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Experimental Conversion of Liver to Pancreas

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Summary

Background: The liver and the pancreas arise from adjacent regions of endoderm in embryonic development. Pdx1 is a key transcription factor that is essential for the development of the pancreas and is not expressed in the liver. The aim of this study was to determine whether a gene overexpression protocol based on Pdx1 would be able to cause conversion of liver to pancreas.
Results: We show that a modified form of Pdx1, carrying the VP16 transcriptional activation domain, can cause conversion of liver to pancreas, both in vivo and in vitro. Transgenic *Xenopus* tadpoles carrying the construct *TTR-Xlhbox8-VP16:Elas-GFP* were prepared. *Xlhbox8* is the *Xenopus* homolog of *Pdx1*, the *TTR* (*transthyretin*) promoter directs expression to the liver, and the *GFP* is under the control of an elastase promoter and provides a real-time visible marker of pancreatic differentiation. In the transgenic tadpoles, part or all of the liver is converted to pancreas, containing both exocrine and endocrine cells, while liver differentiation products are lost from the regions converted to pancreas. The timing of events is such that the liver is differentiating by the time *Xlhbox8-VP16* is expressed, so we consider this a transdifferentiation event rather than a reprogramming of embryonic development. Furthermore, this same construct will bring about transdifferentiation of human hepatocytes in culture, with formation of both exocrine and endocrine cells.
Conclusions: We consider that the conversion of liver to pancreas could be the basis of a new type of therapy for insulin-dependent diabetes. Although expression of the transgene is transient, once the ectopic pancreas is established, it persists thereafter.

Introduction

A total of 150 million people worldwide currently suffer from diabetes, and that number is predicted to rise to 300 million over the next 20 years [1]. A minority are type 1 diabetics who usually require insulin treatment for survival. The majority are type 2 diabetics, a proportion of who have some element of pancreatic pathology and may also require insulin treatment [2]. Transplantation therapies for diabetes have been explored for many years, but islet cell transplants still involve serious problems of immune rejection and donor supply [3]. One way forward is to produce β cells in vitro for transplantation. Although this may eventually become possible with islet progenitor cells, adult stem cells, or embryonic stem cells, this work is currently at an early stage. An alternative method, which involves reprogramming liver cells into pancreas cells, would overcome the problems, but recent attempts to achieve this have also been unsuccessful.

"Transdifferentiation" is the name used to describe the conversion of one differentiated cell type to another [4–7]. It belongs to a wider class of cell type conversions known as "metaplasias," which also include switches in fate between stem cells of different tissues [4, 5]. Transdifferentiation and metaplasia are important for a number of reasons. Firstly, identification of the switches involved may provide information on the molecular basis of normal developmental mechanisms. Secondly, some metaplasias predispose to neoplasia, and so their prevention has a direct health benefit. For example, in Barrett's esophagus, the lower end of the esophagus is converted to stomach or intestinal-type tissue, and this can be a precursor to oesophageal adenocarcinoma [8]. Thirdly, and of much current interest, understanding the rules for transdifferentiation between cell types will improve our ability to reprogram stem cells or differentiated adult cells for the purposes of therapeutic transplantation.

An example of transdifferentiation that has been well studied is the conversion of pancreas to liver [9]. This has been described in both in vivo and in vitro experimental models. For example, copper deprivation in the rat induces hepatocytes in the pancreas [10, 11]. Our labs have recently produced an in vitro model for the transdifferentiation of pancreatic cells to hepatocytes based on culture with the glucocorticoid dexamethasone [12]. The hepatocytes are formed directly from exocrine cells, and this switch can be activated by the transcription factor C/EBPβ [12]. The reverse transdifferentiation of liver to pancreas has been found to occur in the livers of rats treated with polychlorinated biphenyls [13], in fish liver tumors induced by chemical carcinogens [14], and in the liver of a human patient with hepatic cirrhosis [15]. Endocrine tissue, however, was not present in any of these examples.

The pancreas and liver arise from adjacent areas in the anterior endoderm of the developing embryo [16]. It has been proposed that a common precursor cell exists and that signals from the cardiac mesoderm are responsible for specifying the two organs [17, 18]. In this model, the default state of the ventral foregut endoderm is to become pancreas, but an FGF-like signal released from the cardiac mesoderm diverts the fate of some cells into liver. These results suggest that the liver and pancreas differ by a single developmental decision, presumably affecting the expression of a relatively small number of genes, and that experimental interconversion between the two tissues should therefore be possible.

Numerous transcription factors have been identified that are known to play an important role in the specifica-

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tion of the different cell types within the pancreas [19–21]. Of these, the one that has the most likelihood of encoding the difference between liver and pancreas is Pdx1. It is expressed early in the endoderm prior to overt morphological development of the pancreas and has been shown to play a fundamental role in pancreatic development [22]. In *Pdx1* knockout mice, pancreatic tissue is missing. Although initial specification does occur, no mature pancreas is formed [23, 24]. In humans, the lack of a functional Pdx1 protein also results in agenesis of the pancreas [25]. Several groups have overexpressed *Pdx1* in various sites but have not so far succeeded in producing ectopic pancreas [26–28]. We felt that the lack of success in these experiments might be due to the lack of appropriate protein partners in the target tissues and have therefore taken a different approach by using a modified form of Pdx1 for which protein partners are unlikely to be necessary.

Using a tissue-specific promoter, we have produced transgenic *Xenopus* tadpoles that overexpress an activated form of the *Xenopus* homolog of *Pdx1*, *Xlhbox8-VP16*, in the liver. This causes a conversion of liver to pancreatic tissue, containing both exocrine and endocrine cell types. To confirm that this result is not limited to *Xenopus*, or to larval stages of development, we have also transfected *Xlhbox8-VP16* into the human hepatoma cell line, HepG2. This is also converted into pancreatic cells, which express either amylase or insulin. These results show that overexpression of an activated form of Pdx1 can induce the formation of pancreas from liver, in a process that we consider to be a transdifferentiation event. They demonstrate that it is possible to switch one tissue to another by overexpression of a single transcription factor and suggest a possible novel therapy for diabetes based on the transdifferentiation of liver cells.

Results

Xlhbox8-VP16 Causes Conversion of Liver to Pancreas

In order to produce a version of Pdx1 that would activate target genes without the need for protein partners, the VP16 activation domain from *Herpes simplex* virus [29–31] was fused to the C terminus of Xlhbox8, the *Xenopus* homolog of Pdx1 [32]. We then used a transgenic method [33] to express *Xlhbox8-VP16* in the liver of *Xenopus* tadpoles. The *Xlhbox8-VP16* is driven by the murine transthyretin (*TTR*) promoter [34], which we have previously shown to be liver-specific in *Xenopus* and to become active only after liver differentiation has begun [35]. To make it easier to identify the transgenic tadpoles and to monitor the efficiency of the process, GFP was placed under the control of an *elastase* promoter (*Elas-GFP*) and was included in the same transgene (Figure 1A). The *elastase* element used is a 213-bp fragment of the complete promoter that drives expression in both endocrine and exocrine cells [36]. Hence, all cell types in regions of pancreatic tissue are expected to express GFP. As shown previously in our lab, this *elastase* promoter drives expression of transgenes in both the dorsal and ventral pancreatic buds from stage 35 (2 days) [35].

