EARLY PANCREAS DEVELOPMENT AND ENDOCRINE INDUCTION:

PTF1A AND VEGF

By

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CHAPTER I

BACKGROUND AND SIGNIFICANCE

Diabetes mellitus is a devastating disease suffered by 150 million people worldwide. This can be the caused lack of sufficient pancreatic beta cell mass, which are crucial for maintaining glucose homeostasis. Fortunately, there is a treatment in human clinical trials that may help free diabetics from their insulin injection regiment and prevent diabetic complications: islet transplantation. The procedure itself requires more than one donor for transplantation for recipients to be insulin-injection free, and due to an inadequate supply of islets, transplantation will not be available to all diabetics (Ryan et al. 2002). There has been some recent limited success making islet-like tissue from stem cells, which provides promise for this as a future islet-like source for transplantation, but more effort needs to be placed on understanding pancreatic development and islet differentiation before this can become a realistic resource for transplantation.

The pancreas arises from the foregut endoderm and the accompanying mesodermally derived mesenchyme just caudal to the liver as one dorsal bud and one ventral bud in the human, mouse, and zebrafish (Wessels 1967; Laitio et al. 1974; Field et al. 2003). Pancreatic bud formation can be observed as early as eight weeks gestation in humans (Wang et al. 2005), embryonic day 9.5 (Wessels 1967), in the mouse, and by 24 h postfertilization (hpf) in zebrafish (Field et al. 2003). The endoderm of the pancreatic bud gives rise to both

endocrine and exocrine tissue (Figure 1). The endocrine tissue is composed of at least five cell types producing insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), pancreatic polypeptide (PP-cells) and ghrelin (ϵ-cells), which constitute clusters of cells called islets of Langerhans. The exocrine tissue is composed of acini that secrete digestive enzymes and ducts that transport the acinar secretions into the intestine. In mammals, both pancreatic buds can give rise to both tissue types.

During initial pancreatic outgrowth, both cells expressing insulin and glucagon can be detected. The immature insulin cells are unable to properly secrete their hormones in response to glucose (Hellerstrom and Swenne 1991). These immature cells are not necessary for the development of normal islet induction and differentiation (Herrera et al. 1994). It is likely that the pancreatic bud provides an environment that allows endocrine cell specification to be permitted, but lacks the cues necessary for these cells to properly mature. Later during pancreatogenesis, there is a phase of endocrine cell differentiation expansion termed the secondary transition. These cells give rise to mature endocrine mass and comprise postnatal islets (Pictet et al. 1972; Slack 1995; Jensen 2004). The signaling events and transcription factors that are responsible for this process are not yet completely understood, but are important for determining the process by which β cells are formed.
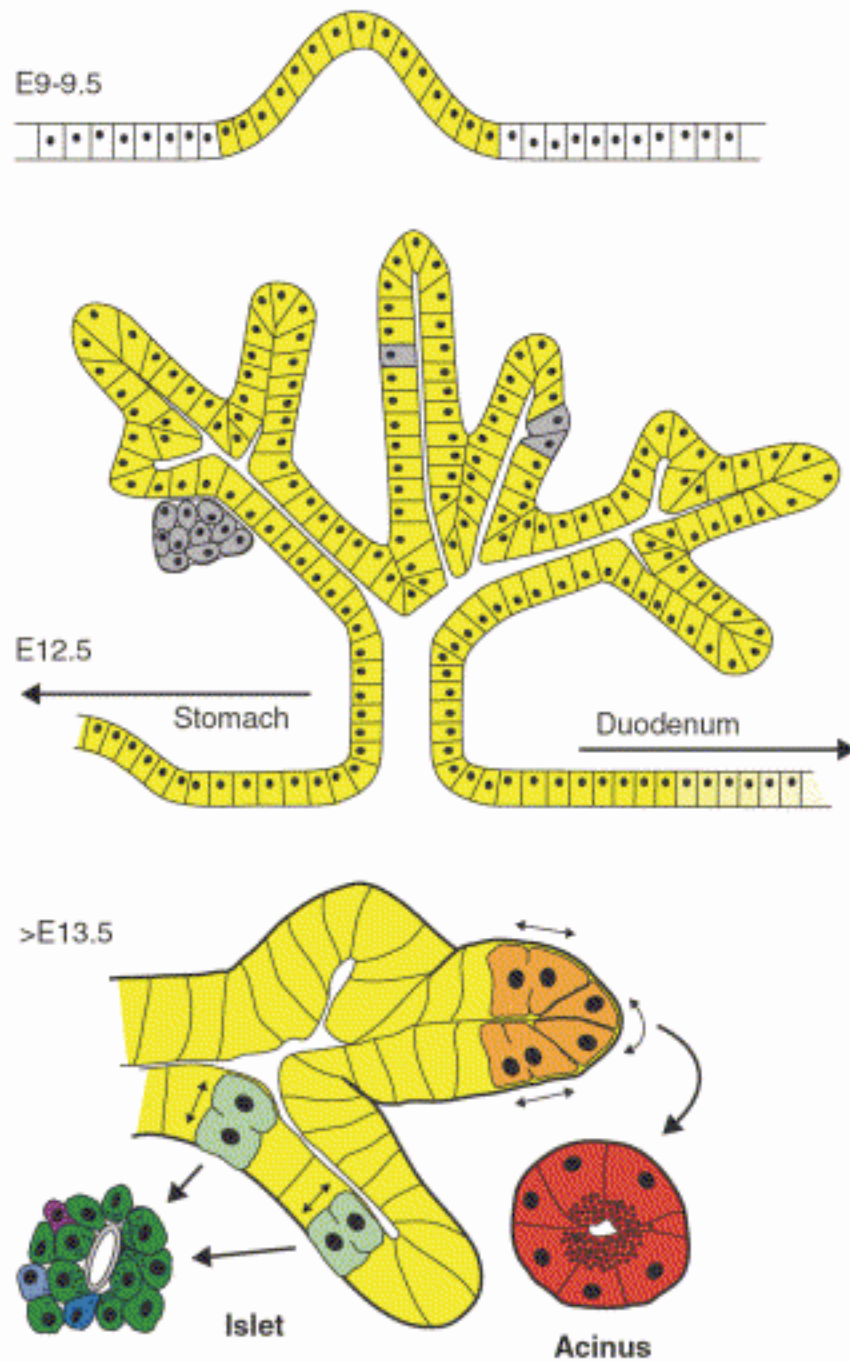
Figure 1. Events of pancreatic organogenesis. Development of the dorsal rudiment only is depicted after E9.0. Yellow shading indicates the extent of Pdx1 expression and gray indicates the early-differentiated endocrine cell clusters. Differentiation into mature β cells in green and mature acinar cells in red (Kim and MacDonald 2002).

Characterization of genetic regulatory elements and the transcription factors operating through these elements during pancreas development contributes to our understanding of insulin-producing β cell formation. Several transcription factors have been described to be involved at different time points during pancreatic differentiation (Figure 2). Perturbations in the transcription factors that bind important regulatory control elements can lead to defective pancreatic function and diabetes (Stoffers et al. 1997; Hansen et al. 2002; Schwitzgebel et al. 2003; Beres et al. 2006). Proper development of the endocrine and exocrine compartments of the pancreas requires several characterized transcription factors, including the homeodomain transcription factor *pancreas and duodenum homeobox 1* (*Pdx1*) and pancreas transcription factor-1a (*Ptf1a*).
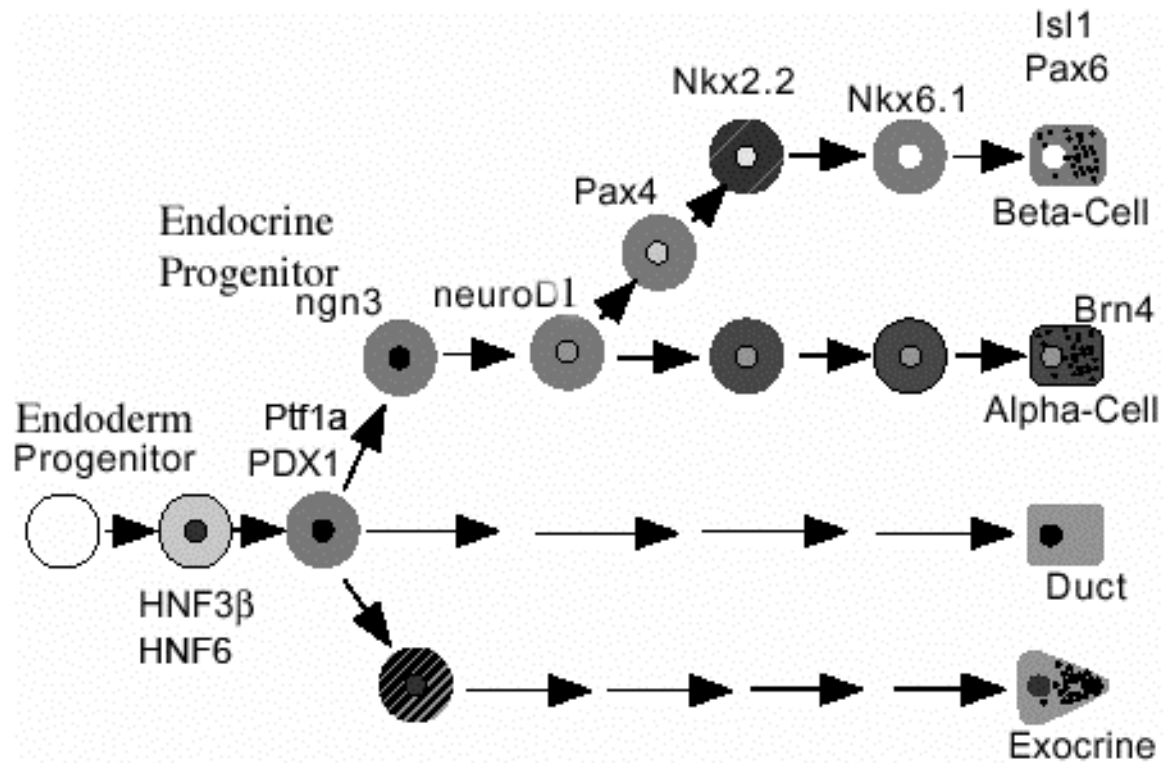
Figure 2. A simplified model for the role of islet transcription factors in endocrine differentiation in the developing pancreas. The proposed position for each transcription factor is based on its timing of expression, timing of predominant functional role, or both. Clearly some factors function at several steps, but a single step is shown for simplicity adapted from (Wilson et al. 2003).

