosophila)	cell adhesion	P	Xl.76492	XL088m05
MDK	midkine (neurite growth-promoting factor 2)	signal transduction	PS	Xl.897	XL091d20
NR5A2	nuclear receptor subfamily 5, group A, member 2	signal transduction	P	Xl.12129	XL106i11
PCTK2	PCTAIRE protein kinase 2	cell cycle	P	Xl.55323	XL058l19
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	apoptosis	P	Xl.14956	XL214b12
PLXNB1	plexin B1	signal transduction	P	Xl.72590	XL084f22
PLXND1	plexin D1	signal transduction	PL	Xl.18576	XL073a19
RBPJ	recombining binding protein suppressor of hairless (Drosophila)	transcription	P	Xl.12006	XL202i12
REEP5	receptor accessory protein 5	signal transduction	P	Xl.8593	XL174p24
RND1	Rho family GTPase 1	signal transduction	P	Xl.517	XL001c12
STXBP6	syntaxin binding protein 6 (amisyn)	transporter	P	Xl.5823	XL004n21
TRIM66	tripartite motif-containing 66	transcription	P	Xl.24102	XL084d03