Initially, liver and pancreas development occur normally in transgenic tadpoles containing *TTR-Xlhbox8-VP16*, with *Elas-GFP* only being expressed in the dorsal and ventral pancreatic buds (Figure 1D). From stage 45 (5 days), many individuals also show an anterior patch of fluorescence in the position normally occupied by the liver (Figures 1E–1I). In the method used, each transgenic tadpole represents a separate integration event, with differing gene copy numbers integrating into different sites in the host genome; thus, some variation in results is to be expected. The proportion of transgenic individuals showing ectopic pancreas is 61%, and these may be complete or partial transformations of liver to pancreas, with the rest of the tadpole appearing normal. In all positive cases, the ectopic pancreatic tissue, visualized by an ectopic patch of GFP fluorescence, is clearly distinguishable from the endogenous pancreas (Figure 1G).

To determine unequivocally whether the ectopic *Elas-GFP* expression represents true conversion of liver to pancreas, we examined the expression of several pancreatic differentiation products. Insulin and glucagon are the principal hormones produced, respectively, by β- and α-type endocrine cells. In normal tadpoles, *insulin* mRNA is detected in the dorsal pancreas prior to stage 47 (7 days) (Figure 2A) [37, 38]. In *TTR-Xlhbox8-VP16* transgenic tadpoles, ectopic *insulin* is also detected in the region of the liver (Figures 2B and 2C). *Glucagon* expression is normally found in small groups of cells in the pancreas beginning at stage 45, as well as in the enteroendocrine cells of the stomach and small intestine (Figure 2D) [37–39]. In *TTR-Xlhbox8-VP16* transgenic tadpoles, ectopic *glucagon* expression can also be seen in the normal position of the liver (Figures 2E and 2F). Amylase is a major product of the pancreatic exocrine cells [40]. In *TTR-Xlhbox8-VP16* transgenic tadpoles, *amylase* mRNA is expressed in the ectopic pancreatic tissue in identical regions to the *Elas-GFP* expression (Figures 2G–2I). In the example shown here, part of the liver has become pancreas, but the portion marked L does not express either GFP or *amylase* and is presumably unchanged. These results demonstrate that the region expressing GFP in the transgenics is differentiated pancreas, containing both exocrine and endocrine tissues.

To begin to understand the mechanism of the process, we have used RT-PCR to examine whether the endogenous *Xlhbox8* is activated in the liver upon transient expression of *Pdx1-VP16*. As shown below, the mouse Pdx1-VP16 is also active, and use of this transgene enables specific detection of the endogenous Xlhbox8 by RT-PCR. In *TTR-Pdx1-VP16:Elas-GFP* transgenic tadpoles, we find that the endogenous *Xlhbox8* is activated in the ectopic pancreas, but not in the normal liver (data not shown). This result suggests that conversion of liver to pancreas by the transgene occurs by activating the endogenous pancreatic transcription program.

Structure-Activity Relationship

In order to check that the result was not specific to the *Xenopus* form of *Pdx1*, we tested the activity of the mouse *Pdx1*. Like Xlhbox8, when Pdx1 is fused to VP16,

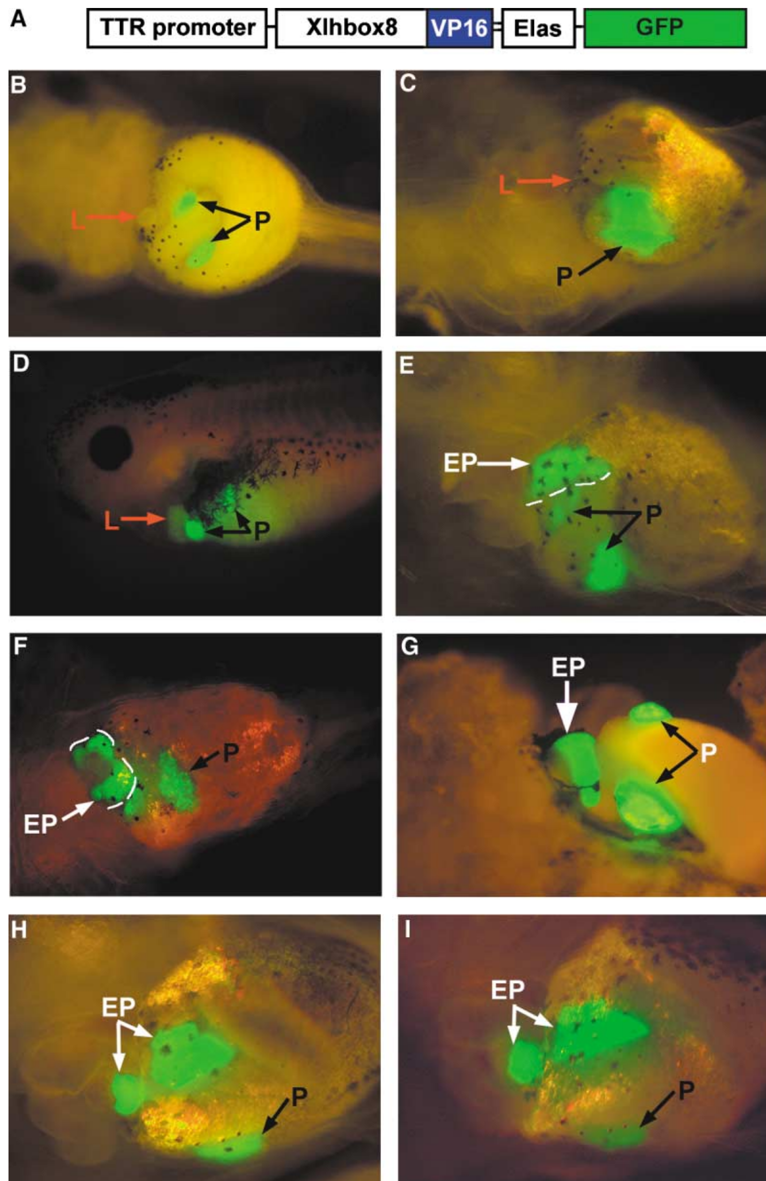

Figure 1. Xlhbox8-VP16 Is Able to Cause Conversion of the Liver into Pancreas

(A) A schematic of the transgene construct used. The 3-kb liver-specific promoter for *transthyretin* (*TTR*) is used to overexpress *Xlhbox8-VP16* in the liver. The 200-bp pancreas-specific elastase promoter driving GFP expression is used to distinguish transgenic from nontransgenic animals and to easily identify the ectopic pancreatic tissue in the liver.

(B and C) *Elastase-GFP* transgenic tadpoles at stage 42 (4 days) and stage 46 (6 days).