Pdx1

Pdx1 is a homeodomain containing protein initially described in Xenopus (Wright et al. 1989). It contains 283 amino acids and has a prediction molecular weight of 31 kDa (Figure 3)(McKinnon and Docherty 2001). Pdx1 is modular in structure with it's homeodomain flanked by proline rich domains, while the transactivation domain is at the N-terminus.
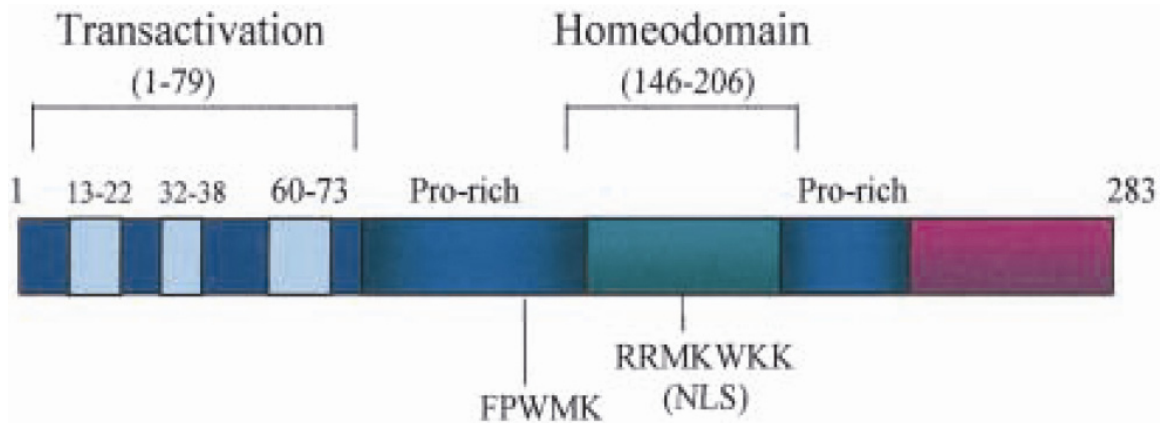
Figure 3. Structure of Pdx1. Schematic representation of the predicted structure of Pdx1 showing the antennapedia-like homeodomain flanked by two proline rich regions. The NH2-terminal activation domain comprising three sub-domains is also indicated as are a basic residue-rich nuclear localization signal (NLS) within helix three of the homeodomain and a pentapeptide motif that interacts with PBX located close to the homeodomain (McKinnon and Docherty 2001).

Pdx1 is also known as Ipf1, or insulin promoting factor, as it has been described a major transcriptional regulator of the insulin gene (Ohlsson et al. 1993). Pdx1 regulates the expression of additional islet-enriched genes through its interaction with their promoter regions. These include GLUT2, glucokinase, amyloid polypeptide (IAPP) and somatostatin (reviewed in Melloul 2004). Pdx1 also regulates genes encoding transcription factors, such as MafA (Raum et al. 2006) and *Pdx1* itself (Gerrish et al. 2004). *Pdx1* is expressed very early in pancreas development throughout both the dorsal and ventral pancreatic buds (Figure 4)(Guz et al. 1995; Gannon and Wright 1999; Li et al. 1999). After birth, *Pdx1* is expressed at high levels in the insulin-producing β cells within the islets, in some somatostatin-producing δ cells, and at lower levels in subpopulations of exocrine cells (Figure 4)(Guz et al. 1995; Offield et al. 1996; Wu et al. 1997; Gannon et al. 2001).
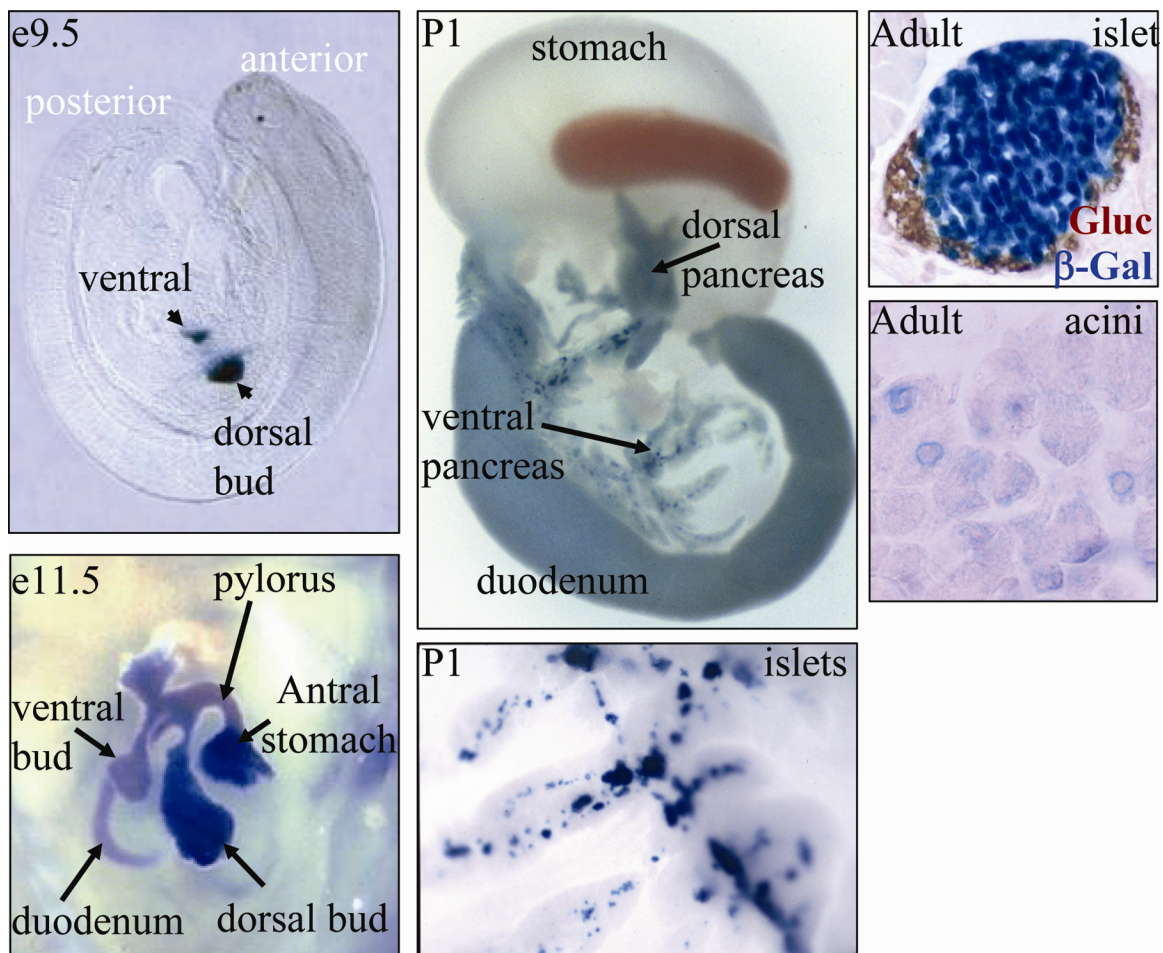
Figure 4. Dynamic expression pattern of Pdx1 in the developing pancreas and foregut. Tracking of Pdx1 expressing tissues in *Pdx1*$^{+/-(lacZ)}$ embryos. At E9.5, dorsal and ventral buds stain for β-galactosidase expression in *Pdx1*$^{+/-(lacZ)}$ embryos. The presumptive duodenum between the buds is also stained. The ventral and dorsal buds stain throughout with X-gal by E11.5 in *Pdx1*$^{+/-(lacZ)}$ animals and X-gal staining labels the antral stomach, duodenum, and common bile duct (Offield et al. 1996).Digestive organs from *Pdx1*$^{-(lacZ)/-(lacZ)}$;*Pdx1*resc animals at P1. *Pdx1*-driven lacZ expression from the *Pdx1*$^{-(lacZ)}$ null allele is detected by X-gal staining (blue). Islets are present along the pancreatic duct at P1. Glucagon expression (brown) marking islets from three-month-old *Pdx1*$^{-(lacZ)/-(lacZ)}$;*Pdx1*resc animals (Gannon et al. 2001). *Pdx1* driven β-gal expression was also detected at lower levels in subpopulations of acinar cells (Wu et al. 1997).

Null mutations in *Pdx1* in humans and mice result in an apancreatic

phenotype (Jonsson et al. 1994; Offield et al. 1996; Stoffers et al. 1997).

Mutations in *Pdx1* have also been identified in a subset of patients with a monogenic dominant form of diabetes, maturity onset diabetes of the young (MODY4)(Stoffers et al. 1997; Dutta et al. 1998). In mice, *Pdx1* haploinsufficiency results in decreased β cell function (Dutta et al. 1998; Clocquet et al. 2000; Brissova et al. 2002), increased β cell apoptosis (Johnson et al. 2003), and loss of *Pdx1* specifically from adult β cells leads to diabetes (Dutta et al. 1998). Taken together, these studies demonstrate that Pdx1 functions both early in pancreas development and later in mature islets. Characterization of *Pdx1* regulatory regions and the factors that bind to these regions should lead to a better understanding of how the pancreatic program is initiated and is investigated in Chapter I.

PTF1

The heterotrimeric pancreas transcription factor-1 complex (PTF1) was first identified as an activator of exocrine-specific genes (Rose et al. 1994), and is comprised of an acinar cell-enriched basic helix loop helix protein (bHLH), Ptf1a (p48), a ubiquitous bHLH protein (Krapp et al. 1996), HEB, and the distinct mammalian Suppressor of Hairless (RBP-Jκ/CBF1)(Obata et al. 2001) or its paralogue (RBP-L)(Beres et al. 2006). The PTF1 complex controls the selective transcription of acinar secretory enzymes, such as elastases, carboxypeptidases, amylases, and chymotrypsinogens (Harding et al. 1977; Cockell et al. 1989).

Ptf1a is expressed as early as E9.5 throughout the developing pancreas and is essential for pancreas formation and function in both mouse and human

(Krapp et al. 1998; Obata et al. 2001; Kawaguchi et al. 2002; Sellick et al. 2004).