(D–I) *TTR-Xlhbox8:Elas-GFP* transgenic tadpoles. (D) At stage 40, GFP fluorescence is only seen in the pancreas. (The fluorescence visible in the yolk is background). (E–G) Stage-45–48 tadpoles (5–7 days). (E) Extra Elastase-GFP fluorescence (EP) can be clearly seen anterior and above the endogenous pancreas (P). The dotted white line separates the two. (F) Ectopic pancreatic tissue encircles a part of the liver that has not transdifferentiated and is located anterior to the endogenous pancreas. (G) Half of the former liver is expressing GFP. The dorsal and ventral pancreas buds are visible on either side of the gut tube. (H and I) A specimen at 6 days and 11 days, indicating long-term stability of the ectopic pancreas. L, liver; P, normal pancreas; EP, ectopic pancreas.

it is able to bring about conversion of liver to pancreas, while the unmodified Pdx1 cannot (Figure 3 and data not shown). To determine what portions of the coding region of Xlhbox8 are necessary, we investigated the biological activity of various modifications of Xlhbox8 (Figure 3). Two different DNA binding domain (DBD) mutants were constructed: first, the complete homeodomain was removed, and, second, a single amino acid change, which is known to abolish DNA binding, was produced within helix three of the homeodomain (WFQ**N**RR → WFQ**S**RR). When these constructs were overexpressed in the liver, ectopic pancreas was not produced in either case (Figure 3). We established that these fusion proteins are expressed, as shown by VP16 staining in HepG2 cells (see below).

Xlhbox8 belongs to the ParaHox subfamily, and numerous experiments have shown that the specificity of homeodomain proteins is generated by interactions with other transcription factors, including Pbx and Meis proteins [41, 42]. In fact, Pdx:Pbx binding has been shown to be important for making exocrine cells [43], as well as for the normal proliferation of pancreatic cells [44] and for transcriptional activity [45, 46]. The interaction of Pdx1 with Pbx occurs through the pentapeptide motif FPWMK located upstream of the homeodomain [41, 47]. We eliminated Pbx binding by changing FP**WMK** to FP**AAA,** but this did not reduce the biological activity (Figure 3).

The N-terminal activation domains of Xlhbox8 and Pdx1 have been shown to be essential to their functions [48]. We wished to determine the importance of this endogenous activation domain in the ability of *Xlhbox8-VP16* to convert liver to pancreas, especially since we had added on an extra activation domain. To test this, we made a construct in which the VP16 was placed at the N-terminal end and substituted for the first 90 amino acids. When overexpressed in the liver, this construct is active and is able to induce ectopic pancreas, but at a

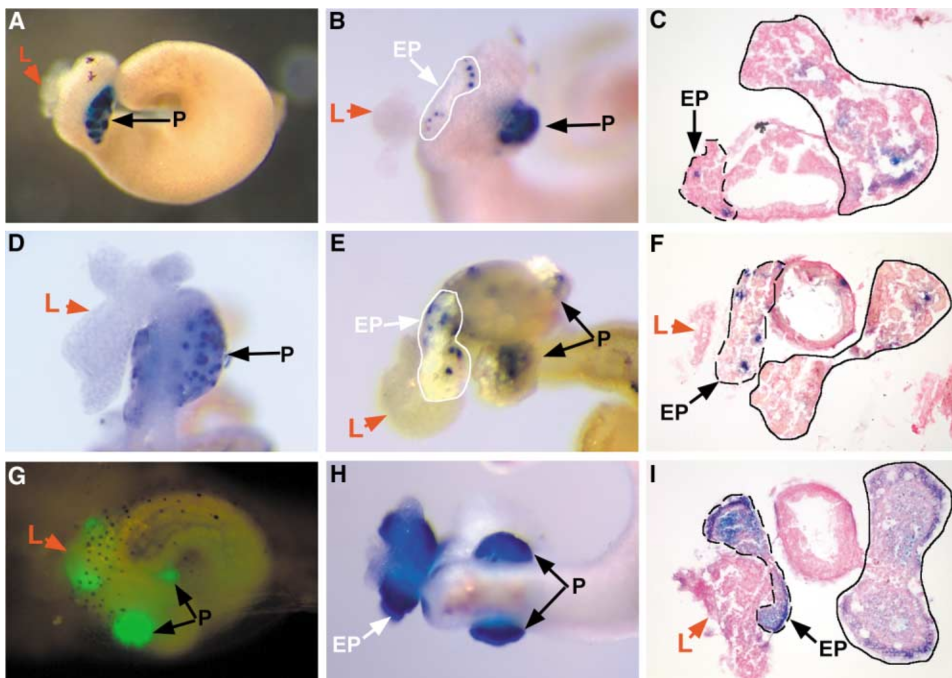

Figure 2. Endocrine and Exocrine Cells Are Both Present in Ectopic Pancreas

(A–F) Expression of endocrine genes. (A–C) *Insulin* RNA expression; (D–F) *glucagon* RNA expression. (A) Wild-type *insulin* expression. Expression is only seen in the dorsal pancreas (black arrow) at this stage in several prominent clusters. The liver (L) is located on the opposite side of the duodenum (red arrowhead). The two dark spots on the stomach are melanocytes and not insulin-stained cells. (B) *TTR-Xlhbox8-VP16* transgenic whole gut. Several ectopic clusters of *insulin* expression are seen in the ectopic pancreatic tissue (EP) opposite the pancreas (outlined in white). (C) Section of (B) showing the ectopic pancreas (EP, dotted line) opposite the endogenous pancreas (solid line) with two clusters of *insulin*-expressing cells. (D) Wild-type *glucagon* expression. Expression is seen in several spots within the pancreas. (E) *TTR-Xlhbox8-VP16* transgenic gut. Ectopic *glucagon* expression is found in the position of the liver (EP, outlined in white) opposite the normal pancreas. Note that glucagon cells are normally found in stomach and intestine, as well as pancreas, but not in the liver [37–39]. (F) Section of (E) showing glucagon expression within the ectopic pancreas (dotted line).

(G–I) Ectopic expression of exocrine markers in the liver of transgenic tadpoles. (G) Live image of a *TTR-Xlhbox8-VP16:Elas-GFP* transgenic tadpole, showing ectopic elastase-GFP fluorescence within the liver (red arrowhead), anterior to the normal pancreas (P). (H) *Amylase* RNA expression. Ectopic expression of *amylase* is seen in the position of the liver (EP) as well as in the normal pancreas (P), in an identical pattern to the Elas-GFP. (I) Section of (H) showing expression of *amylase* in a portion of the liver (L), as highlighted by the dotted line. Normal amylase RNA expression is seen throughout the pancreas (solid black line). L, liver; P, normal pancreas; EP, ectopic pancreas.

somewhat lower frequency than the construct described above, which contains the endogenous Xlhbox8 activation domain (Figure 3). Taken together, the results demonstrate that the DNA binding domain and the VP16 domain are essential for conversion of liver to pancreas, while the endogenous activation domain and the Pbx binding region are not necessary.