Ptf1a null mice lack a ventral pancreatic bud and show an early arrest in dorsal bud outgrowth (Figure 5)(Kawaguchi et al. 2002). Exocrine cells do not develop and there is limited endocrine development. The endocrine cells that do form are found alongside the pancreatic rudiment (Figure 5)(Kawaguchi et al. 2002), or mislocalized to the spleen (Krapp et al. 1998).
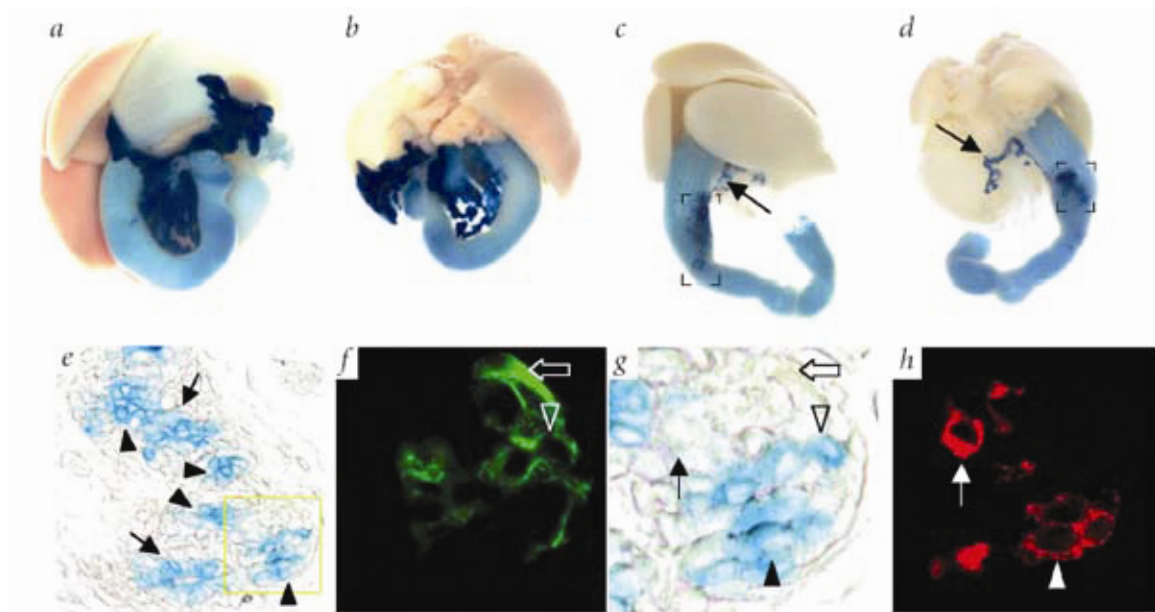
Figure 5. Conversion from pancreatic to duodenal fates by inactivation of Ptf1a. a–d, Macroscopic phenotypes of 18.5-d.p.c. embryos, viewed ventrally (a,c) or dorsally (b,d), to observe dorsal or ventral bud−derived cells, respectively. a,b, A Ptf1a−cre R26R wildtype control. c,d, A Ptf1a Cre/Cre R26R, Ptf1a homozygous null mutant. A dorsal bud−derived pancreatic rudiment is observed (arrows in c,d) and expansive areas of cells expressing β-galactosidase cells are found in the duodenum (framed). e–h, Histological analysis of pancreatic rudiment from a Ptf1a Cre/Cre R26R embryo using thin sections (2 m) to determine the degree of overlap of -galactosidase expression and endocrine markers. e, Endocrine clusters (arrowheads) are found alongside a pancreatic duct−like epithelium (arrows). Almost all duct cells express -galactosidase; insulin- (f) or glucagon-producing cells (h) are either labeled (arrowheads) or unlabeled (arrows) by X-gal staining (g) (Kawaguchi et al. 2002).

Ptf1a expression in the early pancreatic primordium requires the adjacent mesenchyme including vascular endothelial cells (Yoshitomi and Zaret 2004; Jacquemin et al. 2006). The dependence of this transcription factor's expression suggests an early signaling event from the neighboring endothelial cells. Such a connection underscores the importance of orchestrating transcription factor expression, and that these events may not be cell autonomous, but instead rely on extracellular signaling for proper patterning and differentiation. The relationship between endothelial cells and pancreatic endoderm is the subject of investigation in Chapters II and III.

Pancreatic endothelial cells

Pancreatic bud outgrowth is initiated at sites in the posterior foregut endoderm where it contacts the endothelium of major blood vessels and endocrine differentiation initially occurs in cells that have direct contact with endothelial cells (Figure 6)(Lammert et al. 2001). For example, at E10.5, insulin expression is detected at sites where the dorsal pancreatic bud contacts portal vein endothelium (Figure 6). The importance of VECs in pancreatic endocrine differentiation has been demonstrated both in tissue recombination experiments and in genetically modified mice. For example, E8.5 endoderm cultured in the absence of endothelial cells failed to activate either Pdx1 or insulin protein expression, whereas when undifferentiated endoderm was cultured in combination with dorsal aorta, both Pdx1 and insulin were induced (Lammert et al. 2001). Examination of VEGFR2 null mutant mice, which die prior to the second wave of endocrine differentiation, revealed that early insulin and

glucagon cells fail to develop (Lammert et al. 2001; Yoshitomi and Zaret 2004). These mice express most pancreatic/endocrine transcription factors (Pdx1, Hnf6, Ngn3, NeuroD, Prox1, and Hb9) with the exception of Ptf1a (Yoshitomi and Zaret 2004). Taken together, these data provide support for an endothelial-derived endocrine inducing factor(s). Although the identity of this factor(s) is currently unknown, it follows that if endothelial cell numbers were to increase, the amount of the inducer, and thus the amount of endocrine cells, would also increase. To this end, the Melton laboratory generated transgenic mice expressing the $VEGF_{164}$ isoform throughout the entire pancreatic bud using a fragment of the *Pdx1* promoter (Lammert et al. 2001). *Pdx1*-VEGF transgenic embryos have greatly increased vasculature in the pancreas and a corresponding 3-fold increase in islet number and islet area. Ectopic insulin expression was found adjacent to the ectopically induced endothelial cells.

In collaboration with the Powers lab we have found that early differentiating pancreatic endocrine cells produce angiogenic factors including vascular endothelial growth factor (VEGF) and angiopoietin 1 (Ang-1) and that expression of these is maintained in adult islets (Brissova et al. 2006). Other investigators have also observed VEGF expression in mature islets (Christofori et al. 1995; Rooman et al. 1997; Lammert et al. 2001). These factors are important to recruit vascular endothelial cells (VECs) and direct their differentiation into a porous fenestrated vasculature. This vasculature associated with the pancreatic endocrine tissue is fenestrated to allow for exchange with and sensing of the blood, so appropriate amounts of hormones can be rapidly secreted. The

continued expression of these factors in adult islets suggests that maintenance of

a fenestrated endothelium is critical for mature islet function. In addition to islet

endocrine cells communicating with VECs via secreted growth factors,

endothelial cells have been shown to signal to the pancreatic epithelium,

influencing the differentiation of endocrine cells (Lammert et al. 2003; Lammert et

al. 2003; Yoshitomi and Zaret 2004). Also, analysis of the islet microenvironment

reveals differences in the basement membranes of islets in association with

vascular endothelial cells (Nikolova et al. 2006; Nikolova et al. 2007). Together,

the association between pancreatic endocrine cells and endothelial cells is

important for both differentiation and function of islet cells.
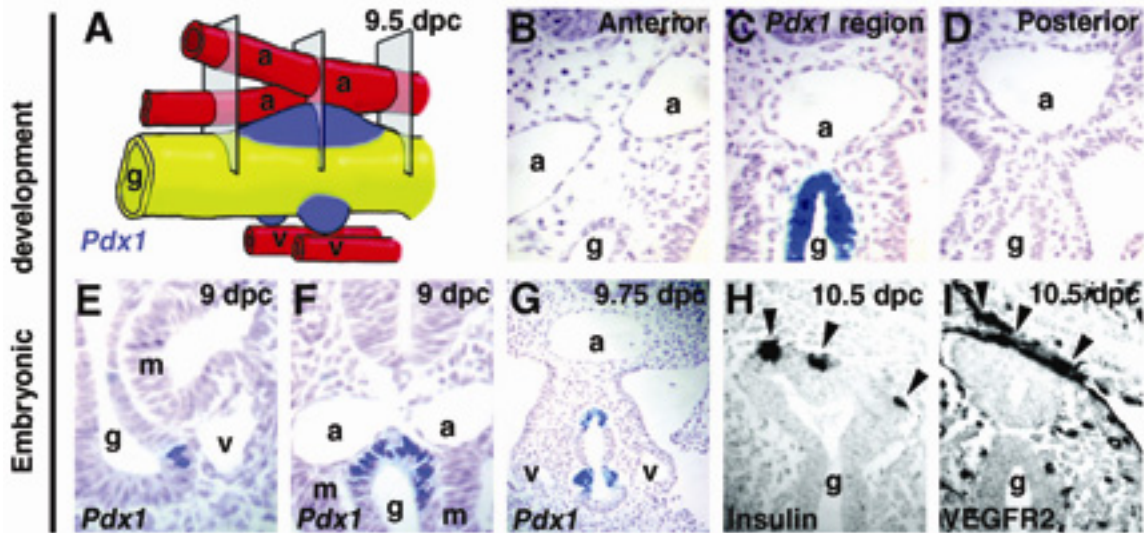
Figure 6. Pancreatic budding and endocrine differentiation take place adjacent to blood vessels. (A) Schematic representation of blood vessels (red) relative to pancreatic endoderm expressing Pdx1 (blue). The gut tube endoderm is yellow. Aortae (a), vitelline veins (v), and gut endoderm (g) are indicated. (B to D) Transverse sections through mouse foregut, as indicated in (A). Sections are taken from the same 9.5-dpc mouse embryo. (B) Section anterior to the Pdx1 region. (C) Section through the Pdx1 region. (D) Section posterior to the Pdx1 region. (E) In the ventral gut tube (g) at E9, Pdx1 expression begins in two ventral domains. One domain is shown here. Pdx1 is initiated in the endoderm adjacent to the vitelline vein endothelium (v) and not in endoderm adjacent to mesenchyme (m). (F) Pdx1 expression begins slightly later in the dorsal endoderm adjacent to the fusing dorsal aortae (a). (G) These Pdx1-expressing domains are budding from the gut tube at E9.75. (H and I) Adjacent transverse sections of the mouse gut tube at E10.5. Scattered cells expressing insulin develop in the dorsal pancreatic bud [arrowheads in (H)] adjacent to the right vitelline or portal vein as marked by VEGFR2 expression [sites of contact with insulin-expressing cells are marked with arrowheads in (I)] (Lammert et al. 2001).