Liver Gene Expression Is Turned Off in the Ectopic Pancreas

To confirm that the ectopic pancreatic tissue has lost expression of liver genes, we examined the transgenic tadpoles for expression of the endogenous *Xenopus transthyretin* (*TTR*) mRNA [49]. At stage 45 in normal tadpoles, *TTR* mRNA is present only in the liver (Figure 4A) [50]. When individual *TTR-Xlhbox8-VP16* transgenic tadpoles are examined (Figures 4B and 4C), we find variations in endogenous *TTR* expression in a precise complementary pattern to the ectopic pancreatic tissue seen with *Elas-GFP* (see Figure 1). When the whole liver is converted to pancreas, no expression of endogenous *TTR* is detected (Figure 4B). However, when only half the liver has undergone conversion, we find *TTR* expression in the half that has not been changed to pancreas (Figure 4C). These results demonstrate that endogenous liver markers are quickly downregulated in the ectopic pancreas.

Pancreatic Tissue Arises from Differentiating Liver Cells

Because the experiments concern a larval form, the *Xenopus* tadpole, in which the tissues are not fully differentiated, we have investigated carefully whether the effect represents a transdifferentiation event or the alteration of the developmental fate of uncommitted endodermal tissue. Previous work has shown that liver differentiation products begin to be expressed at stage 32, about 2 days of development [50], and that by stage 40/41, 3 days of development, the liver has formed as a separate organ. We have compared the timing of *TTR* promoter activation with the temporal expression of two liver markers, *Xhex* (a homeobox gene) [51] and *AMBP* (α1-microglobulin/bikunin precursor) [52]. Both *Xhex* and *AMBP* mRNAs are specifically expressed in a region

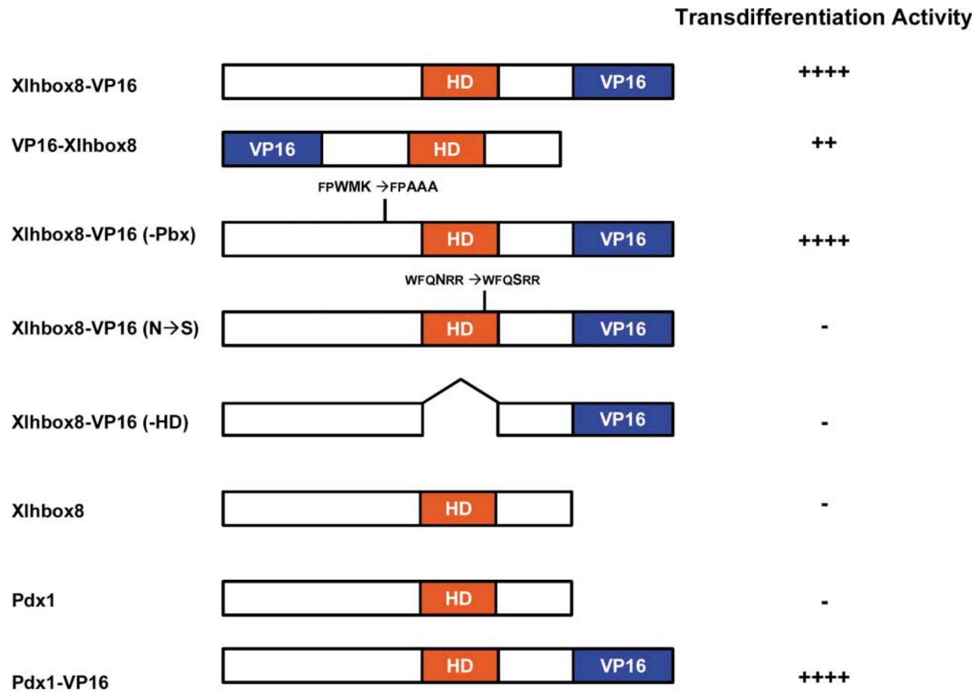

Figure 3. Domains Necessary for *Xlhbox8* to Convert Liver to Pancreas

This is a schematic diagram illustrating the different *Xlhbox8* mutants made with their biological activity. *Xlhbox8-VP16* is able to convert the liver to pancreas (68/112 cases), while *Xlhbox8* alone is not (0/20 cases). Replacement of the endogenous *Xlhbox8* activation domain with *VP16* reduces the ability of *Xlhbox8-VP16* to cause conversion of the liver by about 50% (6/18 cases). By mutating the pentapeptide motif FPWMK to FPAAA, Pbx binding is disrupted. This mutation has little effect on transdifferentiation (11/17 cases). Two DNA binding mutants were made: the first was a point mutation in helix 3 of the homeodomain changing the amino acid Asparagine to Serine, and the second removed the entire homeodomain. Neither of these mutants is able to convert the liver to pancreas (both 0/20 cases). The murine Pdx1 gene was also tested, and similar results were obtained to those with Xlhbox8. Pdx1-VP16 was able to cause conversion of liver to pancreas to a slightly lesser degree than Xlhbox8-VP16 (7/14 cases). The unmodified Pdx1 cDNA, however, was inactive (0/20 cases).

just posterior to the heart, and their presence identifies the future liver as early as stage 35 (2 days) (Figures 4E–4H). In contrast, the *TTR* promoter does not become active until stage 44 (4 days) (Figures 4I and 4J). This means that, prior to *TTR* promoter activation, both *Xhex* and *AMBP* have been expressed for 2 days, and the liver has been separated as a discrete organ for 1 day. Note that the expression of endogenous *TTR* is not quite the same as that of the *TTR* promoter we have used. In addition to the pattern shown here, the endogenous gene also shows an early phase of activation in the whole endoderm, excluding the liver [50].

When *TTR-Xlhbox8-VP16:Elas-GFP* transgenic tadpoles are examined at stage 40/41, before activation of the *TTR* promoter, the GFP fluorescence can be seen in the normal pancreatic buds, but not in the liver (Figure 1D). Cases fixed at this stage showed the presence of *Xhex* mRNA throughout the liver as normal and showed the presence of *GFP* mRNA only in the pancreas (Figure 4D).

In addition to this evidence that the liver is already differentiating, it is significant that, in some cases, there is a complete transformation of liver to pancreas. Although we cannot exclude the participation of some persisting undifferentiated endodermal cells in this process, the fact that the whole organ can be transformed means that at least some of the ectopic pancreas must have arisen from the differentiating hepatocytes.

Although it is true that the hepatocytes in the 4-day-old tadpole are not fully mature, and other liver cell types are not yet visible, these results all show that the liver is already differentiating by the time that expression of the transgene is initiated. We therefore feel that the effect is appropriately described by the term "transdifferentiation."

Xlhbox8-VP16 Causes Conversion of Human Liver Cells to Pancreas

In order to determine that the biological activity of *Xlhbox8-VP16* is not confined to *Xenopus*, or to the larval stages of development, we investigated the effects of transfecting the same construct into the human liver cell line, HepG2. This cell line shows a large number of properties of differentiated hepatocytes, including expression of albumin, transferrin, and transthyretin [53, 54], and it does not express specific pancreatic markers. As expected, the *elastase* promoter is not active in these cells (data not shown), but it is active in pancreatic cell lines such as AR42J [12]; however, the *TTR* promoter is expressed in HepG2 cells, but not in AR42J cells (see Figure 5D).

A total of 4–5 days after transfection of HepG2 cells with *TTR-Xlhbox8-VP16:Elas-GFP*, a number of cells positive for GFP fluorescence were clearly visible, indicating the activation of the *elastase* promoter. As mentioned above, the element used is active in both exocrine

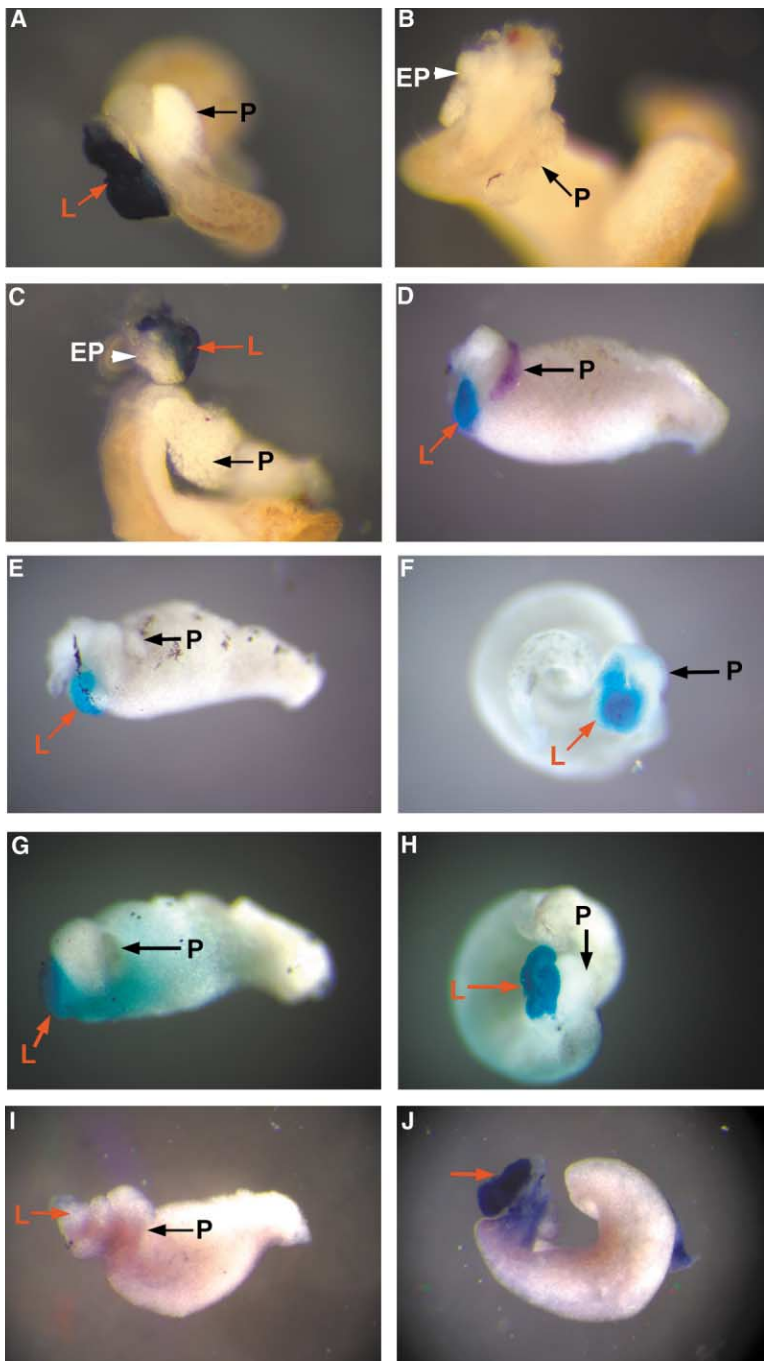

Figure 4. Liver Gene Expression in Transgenic Gut Preparations

(A–C) *Transthyretin* (*TTR*) RNA expression. (A) In wild-type preparations, expression is only seen within the liver. (B and C) *TTR-Xlhbox8-VP16* transgenics. (B) In this case, there is complete conversion of liver to pancreas, and *transthyretin* expression has been completely suppressed. (C) In this case, half the liver has changed into pancreas, and *transthyretin* expression is seen only in the other half. In all examples, nontransgenic guts were stained simultaneously with transgenic guts, and the staining reaction was stopped in both when the nontransgenic expression was clearly evident.

(D) Liver differentiation begins normally in *TTR-Xlhbox8-VP16* tadpoles. *Xhex* (blue) and *GFP* (magenta, driven by the elastase promoter) expression. *GFP* RNA is seen only in the dorsal and ventral pancreas at stage 40; no *GFP* expression is detected in the liver. *Xhex* expression is normal at this stage and is present throughout the whole liver.

(E–H) Timing of liver differentiation in normal nontransgenic tadpoles: from 3 days, the liver has become a separate organ, with *Xhex* and *AMBP* expressed throughout. (E) *Xhex* RNA at stage 40 (3 days). (F) *Xhex* RNA at stage 45 (5 days). (G) *AMBP* RNA at stage 40 (3 days). (H) *AMBP* expression at stage 45 (5 days).

(I and J) The *TTR* promoter becomes active in *Xenopus* tadpoles only after the liver has differentiated. This shows RNA in situ hybridization for GFP driven by the *TTR* promoter. (I) At stage 40 (3 days), no GFP expression is detected. (J) GFP expression is first detected at stage 44 (4 days).

and endocrine cells [36]. Immunostaining for amylase and for insulin showed that a proportion of the GFP-positive cells expressed one of these differentiation products (Figures 5B, 5C, 5E, and 5F), thus demonstrating that they are now differentiated pancreatic cells. This shows that the activity of the *Xlhbox8-VP16* construct is not confined to *Xenopus* but can also drive human liver cells to form both pancreatic exocrine and endocrine cells. A similar result has also been obtained for the rat FAO hepatoma line (data not shown).

To confirm that hepatocyte gene expression is being downregulated in HepG2 cells during the process, we have examined the expression of a liver protein, transferrin. HepG2 cells transfected with *TTR-Xlhbox8-VP16:Elas-GFP* were double stained for GFP (pancreatic fate) and transferrin (hepatic fate). Transferrin is abundantly expressed in nontransfected cells (Figure 6A). In cells undergoing conversion to pancreas, however, transferrin expression is downregulated (Figure 6B). These results are in agreement with the *Xenopus* results and show that, upon conversion to pancreas, hepatocyte gene expression is shut off.