VEGF

VEGF is a secreted heparin binding, 45 kDa homodimeric glycoprotein that acts as a potent endothelial cell-specific mitogen, capable of promoting growth and chemotaxis of VECs (reviewed in Ferrara 1999). VEGF belongs to a family of proteins that includes six members: VEGF (VEGF-A), placenta growth factor (PlGF), and VEGF-B through VEGF-E. All family members contain a

13

characteristic cysteine knot motif which consists of eight regularly-spaced

cysteine residues (reviewed in Veikkola and Alitalo 1999). VEGF is the most

abundant family member and is composed of at least four isoforms, of which

$VEGF_{165}$ predominates (Cross et al. 2003).
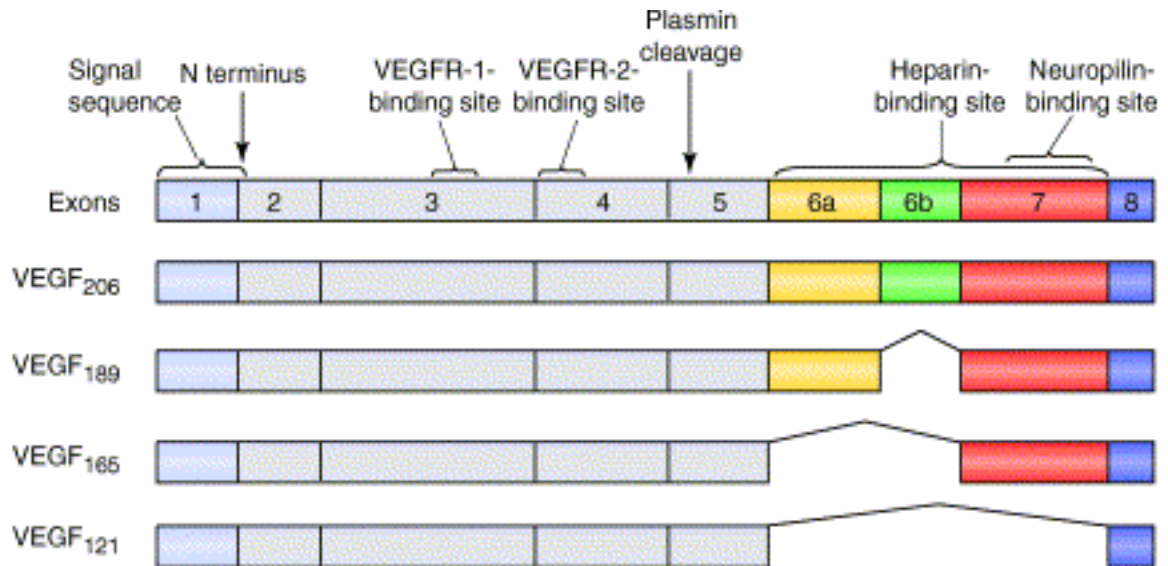
Figure 7. Exon structures of the vascular endothelial growth factor (VEGF)-A mRNA splice variants. The gene encoding VEGF-A consists of eight exons that encode several different structural motifs. Alternative splicing of a single pre-mRNA species produces at least four different VEGF-A isoforms that vary in total amino-acid number. In humans these correspond to VEGF-A$_{121}$, VEGF-A$_{165}$, VEGF-A$_{189}$ and VEGF-A$_{206}$, of which VEGF-A$_{165}$ is the predominant form (Cross et al. 2003).

There is significant genetic evidence supporting a role for VEGF in

angiogenesis. Mice that lack one allele of the VEGF gene (Carmeliet et al. 1996),

mice that can only express the $VEGF_{120}$ isoform (Carmeliet and Collen 1999),

and mice that have a modest increase in VEGF expression (Miquerol et al. 2000)

all die during gestation due to vascular defects. Additionally, the VEGFR1 and

VEGFR2 knock out mice also die during gestation due to vascular defects (Fong et al. 1995; Shalaby et al. 1995). Both VEGF ligand and receptor expression are imperative for proper development and function of VECs.

There are three receptors for VEGF family members, VEGFR1-3, and a coreceptor, neuropilin-1. VEGFR1, VEGFR2 and neuropilin-1 are expressed on VECs (Figure 8), while VEGFR3 is restricted to lymphatic endothelial cells (Makinen and Alitalo 2002). Both VEGFR1 and VEGFR2 have seven immunoglobin-like domains in the extracellular region, a single transmembrane region, and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain (Figure 9,Figure 10)(reviewed in Ferrara et al. 2003).
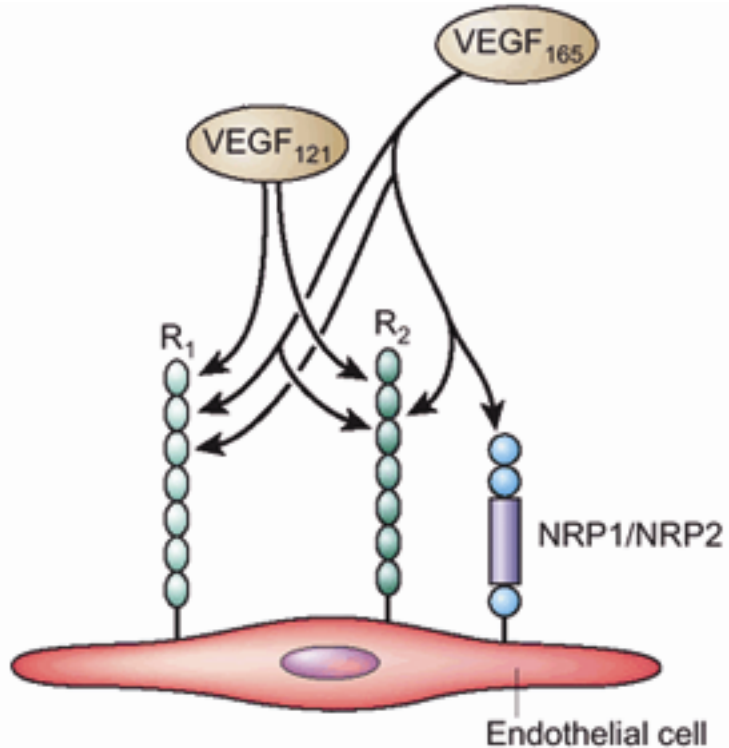
Figure 8. Diagram showing the receptors expressed on VECs and their VEGF isoform specificities. $VEGF_{121}$ lacks the heparin and neuropilin binding domain and does not bind to the extracellular matrix. $VEGF_{165}$ and the larger isoforms have both the heparin and neuropilin binding sites. The larger the isoform, the greater affinity it has for the extracellular matrix. VEGF can be cleaved by plasmin during extracellular matrix remodeling producing a soluble $VEGF_{110}$ N-terminal isoform that behaves like the $VEGF_{121}$ isoform (modified from Ferrara et al. 2003).
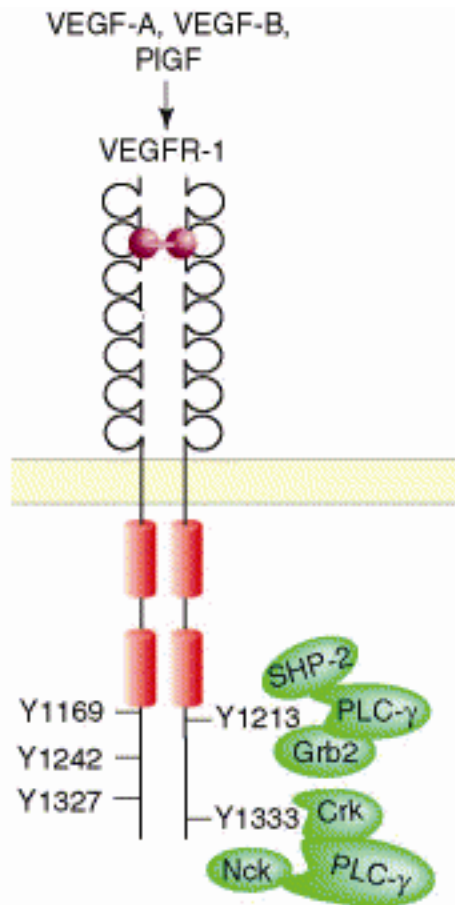
Figure 9. Diagram of VEGFR1 showing cytoplasmic tyrosine phosphorylation sites and signaling molecules that bind these sites via their SH2-domains. (modified from Cross and Claesson-Welsh 2001).

VEGFR1

VEGFR1 (flt1) is necessary for survival, which was demonstrated by the creation of a genetic knock-out mouse. The mice homozygous for this knock-out die during embryogenesis due to an overgrowth of endothelial cells (Fong et al. 1995), which suggests that VEGFR1 might play a negative regulatory role during development. Studies in tissue culture cells demonstrated that VEGFR1 activation by VEGF caused receptor dimerization and transphosphorylation, but maximally by only two fold despite the multiple phosphorylation sites (Sawano et

al. 1997; Ito et al. 1998). Additionally, mice genetically engineered to lack the kinase domain from VEGFR1 survived and had no overt phenotype (Hiratsuka et al. 1998).

Although signaling through VEGFR1 has been demonstrated, it is not necessary for survival, suggesting that VEGFR1 must have some other function independent of its kinase domain. Indeed, Clark et al demonstrated that the extracellular domain of VEGFR1 is produced in vivo and acts as an antagonist of VEGF signaling (1998). VEGFR1 has a k_d for VEGF of 37 pM (Gille et al. 2001), and it has been suggested that since VEGFR1 has a higher affinity for VEGF than VEGFR2, that VEGFR1 can act to sequester VEGF and therefore negatively regulate endothelial cell growth (Carmeliet et al. 2001).

In the absence of the VEGFR1 kinase domain, macrophages are unable to migrate normally via a VEGF mediated signal (Hiratsuka et al. 1998), suggesting that VEGFR1 may have multiple roles depending on the developmental stage and cell type expressing the receptor. Additionally, hepatic sinusoidal endothelial cells express VEGFR1, and when an agonist specific for this receptor is present it causes theses cells to secrete HGF and other growth factors in a paracrine fashion (LeCouter et al. 2003).

Analyses of VEGFR1 activation by VEGF demonstrated that the receptor becomes phosphorylated at two major sites, Tyr-1213 and Tyr-1242, and two minor sites, Tyr-1327 and Tyr-1333 (Figure 9). Phospholipase C-gamma binds to both Tyr-1213 and Tyr-1333, while Grb2 and SHP-2 bind to Tyr-1213, and Nck and Crk bind to Tyr-1333 (Figure 9), all in a phosphorylation dependent manor *in*

vitro (Ito et al. 1998). The significance of these interactions has yet to be resolved, but it seems likely that they are essential mediators for the cellular response to VEGF via VEGFR1.