In order to quantify the effect, we compared the proportion of green fluorescent cells produced by transfection of *TTR-Xlhbox8-VP16:Elas-GFP* with the proportion obtained by transfection of *CMV-GFP*. Assuming that

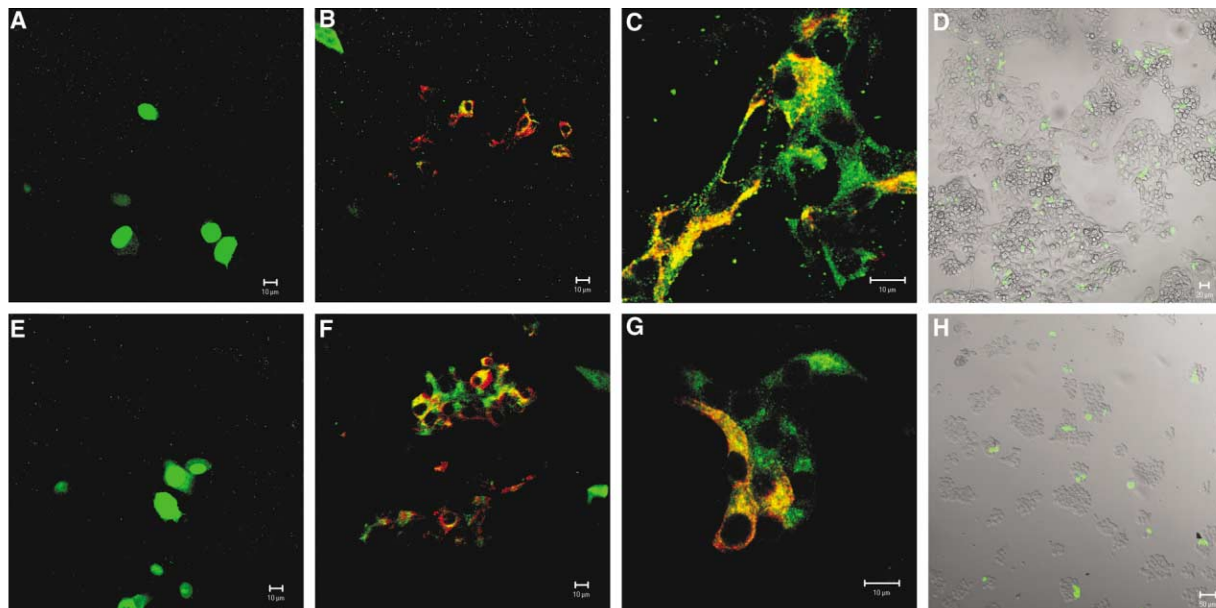

Figure 5. Conversion of Human Liver HepG2 Cells to Pancreatic Endocrine and Exocrine Cells

(A–H) HepG2 cells were transfected with either control plasmid *CMV-nGFP* or the *TTR-Xlhbox8-VP16:Elas-GFP* construct and were immunostained for either (A–C) GFP and Insulin (5 days) or (E–G) GFP and Amylase (4 days). All pictures were taken with the confocal microscope; (D) and (H) have bright field overlay. (A and E) HepG2 cells transfected with *CMV-nGFP* are only positive for GFP. (B, C, F, and G) HepG2 cells transfected with *TTR-Xlhbox8-VP16:Elas-GFP* show both (B and C) Insulin- and (F and G) Amylase-positive cells that colocalize with GFP. A total of 15% (14/94) of the GFP-positive cells were also insulin positive; 12% (11/90) of the GFP-positive cells were also amylase positive. The scale bars for (A)–(C) and (E)–(G) represent 10 μm. (D) HepG2 cells transfected with *TTR-nucGFP* demonstrating activation of the TTR promoter. The scale bar represents 20 μm. (H) Live image of AR42J-B13 cells transfected with *TTR-Xlhbox8-VP16:Elas-GFP* showing that the elastase promoter is active in AR42J cells. The scale bar represents 50 μm.

the transfection efficiency is the same for the two constructs, this should give the proportion of cells receiving *TTR-Xlhbox8-VP16:Elas-GFP* that are converted from a hepatic to a pancreatic phenotype (Figures 6C–6E). This proportion is about 65%, similar to the proportion of *Xenopus* tadpoles in which part or all of the liver is converted to pancreas (Figure 6C). In Figures 6F and 6G, cells transfected with *TTR-Xlhbox8-VP16:Elas-GFP* and coimmunostained for VP16 protein and GFP are shown. These results concur in showing that a high proportion of differentiated liver cells receiving the construct are converted into pancreatic cell types.

Discussion

Our results demonstrate that it is possible to bring about conversion of liver to pancreas by overexpression of an activated form of Pdx1, both in vivo and in vitro. The *Xenopus* experiments clearly show the formation of ectopic whole pancreas in a normal, intact, vertebrate organism. The HepG2 experiments show that the same construct can produce a very similar effect in human liver cells, and the results from these experiments demonstrate that the result is a general one and not restricted to *Xenopus*, or to the larval stage of vertebrate development. A recent paper [55] has shown formation of pancreatic cells from a type of liver stem cell, hepatic oval cell, following long term culture in a high-glucose medium.

We believe that the process is a genuine transdifferentiation. In the *Xenopus* tadpole, the cells we are changing from liver to pancreas have already commenced differentiation, and we have confirmed here that liver differentiation products are already expressed for about 2 days prior to the activation of the *TTR* promoter (Figure 4). We cannot exclude the presence of some residual undifferentiated cells in the liver in addition to the differentiated cells, but the fact that a complete organ transformation is possible in some cases argues against pancreatic tissue arising only from undifferentiated stem cells in the liver.

The *TTR* promoter used is inactive in the pancreas, and, when it is used to drive *Xlhbox8-VP16* in the liver, it rapidly becomes shut off as the cells are converting to pancreas. This means that the expression of the Xlhbox8-VP16 protein is only transient. The ectopic pancreas, however, persists, showing that, once the ensemble of endogenous pancreatic regulatory genes have been activated by Xlhbox8-VP16, the transdifferentiation becomes irreversible. If the procedure were one day developed into a therapeutic method, this fact indicates that only a transient expression of the construct would be necessary. This would enable a wide variety of gene delivery methods to be employed, and it means that the procedure is more likely to be a safe one, since no permanent genetic changes would be required.

An Extra Activation Domain Is Essential to the Activity of Pdx1

We have not succeeded in obtaining pancreatic transdifferentiation of the liver in either *Xenopus* or mammalian cells by using unmodified *Pdx1*, and this accords with