VEGFR2

VEGFR2 (kdr; flk1) is also essential for survival, demonstrated by the creation of mice with a null allele of this gene. Mice homozygous for VEGFR2 null allele die embryonically due to the failure of endothelial cells to organize into blood vessels (Shalaby et al. 1995). VEGFR2 is the main receptor responsible for proliferation, migration and permeability of endothelial cells (Figure 10)(reviewed in Ferrara et al. 2003). VEGF binds to the second and third extracellular IgG-loop of VEGFR2 with a K_d of 75-125 pM (Waltenberger et al. 1994). It is phosphorylated in several places in response to VEGF ligand binding (Figure 10), and the signaling cascade has been more extensively investigated than for VEGFR1 (Matsumoto and Claesson-Welsh 2001).

Proliferation following VEGFR2 activation in endothelial cells is mediated by mitogen activated protein kinase (MAPK) pathway via phospholipase C (PLC) gamma binding the tyrosine phosphorylated receptor, leading to diacylglycerol (DAG) production from phosphatidylinositol, and activation of protein kinase C (PKC), which activates Raf via both Ras dependent (Figure 10)(Takahashi et al. 1999) and independent pathway (Meadows et al. 2001). Ultimately, this results in alterations in gene transcription and proliferation (Figure 10). VEGFR2 activation also affects endothelial cell permeability. Phosphoinositide 3-kinase (PI3K) is

activated by VEGFR2 via focal adhesion kinase (FAK) increasing

phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and activating Rac, which leads

to increased permeability (Figure 10)(Eriksson et al. 2003). The increased PIP3

also leads to protein kinase B (PKB; Akt) activation, which activates endothelial

nitric oxide synthase (eNOS), and the NO production resulting in increased

permeability (Figure 10)(Dimmeler et al. 1999; Fulton et al. 1999).
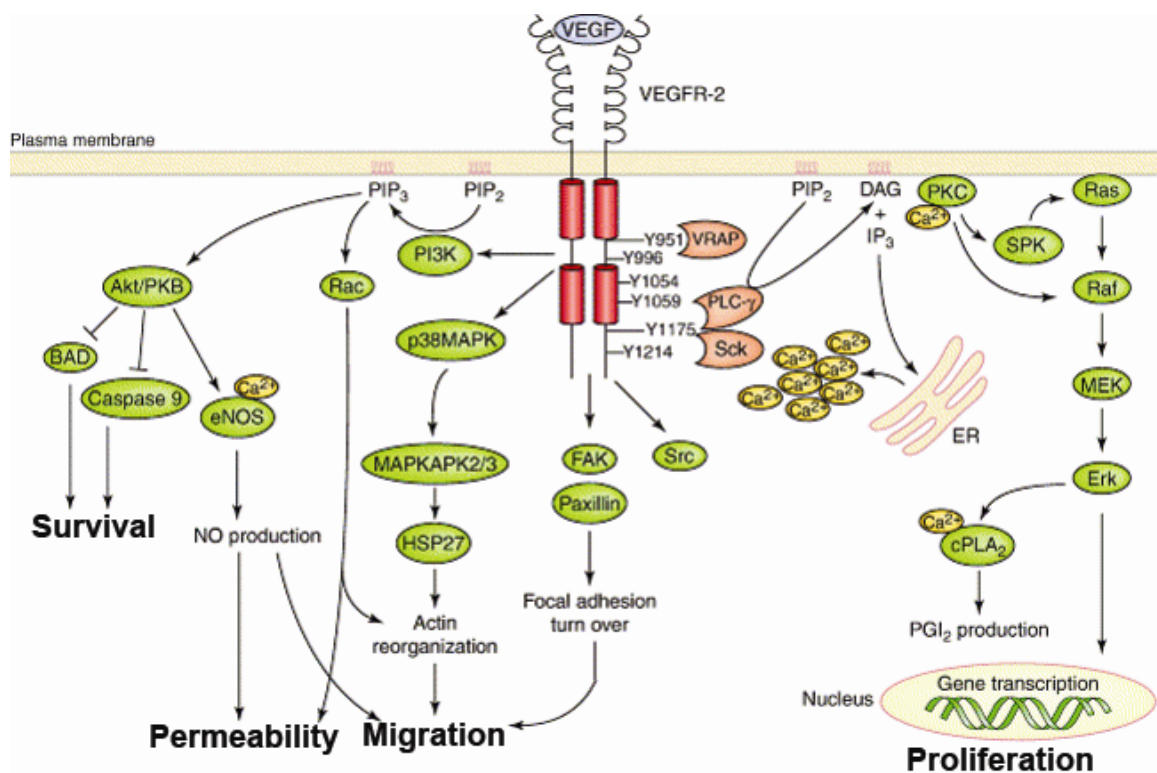
Figure 10. Diagram of VEGFR2 intracellular signaling. Ligand binding the extracellular domain causes receptor dimerization and autophosphorylation of specific tyrosine residues. The phosphorylated amino acids provide binding sites for SH2-domain containing signaling molecules, which mediate the indicated effects on VECs (Cross et al. 2003).

There are several mediators of migration of endothelial cells by VEGFR2

activation. Activated Rac via PI3K has been implicated in migration (Figure

10)(Eriksson et al. 2003) as well as increased NO production from eNOS via

PI3K and Akt/PKB activation (Figure 10)(Dimmeler et al. 1999; Fulton et al.

1999), which are also involved in permeability via fenestrations and intercellular

porosity. Cytoskeletal rearrangement necessary for migration includes

involvement of p38 mitogen-activated protein kinase (MAPK)(Rousseau et al.

1997), FAK (Qi and Claesson-Welsh 2001), and stress-activated protein kinase-2

(SAPK)(Rousseau et al. 2000)(Figure 10).

VEGFR1 and VEGFR2 have been shown to heterodimerize and

transphosphorylate each other, suggesting that there is some cross-talk between

these two signaling pathways (Autiero et al. 2003). This initially might seem

irrelevant since VEGF activates both of these receptors, except that VEGF is a

family of proteins that each have different affinities for both of these receptors.

For example, PIGF, a VEGF homolog and family member, binds specifically to

VEGFR1 and not to VEGFR2 (De Falco et al. 2002), while VEGF-E binds

specifically to VEGFR2 and not to VEGFR1, and VEGF-A (VEGF) binds to both

(Figure 11)(reviewed in Clauss 2000).
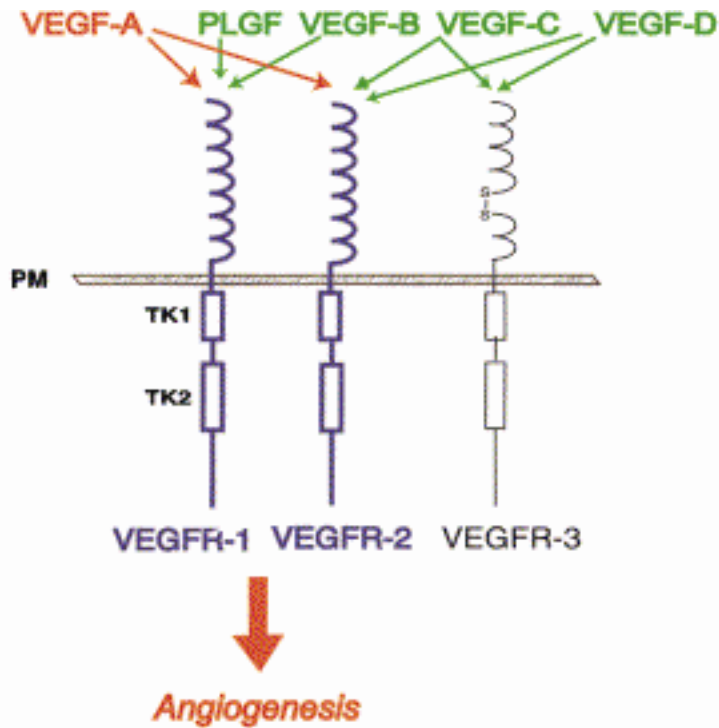
Figure 11. The complex and overlapping signaling potential of the VEGF family of angiogenesis regulators. VEGF-A, -B, -C, -D and PLGF bind as indicated to VEGFR-1 and/or VEGFR-2, receptor tyrosine kinases whose expression is largely restricted to endothelial cells. In adults, the related VEGFR-3 is expressed primarily in lymphatic vessels and can demonstrably regulate lymphangiogenesis; it may also have a role in developmental angiogenesis (Inoue et al. 2002).

The evidence is increasing that during both liver and pancreas development, endothelial cells produce an instructive signal that induces pancreatic endocrine differentiation and morphogenesis (Lammert et al. 2001; Matsumoto et al. 2001; Yoshitomi and Zaret 2004). VECs are known to produce several different secreted growth factors including: fibroblast growth factor (FGF), transforming growth factor β (TGFβ), Wingless (Wnt), and hepatocyte growth factor (HGF)(Lammert et al. 2003). In the liver, a VEGFR1 receptor-selective agonist causes these cells to express growth factors in a paracrine fashion.

Thus, VEGF signaling through this particular receptor may induce the expression of growth factors known to be mitogenic to β cells (Hayek et al. 1995; Otonkoski et al. 1996; Garcia-Ocana et al. 2000). These studies highlight the reciprocal communication between pancreatic endocrine cells and endothelial cells (Figure 12). Chapter III and IV present experiments wherein signaling in the pancreatic through the VEGF pathway was modified to elucidate its role in pancreatic endocrine cell specification and proliferation.
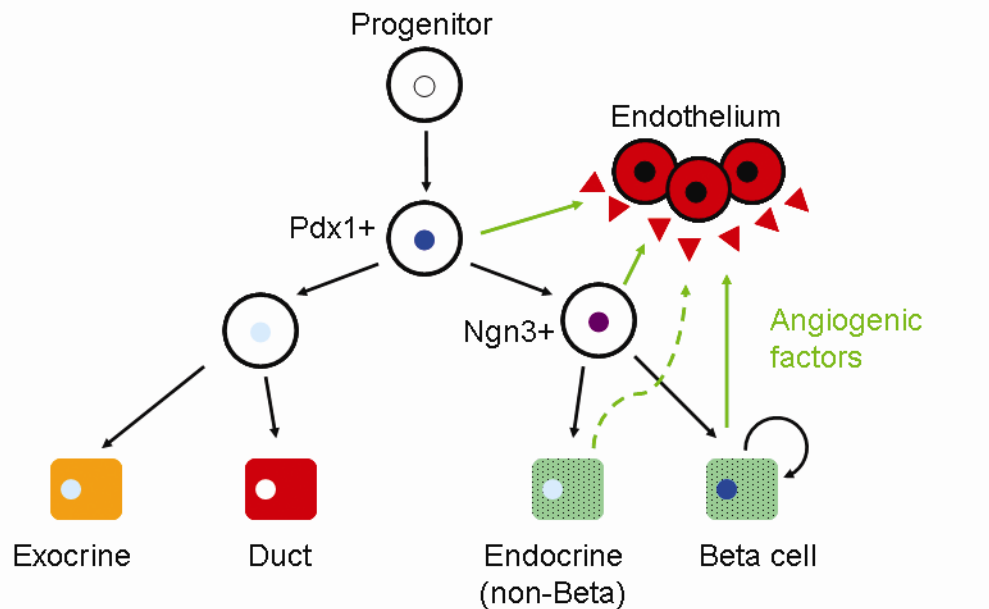
Figure 12. Model outlining the reciprocal signaling between the vascular endothelium and pancreatic cell types during development. Endocrine cells express angiogenic factors that influence migration and proliferation. Endothelial cells respond to these signals and preferentially influence endocrine cells in the pancreas. The developmental time frame when this can occur and the signaling events arising from the endothelium remain to be elucidated.

CHAPTER II

PTF1A BINDS TO AND ACTIVATES AREA III, A HIGHLY CONSERVED REGION OF THE *Pdx1* PROMOTER THAT MEDIATES EARLY PANCREAS-WIDE *Pdx1* EXPRESSION

Introduction

Previous analysis of *Pdx1* non-coding regulatory regions identified four areas of highly conserved sequence (termed Areas I through IV; each ~300 bp) within 7 kb upstream of the promoter (Figure 13)(Sharma et al. 1996; Gerrish et al. 2000; Gerrish et al. 2004). Areas I (-2852 to -2547), III (-1973 to -1694), and IV (-6422 to -5931) are conserved among humans, rodents, and chickens, while Area II (-2247 to -2071) has only been identified within the mammalian orthologs (Gerrish et al. 2000; Gerrish et al. 2004). A β-galactosidase (β-gal) reporter transgene driven by a 4.6 kb (-4617 to -33) or a 4.3 kb (-4617 to -320) promoter fragment, containing Areas I, II, and III, but not Area IV (Figure 13), recapitulates the endogenous *Pdx1* expression pattern (Stoffers 1999; Gannon et al. 2001). Transgene-based complementation experiments on *Pdx1* null mice reveal that the proximal promoter/enhancer region, excluding Area IV, rescues the pancreatic defects caused by Pdx1 deficiency (Boyer et al. 2006). Thus, Area IV does not appear to be essential for appropriate temporal control of pancreatic *Pdx1* expression. In cell lines, Areas I and II are each capable of driving β cell-specific reporter gene expression independently, while together their β cell-specific activity is greatly enhanced, suggesting that synergistic interactions

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between the two regions mediate high level *Pdx1* gene expression in islets

(Gerrish et al. 2000; Van Velkinburgh et al. 2005). Area III does not drive β cell-

selective activity in cell lines (Gerrish et al. 2000).
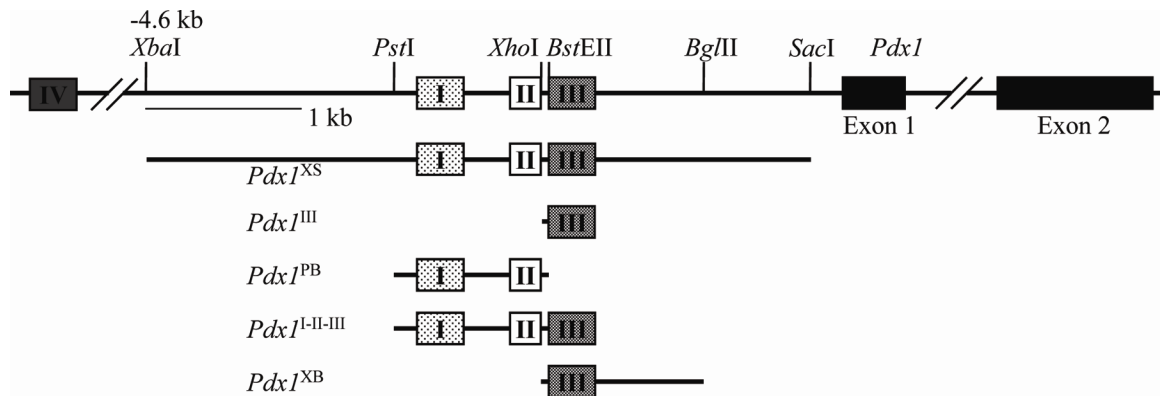
Figure 13. Diagram of highly conserved areas within the *Pdx1* promoter/enhancer region. Area I (stipple, -2852 to -2547 bp), Area II (white, -2247 to -2071 bp), Area III (gray, -1973 to -1694 bp), and Area IV (dark gray, -6422 to -5931) are conserved regions among species (Gerrish et al. 2000). Labeled horizontal lines below the *Pdx1* promoter indicate regions of relevance in this investigation. PstI (-3007), XhoI (-2046), BstEII (-2011), and BgIII (-994) are relevant restriction sites. Numbering is relative to the mouse *Pdx1* gene translation start site.

Transgenic analysis of the *Pdx1* upstream region identified modules that

were capable of driving endocrine expression *in vivo* (Figure 13)(Wu et al. 1997;

Gannon et al. 2001). The 1 kb PstI-BstEII fragment (*Pdx1*[PB]; -3007 to -2011),

which contains Areas I and II (and none of Area III), drives expression of a β-gal

reporter transgene exclusively in endocrine cells of embryonic and adult

pancreas (Gannon et al. 2001). In contrast, the adjacent 3' 1 kb XhoI-BgIII

fragment (*Pdx1*[XB]; -2046 to -994) drives β-gal expression to only β cells of the

late embryonic and neonatal pancreata, but is not active in the mature adult

organ (Gannon et al. 2001). Of the four conserved regions, only Area II showed islet-specific activity in transgenic reporter assays when analyzed individually, although this activity was detected in only a subset of β cells (Gannon et al. 2001; Samaras et al. 2002; Van Velkinburgh et al. 2005). Thus, as observed in cell lines, Area I and II seem to act together *in vivo* for optimal *Pdx1* expression in β cells.

Combined cell line and transgenic analyses had revealed only a transient endocrine role for the highly conserved Area III in *Pdx1* gene regulation. In one of four transgenic lines, Area III alone drove transient reporter gene expression in developing endocrine cells, and drove expression in scattered cells throughout the pancreas in adults (Gannon et al. 2001). Interestingly, deletion of these conserved *cis* regulatory regions, Area I, II, and III, from the endogenous *Pdx1* locus, results in a dramatic impairment in endocrine as well as exocrine tissue development at early stages of pancreatic outgrowth (Fujitani et al. 2006). Otherwise, prior to the current study, there had been no evidence for a role for any of these regions in regulation of *Pdx1* expression outside of the pancreatic endocrine lineage. Using a combination of biochemical and *in vivo* lineage tracing approaches, we uncovered a role for Area III in *Pdx1* expression, identified a critical site within this region that binds a Ptf1a-containing complex, and established the relevance of this activator for proper pancreatic development.

Materials and Methods

Transgenic Constructs and Generation of Transgenic Mice

Pdx1[I-II-III]lacZ: Areas I, II, and III of mouse *Pdx1* (*Pdx1*[I-II-III]), encompassing nucleotides -3030 through -1696 relative to the start of translation (+1), were PCR amplified from a 9 kb XbaI genomic fragment (Offield et al. 1996) and directionally subcloned into a modified *hsp68lacZpA* vector which contains the heat shock protein minimal promoter and *lacZ* expression cassette (Sasaki and Hogan 1996). Lower case letters indicate base changes introduced to produce 5' HindIII or 3' PstI restriction enzyme sites for directional cloning (upstream primer: 5'-GTAATCCaAgCTTTGCCTGCcG-3'; downstream primer: 5'-GTCTCTGcagTCTTCAGGGAAAAGAGCCAC-3'). Of six *Pdx1*[I-II-III]lacZ lines, two lines had detectable *lacZ* expression and were propagated for further analysis (see below). Generation of an additional eight transient transgenic mice, collected and analyzed at embryonic stages, yielded two more individuals with detectable expression, which was similar to the established lines.

Pdx1[XB]Cre: the 1 kb XhoI to BglII *Pdx1* fragment contains Area III and an additional 700 bp of 3' non-conserved sequence, encompassing nucleotides -2046 to -994 relative to the start of translation (+1) in the mouse gene (*Pdx1*[XB])(Gannon et al. 2001). The XB fragment was subcloned upstream of the *hsp68* minimal promoter and a Cre recombinase expression cassette (Postic et al. 1999). *Pdx1*[XB]Cre-mediated *R26R* recombination was similar in all four lines generated.

Pdx1[PB]Cre: the Cre recombinase/human growth hormone fusion cDNA (Postic et al. 1999) was subcloned into the *Pdx1*[PB]-HNF6 plasmid vector (Gannon et al. 2000) using *EcoR*I digestion to replace the *HNF6* cDNA, such that the *Pdx1*[PB] was positioned upstream of the *hsp68* minimal promoter and a Cre recombinase expression cassette.

Pronuclei of one-cell embryos from B6D2F1 (Charles River) females were injected with 1–5 pl DNA (3 ng/ml) and embryos implanted into pseudopregnant CD-1 (Charles River) females (Hogan 1994). Some F_0 founders were sacrificed at embryonic stages to analyze the *Pdx1*[I-II-III] driven β-gal expression pattern; transgenic lines were also generated. Genotyping was by Southern blot analysis of genomic DNA from brain (embryos and neonates) or tail (adults) tissue. *Pdx1*[I-II-III]lacZ transgenic mice were identified on *EcoR*I-digested DNA with a *lacZ* cDNA probe. Cre transgenic mice were identified on *EcoR*I-digested DNA with a *Cre* cDNA probe.