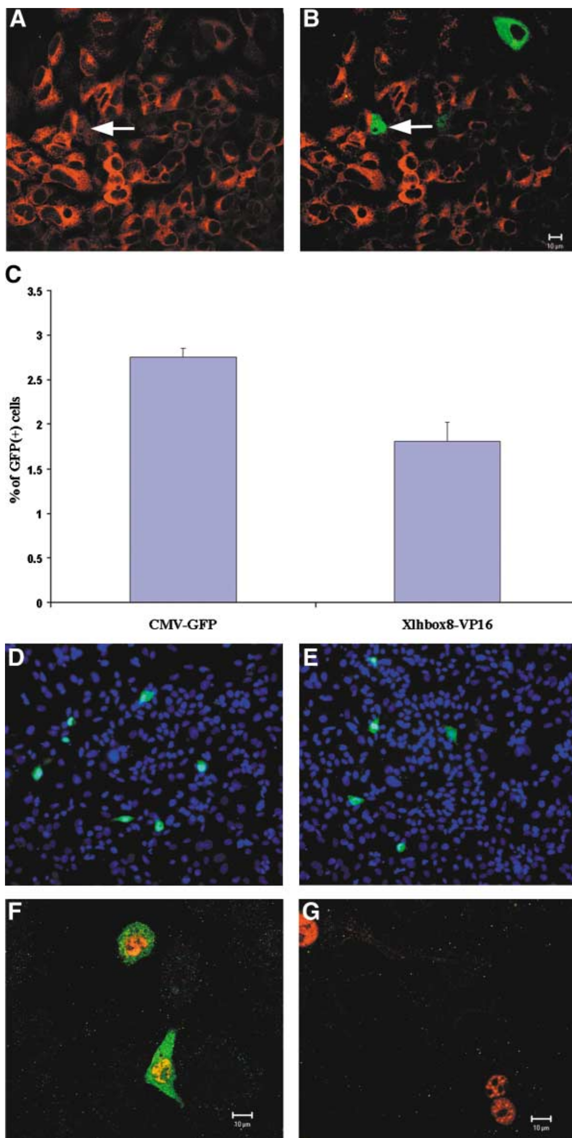

Figure 6. Estimate of the Proportion of Cells Transfected with *TTR-Xlhbox8-VP16:Elas-GFP* that Are Converted to Pancreas

(A and B) *TTR-Xlhbox8-VP16:Elas-GFP*-transfected HepG2 cells immunostained for either (A) Transferrin or (B) Transferrin and GFP. The cells undergoing transdifferentiation to pancreas have downregulated transferrin expression (arrow).

(C–G) (C–F) Estimate of the proportion of cells transfected with *TTR-Xlhbox8-VP16:Elas-GFP* that are transdifferentiated. (C) The histogram shows aggregated counts of GFP-positive cells in five random fields; the bars indicate the standard deviations. Assuming a similar transfection frequency, this shows that the proportion of transdifferentiation of cells transfected with *Xlhbox8-VP16* is about 65%. (D) and (E) show fields of cells stained with DAPI and transfected with either (D) *CMV-nGFP* or (E) *TTR-Xlhbox8-VP16:Elas-GFP*. (F and G) Immunostain for VP16 protein (red) and GFP (green) in cells transfected with either (F) *TTR-Xlhbox8-VP16:Elas-GFP* or (G) *TTR-Xlhbox8-VP16:Elas-GFP(N → S)*. The presence of VP16 staining in (G) demonstrates that the fusion protein is produced. A similar result was also obtained with *TTR-Xlhbox8-VP16:Elas-GFP(−HD)* and *TTR-Xlhbox8-VP16:Elas-GFP(−Pbx)*. (A), (B), (F), and (G) are all confocal images.

the experience of other workers. Grapin-Botton et al. overexpressed *Pdx1* in the chick embryo gut by using electroporation [27]. No ectopic pancreas markers were activated, although endogenous gut markers were downregulated. Heller et al. used the *Hoxa4* promoter to drive expression of *Pdx1* in the mesoderm of the stomach and hindgut [28]. Again, no ectopic pancreas markers were activated, although defects were observed within the *Hoxa4* domain. The experiments by Ferber et al. used an adenovirus to introduce *Pdx1* into the liver [26]. There was some insulin synthesis by a small proportion of liver cells in vivo, but no evidence was presented for any actual transdifferentiation of liver cells to pancreas cells. Furthermore, no insulin was detected in hepatocytes infected with *Pdx1* in vitro. As well as being necessary for early pancreatic development, Pdx1 is also a specific transcription factor for the insulin gene itself [56], and it is possible that the formation of insulin in the in vivo experiments of Ferber et al. simply demonstrates a low ectopic activation of the insulin gene rather than true transdifferentiation.

There are several reasons to explain why our modification of Pdx1 is active while the unmodified Pdx1 is not. First, the necessary transcription partners required for Pdx1 to bring about normal differentiation of the pancreas are absent from the liver. Pdx1 and other homeodomain proteins have been shown to require interactions with other proteins, such as Pbx and Meis, for proper function [42]. Without these protein partners, overexpression of the unmodified Pdx1 is insufficient to activate the pancreatic differentiation program, while VP16 can activate transcription in the absence of protein partners. Second, regulation of transcription by sequence-specific DNA binding proteins requires additional factors, termed coactivators [57]. The activity of Pdx1 may therefore require certain coactivators or adaptors, such as a particular histone acetyltransferase, that are not expressed in the liver. The addition of VP16 may overcome this lack of coactivators, as VP16 is known to interact directly with transcriptional adaptors [58]. Third, the chromatin surrounding the downstream targets of Pdx1 may be in a repressed state. Epigenetic regulation of transcription during development and differentiation maintains the cellular phenotype over many generations, and this is thought to be regulated partly at the level of chromatin [59]. For example, the Polycomb group of proteins is thought to function to maintain a repressive chromatin structure [60]. This regulation is thought to prevent inappropriate activation of one program in an ectopic location; such activation can be harmful, as seen in neoplastic transformations. VP16 is known to interact with chromatin remodeling complexes, resulting in increased transcription and histone acetylation [61, 62]. Fourth, other proteins that act as repressors may bind to Xlhbox8 in the liver and prevent it from activating the pancreatic transcription cascade on its own. Since the liver and pancreas arise from adjacent positions in the early embryo, the presence of a repressor in the liver may be important to restrict the development of the pancreas and prevent ectopic activation of the pancreatic differentiation program in the liver. Fifth, Xlhbox8 may be targeted for destruction by ubiquitination in liver cells, thus again preventing ec-

topic activation. This has been shown to occur with GCN4, E2F-1, and even VP16 [63–67]. All of the above reasons lead to the conclusion that tight control of transcription factors within their allotted domains is essential for correct development and differentiation to occur. By fusing VP16 to Xlhbox8, we have been able to bypass these various checkpoints and induce the ectopic activation of the pancreatic differentiation program in the liver.

Conclusions

Our current results demonstrate that a transient overexpression of Xlhbox8-VP16 is sufficient to convert differentiating or differentiated liver cells into pancreatic cells, producing both endocrine and exocrine cell types. The ability to convert liver to pancreas may provide a useful therapy for diabetes. These results, in combination with our previous results [12], suggest that it may be possible to bring about the transdifferentiation of other tissue types by using specific promoters and transcription factors. Our understanding of the normal hierarchy of cellular commitment [68] indicates that it should be easiest to interconvert tissues that are closely related in development, such as liver and pancreas, since their formation should depend on differential expression of the smallest number of transcription factors. In principle, the most useful factors should be those that normally act at the earliest developmental stage to distinguish two tissue types. But more complex protocols involving modified transcription factors or combinations of factors could bring about transdifferentiation between more distantly related tissues as well. The prospect of tissue replacement by transdifferentiation is a new area of therapy and opens up wide ranging possibilities for regenerative medicine.