Characterization of Transgenic Mice

The morning of vaginal plug was considered to be E0.5. Dissected internal organs were fixed, stained with X-gal to detect β-gal, embedded, and sectioned (Gannon et al. 2000; Zhang et al. 2005). Immunohistochemical staining for glucagon and insulin was as described (Gannon et al. 2000). Pdx1 expression was analyzed using a 1:1000 dilution of rabbit anti-Pdx1 (Peshavaria et al. 1994), and the Vectastain ABC and DAB kits (Vector Labs). Whole mount images of dissected digestive organs and embryos were taken using an Olympus XZS9

bifocal dissecting microscope with a Nikon Coolpix 4300 digital camera and a Nikon UR-E4 eyepiece adaptor. Images of sections were taken under brightfield illumination using an Olympus BX41 microscope and digital camera with Magnafire software (Optronics). Image brightness, contrast, and color variations were minimally adjusted using Adobe Photoshop 6.0 or PowerPoint (Rossner and Yamada 2004). Adjustments were equivalent for all samples that were directly compared.

TRANSFAC Analysis

The highly conserved region of the mouse *Pdx1* fragment ranging from nucleotides -3030 to -1694 was examined *in silico* using default setting for putative transcription factor binding sites using the TRANSFAC Matrix table and Tfsearch software version 1.3 (http://www.cbrc.jp/htbin/nph-tfsearch).

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts were prepared from 8 week old male B6D2F1/J (Jackson Labs) mouse pancreata as described in (Shapiro et al. 1988; O'Brien et al. 1995), modified for use with whole tissue (Rose et al. 1994). Briefly, whole pancreata were homogenized with a Tissuemiser homogenizer in ice-cold lysis buffer [10 mM Tris-HCl (pH 7.4), 0.1M NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mg/L N-α-p-tosyl-L-phenyl chloromethyl ketone (TPCK), 0.5mg/L N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), 0.6 μM leupeptin, and 2 μM pepstatin]. Homogenate was centrifuged

(1000xg, 2 min, 4°C); pellets were resuspended in ice-cold lysis buffer, and centrifuged (1000xg, 1 min, 4°C). The pellet was resuspended in ice-cold nuclear extraction buffer [20 mM HEPES (pH 7.9), 0.4 M NaCl, 0.90 mM spermidine, 0.2 mM EDTA, 2 mM EGTA, 25% glycerol, 2 mM DTT, 1 mM PMSF, 0.5 mg/L TPCK, 0.5 mg/L TLCK, 0.6 μM leupeptin, and 2 μM lepstatin]. The nuclear extract was centrifuged (16,000xg, 1 min, 4°C), and the supernatant was collected and stored at -80°C.

EMSAs, including antibody supershifts, were performed as described (Sawada and Littman 1993; Rose et al. 2001). PTF1 control (PTF1 *Ela1*; -115 to -96 of the rat *elastase 1* promoter) upper: 5'-gatcGTCACCTGTGCTTTTCCCTGC-3', lower: 5'-gatcGCAGGGAAAAGCACAGGTGAC-3'. Putative Area III PTF1 binding site (AIII PTF1; -1730 to -1709 of the *Pdx1* promoter) upper: 5'-gatcCACAGGTGGCTCTTTTCCCTG-3'; lower: 5'-gatcCAGGGAAAAGAGCCACCTGTG-3'. Lower case indicates 5' overhangs for radioactive fill-in labeling. Other oligonucleotides are described in Figure 18C. Mobility shift binding reactions were 20 μL in volume, prepared on ice, and contained: 10 mM HEPES (pH 8.0), 90 mM NaCl, 1 mM EDTA, 0.1 M DTT, 6 μg bovine serum albumin, 2 ug poly dIdC, and 40 fmol of labeled probe. When specified, 5 μg of pancreatic nuclear extract, 5 μg of MIN-6 nuclear extract (gift from Jennifer VanVelkinburg, Vanderbilt University), 1 μL of anti-Ptf1a (Rose et al. 2001), or a specified quantity ranging from 0.4 to 40 pmol of non-radioactive competitor oligonucleotides was added to the binding reaction (Figure 18).

Reactions were incubated for 15 minutes at 30°C. 20 μL of each reaction were loaded and resolved at 4°C on a 4.0% non-denaturing polyacrylamide gel (40:1) in ice-cold Littman running buffer [50 mM Tris, 0.38 M glycine, 2 mM EDTA (pH 8.0)].

Transfection Plasmids

The following were a generous gift of Ray MacDonald (University of Texas, Southwestern Medical Center) and have been described previously (Beres et al. 2006): rat *elastase1* minimal promoter -92 to +8 in the pGL3 basic vector (Promega; *-92rEla1p/pGL3*); a six copy rat Elastase 1 PTF1 site (A element) and *elastase1* minimal promoter (*A26/pGL3*); HEB expressed under the control of cytomegalovirus (CMV) promoter (*HEB/pcDNA1.1*); mouse Ptf1a expressed under the control of CMV (*pCMV-mp48*); human RBP-Jκ expressed under the control of CMV (*Myc-hRbpsuh/pcDNA3*); mouse RBP-L expressed under the control of CMV (*CMV.RBbpsuh-L*).

Oligos annealing in the pBluescript II (Stratagene) vector (ATTAACCCTCACTAAAG) and to the 3' end of Area III (GTCTCTGATTTCTTCAGttAAAAGAGCCACCTtTGCCCGTCAAGGGGCC) (Gannon et al. 2001) were used to generate an E box and TC box mutant of the PTF1 binding site (-1730 to -1709); the lowercase nucleotides correspond to those targeted in other PTF1 binding mutants (Hsieh et al. 1996; Rose et al. 2001; Beres et al. 2006). Wild type and Area III mutant (*Pdx1*[mIII]) sequences were subcloned into the HindIII site just upstream of the herpes simplex virus

thymidine kinase (TK) minimal promoter driving the firefly luciferase expression cassette (TK-Luc) in pGL3 (Promega)(Onuma et al. 2006). Wild type and mutant $Pdx1^{I-II-III}$ TK-Luc were generated by subcloning Area I-II spanning sequences from the $Pdx1^{I-II-III}$ transgene into either $Pdx1^{III}$ TK-Luc or $Pdx1^{mIII}$ TK-Luc. The correctness of the plasmids was verified by restriction digestion and partial DNA sequencing.

Transient Transfections

The *Rattus norvegicus* exocrine-derived cell line (AR42J) was obtained from ATCC (Manassas, VA), and propagated according to specifications provided. Cells were transiently transfected with Lipofectamine 2000 (Invitrogen) in 24-well plates, 2×10^5 cells/well (0.8µg DNA/well) according to manufacturer's instructions. Data was analyzed for significance by One-Way ANOVA and Tukey's post-test.

The *Homo sapiens* embryonic kidney-derived cell line (HEK 293) was propagated in DMEM with 10%FBS according to specifications provided by ATCC. Cells were transiently transfected with Lipofectamine in 24-well plates, 1.5×10^5 cells/well (0.5µg DNA per well). Equimolar amounts of the wild type and Area III mutant TK-Luc plasmids were used at a 1:1 molar ratio with the CMV driven expression plasmids. Data was analyzed for significance by Two-Way ANOVA and Bonferroni's post-test.

TK driven *Renilla* luciferase expression plasmid phRL-TK (Promega; 10ng) was co-introduced into AR42J and HEK 293 cells to control for transfection

efficiency. *Renilla* and firefly luciferase activity were measured using the Dual Luciferase assay (Promega) on cell extracts prepared 40 to 48 hours after transfection. Each transfection condition was performed on at least four independent occasions. Statistical analysis and graph generation were performed using PRISM software (GraphPad).

Embryonic Dorsal Pancreas and Gut Tube Dissections

Dorsal pancreas bud dissections were performed as illustrated in Figure 21 panel A to F. Gut tube dissections were performed as previously described (Bossard and Zaret 2000). Tissue purity was assayed by detection of *Ptf1a* or *alb1* mRNA isolated from dissected tissues. mRNA from dissected tissues was isolated using the RNeasy Micro Kit (Qiagen) and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). PCR analysis was performed using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The following primers were used in 25 μL reactions at a concentration of 0.3 μM: *Ptf1a* GGTTATCATCTGCCATCGAGG, GCTGTTTTTCATCAGTCCAGGA, *alb1* AGCACACAAGAGTGAGATCGCC, TGGCATGCTCATCGTATGAGC and *Hprt* GCTTGCTGGTGAAAAGGACCT, TGCGCTCATCTTAGGCTTTGTA. The iCycler iQ and Optical System Software version 3.1 (Bio-Rad) were used to define the Ct value for each PCR reaction. Ct values of *Hprt* amplification were subtracted from Ct values of *Ptf1a* or *alb1* Ct for normalization. Relative expression was calculated as $2^{(-\Delta Ct)}$.

Ptf1a Chromatin Immunoprecipitation

Chromatin isolation and immunoprecipitation for embryo tissues was modified from established protocols (Weinmann et al. 2001; Chaya and Zaret 2004). Dissected material was fixed for 10 min at 25 ºC in 1% formaldehyde in PBS, pelleted, transferred to 125 mM glycine in PBS and incubated on ice for 5 to 10 min. Fixed cells were pelleted, resuspended in RIPA (50 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.5% Deoxycholic acid, 0.1% SDS, 1% IgePal CA-630, 5mM EDTA, 0.5 mM PMSF, 1mM benzamidine, 5 µg/mL antipain, 5 µg/mL leupeptin, 5 µg/mL trypsin inhibitor) and disrupted in a tissue homogenizer to liberate nuclei. Nuclei were pelleted and resuspended in 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5 mM PMSF, 1 mM benzamidine, 5µg/mL antipain, 5 µg/mL leupeptin, 5 µg/mL trypsin inhibitor and incubated on ice for 10 min prior to being flash frozen and stored at -80 ºC. Crude chromatin preparations were pooled from 163 dissected dorsal pancreatic buds and 77 gut tubes of E11.5 embryos. These nuclear isolates were sonicated to an average size of 150 to 400 bp. Equal amounts of chromatin (1 µg as estimated by DNA concentration) were diluted to into RIPA (modified to 1mM EDTA) and precipitated by Normal Rabbit IgG (Santa Cruz) or anti-Ptf1a rabbit IgG (gift from Ray MacDonald, UT Southwestern). Chromatin-antibody complexes were precipitated with protein A sepharose (Protein A-Sepharose CL-4B Amersham Cat N. 17-0780-01). Protein A beads were washed with RIPA (modified to 0.5 M NaCl) and eluted with 10 mM Tris-HCl pH 8.0, 1 % SDS, 1 mM EDTA for 20 min at 42 ºC. The crosslinks were reversed by incubation at 68 ºC overnight and the DNA was digested in 10 mM TrisHCl pH

8.0, 0.1 M NaCl, 1 mM EDTA, 1 % SDS, 0.1 mg/mL proteinase K, and phenol/chloroform extracted prior to PCR. ChIPed and input material was analyzed by PCR with the following primers: *Pdx1* Area I CTGGGACAGAGTCTCAGCAGAAG and CGTCCTGATAGTCCTCCCTGAT, *Pdx1* Area II AGCAGCGAGCTTGTTTTTCTG and CACTTCTGGTCTAATTGCATGCA, *Pdx1* Area III TGCCCCGGCCTTTCA and GGGAGAGTGTCTCTGATTTCTTCAG, *Pdx1* Area IV GCTCCAATGCCATTTGTCAA and TGGATCCTGACTGCGTCTTG, and *elastase 1* promoter TTGACTTAAAATTTGTTCATTTGT and ACCCTCTTTATACGGCTCTT. PCR reactions were analyzed using the 2100 Bioanalyzer and dsDNA 1000 Lab Chips (Agilent Technologies). PCR product concentration and molarity were calculated using 2100 Bioanalyzer Expert Software (Agilent Technologies).