Experimental Procedures

Transgenesis

Transgenic *Xenopus laevis* tadpoles were produced according to Kroll & Amaya [33] with slight modifications as described in [35], except for the following two changes. First, the high-speed cytoplasmic egg extract was heat treated for 8 min at 80°C and was recentrifuged at 70,000 rpm for 10 min. A total of 10 μl cytoplasmic extract was then used for each reaction. Second, no restriction enzymes were included in the incubation of sperm and transgene DNA. A total of 5–10 μg transgene DNA was linearized with either SacII or NotI, extracted with phenol/chloroform, and ethanol precipitated. The DNA was resuspended to a final concentration of 0.5 μg/μl; 0.5 μg DNA was used to make transgenics. To visualize GFP fluorescence, tadpoles were anaesthetized in a 1:2000 dilution of MS-222 (3-aminobenzoic acid ethyl ester, Sigma) and were visualized by using a Leica Fluo III fluorescent-dissecting microscope.

Transgene Constructs

TTR-Xlhbox8-VP16:Elas-GFP was constructed as follows. Full-length *Xlhbox8* was PCR isolated from *Xlhbox8-CS2* (kind gift of C. Wright) by using the SP6 primer and a 3′ primer with a ClaI site, 5′-TTTATCGATTTCTGCCTGCC-3′. It was cut with ClaI and subcloned into the ClaI site of *VP16-N* (kind gift of D. Kessler). The 3-kb TTR promoter (kind gift of R. Costa) was cut with BamHI and filled in with Klenow and cloned upstream of *Xlhbox8-VP16* in the HindIII (blunt) site. Lastly, the Elastase-GFP (kind gift of G. Swift) was excised with NotI and cloned into the NotI site of *TTR-Xlhbox8-VP16*. The resultant plasmid, *pCS2-TTR-Xlhbox8-VP16:Elas-GFP*, is 10.5 kb. For *TTR-GFP* and *Elas-GFP*, the transgene DNA was digested as described in [35].

The FPWMK → FPAAA PBX binding mutant was made by PCR isolating separate 5′ and 3′ ends of *Xlhbox8-VP16* and then ligating them together. The 5′ end was isolated by PCR with the SP6 primer plus the WMK → AAA reverse primer, 5′-TTTGGTGGATGCGGCCGCTGGGAAAGG-3′. The 3′ end was isolated by PCR with the WMK → AAA forward primer, 5′-CCTTTCCCAGCGGCCGCATCCACCAAA-3′, plus an internal VP16 primer. The 5′ PCR product was cut with ClaI and NotI, and the 3′ PCR product was cut with NotI-SacI and inserted into the ClaI-SacI site of pBS KS. This WMK → AAA mutant was then cut with ClaI and cloned into the ClaI site of *TTR-Xlhbox8-VP16:Elas-GFP*. The DNA binding mutants of Xlhbox8 were made as follows. The N → S amino acid change was made by a similar procedure used for the WMK → AAA mutant. The 5′ end of *Xlhbox8* was PCR isolated with the SP6 primer plus the N → S reverse primer, 5′-CTTCATTCTTCTAGACTGGAACCA-3′, containing an internal XbaI site. The 3′ end was PCR isolated with the N → S forward primer, 5′-TGGTTCCAGTCTAGAAGAATGAAG-3′, containing an internal XbaI site plus an internal VP16 primer. Both PCR products were cut with ClaI and XbaI and inserted into the ClaI site of *TTR-Xlhbox8-VP16:Elas-GFP*. The homeobox deletion was made as follows. The 5′ end of Xlhbox8 was PCR isolated with the X8-160XbaRev primer, 5′-CTCTAGTTCTAGAAGCTGGGCT-3′, which contains an internal XbaI site. The 3′ end was PCR isolated as above with the X8-216XbaFor primer, 5′-GGCAGTGATCTAGAACAAGACTC-3′, which contains an internal XbaI site. Both 5′ and 3′ ends were cut with ClaI and XbaI and inserted into the ClaI site of *TTR-Xlhbox8-VP16:Elas-GFP*.

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed according to [69] by using digoxygenin- and fluorescein-labeled probes. All series included positive controls to ensure consistency in staining intensity between transgenic and nontransgenic whole guts. *Xenopus amylase* was cloned by degenerate PCR into pCR Script as described by Horb and Slack [37]. For antisense RNA, it was linearized with EcoRV and transcribed with T3. *Xenopus insulin* was cloned by PCR based on published sequence (ntds 3–752) into pCR Script and was linearized with EcoRI and transcribed with T3. *Xenopus glucagon* was cloned by PCR based on published sequence (ntds 1–1151) into pCR Script and was linearized with NotI and transcribed with T7. *Xenopus transthyretin* (*TTR*) was cloned by PCR based on published sequence (ntds 10–500) into pCR Script and was linearized with EcoRI and transcribed with T3. *Xenopus hex* (*Xhex*) (kind gift of P. Krieg) was linearized with NotI and transcribed with T7. *Xenopus α1-microglobulin/bikunin* (*AMBP*) (kind gift of A. Kawahara) was linearized with SacI and transcribed with T7. For double-in situ hybridization, fluorescein-labeled probes were detected first with 175 μg/ml magenta phosphate (5-bromo-6-chloro-3-indolyl phosphate, Molecular Probes Europe) and heat inactivated, and then the digoxygenin-labeled probes were detected with 175 μg/ml BCIP.

Transfection of Mammalian Cells

HepG2 cells were obtained from the ECACC (CAMR) and were maintained in Dulbecco's modified Eagle's medium containing penicillin, streptomycin (Sigma), and 10% fetal bovine serum (Invitrogen). Transient transfectants of HepG2s were generated with *TTR-Xlhbox8-VP16:Elas-GFP* or with *CMV-GFP* (control for transfection efficiency). Cells were seeded in 35-mm dishes and grown at 37°C in 5% CO_2 overnight and were then transfected with 2 μg DNA by using GeneJuice transfection reagent (Novagen) and incubated for an additional 5 days. For counting, they were fixed in 4% PFA and immunostained for GFP. Using the 20× objective, five fields were picked randomly from each dish and were photographed with a digital (Spot) camera under DAPI and GFP fluorescence. The cell number in each field was >300. The error bars represent standard deviations. For assessing pancreatic differentiation, they were immunostained for both GFP and either insulin or amylase.

Immunostaining

Cells were cultured on glass coverslips, rinsed with PBS, fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 30 min, blocked in 2% Roche blocking buffer in PBS, and then incubated sequentially with primary and

secondary antibodies. Antibodies were used at the following concentrations: rabbit anti-amylase, Sigma (1/300); guinea pig anti-insulin, Sigma (1/250); rabbit anti-VP16, Clontech (1/100); mouse monoclonal anti-GFP, Clontech (1:200); TRITC goat anti-guinea pig IgG, Sigma (1/200); FITC horse anti-mouse IgG, Vector (1/150); TRITC swine anti-rabbit IgG, Dako (1/150). DAPI was dissolved in PBS at 500 μg/ml and was used at a 1:1000 dilution. Coverslips were incubated with DAPI (500 ng/ml) for 5 min at room temperature. After the immunostaining steps were completed, the coverslips were mounted on slides in gelvatol medium (20% polyvinyl alcohol in 10 mM TrisCl [pH 8.6]).

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