Results

Lineage tracing reveals that Areas I and II drive expression to pancreatic endocrine, but not exocrine tissue

We previously reported that the 1 kb PstI-BstEII fragment (*Pdx1*[PB]; -3007 to -2011), which contains Areas I and II from the *Pdx1* locus (and none of Area III), drives expression of a β-gal reporter transgene exclusively in endocrine cells as early as E11.5 and continuing into adulthood (Gannon et al. 2000; Gannon et al. 2001; Van Velkinburgh et al. 2005; Zhang et al. 2005). A specific role for Areas I and II in regulating endocrine expression is supported here by lineage-tracing analysis using *Pdx1*[PB]Cre mice. *Pdx1*[PB]Cre transgenic mice were crossed with the lineage-independent *Gt(ROSA)26Sor*[tm1sor] (R26R) reporter strain (Soriano 1999) to determine whether *Pdx1*[PB] was activated at an earlier stage of pancreatic development within a common endocrine/exocrine pancreatic progenitor. In this case, we expected to observe β-gal activity in all pancreatic bud derivatives (acini, ducts and islets). As shown in Figure 14, *Pdx1*[PB]Cre-mediated recombination within the pancreas was detected only in islet endocrine cells at postnatal day one (P1). Recombination was also observed in a few scattered cells within the stomach and duodenum (sites of endogenous Pdx1 expression, Figure 14) and ectopically in dorsal root ganglia. When X-gal staining was observed in the duodenal lumen, it was present in both transgenic and non-transgenic neonatal littermates, and is most likely due to endogenous intestinal β-gal activity (Gannon et al. 2001). Within the islets, recombination was throughout the insulin-expressing β cell core and in some glucagon-expressing

cells (Figure 14B). Despite generating only one $Pdx1^{PB}$Cre transgenic line, our findings are consistent with our previously published results regarding the $Pdx1^{PB}$hsplacZ transgenic mice, the $Pdx1^{PB}$Cre-ER^{TM};R26R bigenic mice (Gannon et al. 2001; Zhang et al. 2005), and the expression of endogenous Pdx1 in islets after birth (Guz et al. 1995). Thus, our combined analyses indicate that Areas I and II are not sufficient to direct expression to early pancreatic progenitors or differentiated pancreatic exocrine tissue.
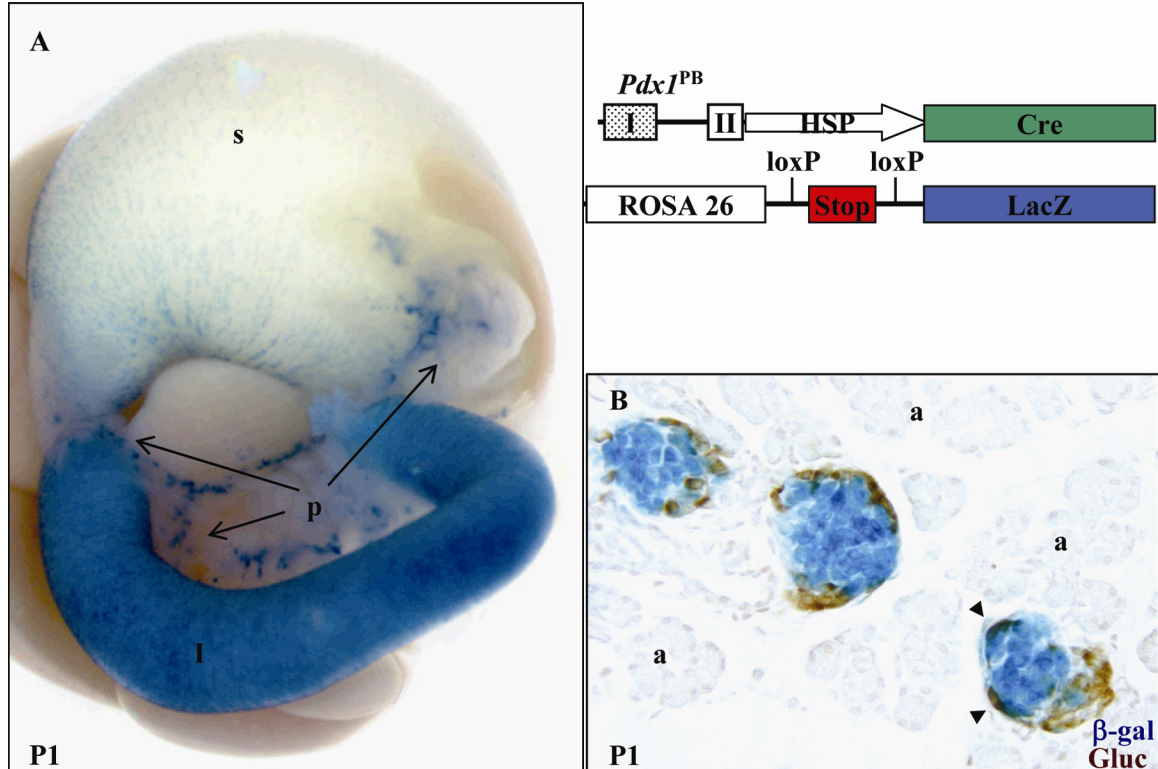
Figure 14. Lineage tracing of a region that contains the conserved Areas I and II (*Pdx1*^{PB}Cre) mediated recombination only in islet endocrine cells of the pancreas at postnatal day one (P1). (A) Whole mount of X-gal stained (blue) P1 digestive organs from *Pdx1*^{PB}Cre;R26R showing high levels of expression in islets and some cells within the stomach. Arrows indicate different regions of the pancreas containing X-gal stained cells consistent with normal islet distribution. X-gal staining was sometimes observed in the duodenal lumen of transgenic and non-transgenic neonatal littermates. (B) Cross-section of X-gal stained P1 pancreas tissue labeled with antibodies to glucagon (Gluc) in brown. Arrowheads point to cells positive for both glucagon and β-gal. Pancreas (p), stomach (s), intestine (I), acinar tissue (a). Top diagram shows schematic of *Pdx1*^{PB}Cre transgene and R26R reporter construct for lineage tracing of Cre expressing cells (not to scale).

Addition of Area III to Areas I and II is sufficient to drive early pancreas-wide expression

Four independent *Pdx1*^{I-II-III}lacZ transgenic mice with detectable

expression were generated, in which lacZ is under the control of *Pdx1*^{PB}

(containing Areas I and II) plus Area III (Figure 13). *Pdx1*[I-II-III]lacZ transgenic mice showed high levels of β cell-enriched expression in the mature pancreas, but at earlier stages showed pancreas-wide expression, at apparently equivalent levels throughout the endodermally derived tissue (Figure 15, Figure 16).
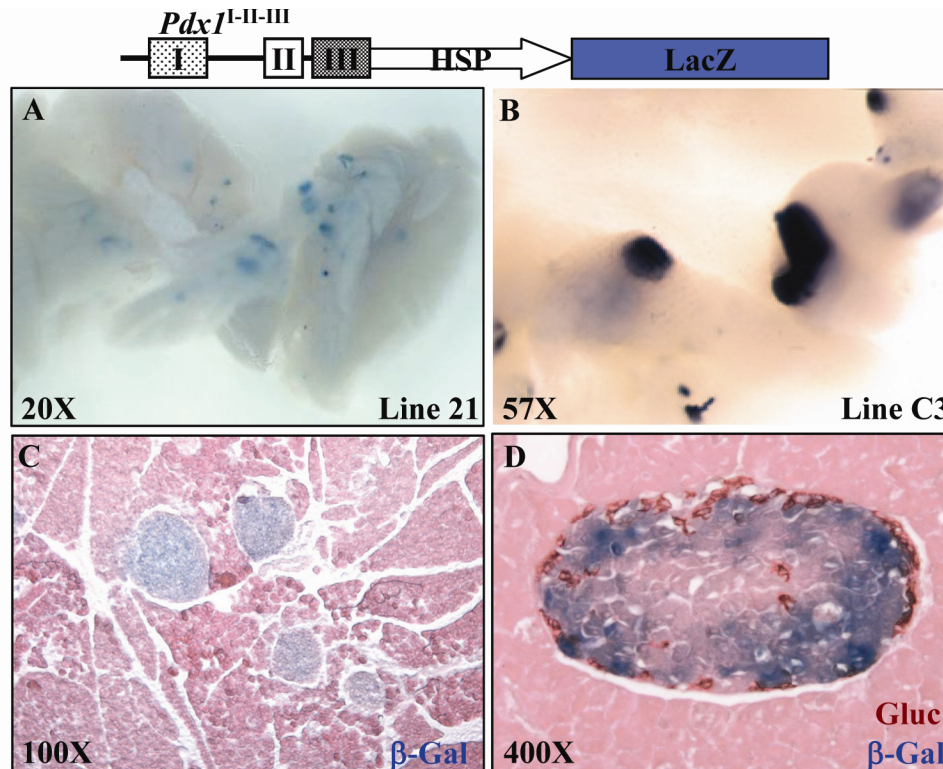
Figure 15. Expression is restricted to β cells in *Pdx1*[I-II-III]lacZ adults. (A, B) Whole mount pictures of X-gal stained (blue) adult pancreata from two independent transgenic lines showing an islet-enriched expression pattern. (C, D) Representative cross-sections of X-gal stained adult pancreata counterstained with eosin, which was typically detected throughout the β cells (C), but sometimes appeared variegated (D). Glucagon-expressing cells are labeled with an antibody in brown (Gluc, D). Top diagram refers to transgene used to create the mice analyzed in this figure (not to scale).

Two *Pdx1*[I-II-III]lacZ lines were propagated and characterized. Adult pancreata from both *Pdx1*[I-II-III]lacZ lines were X-gal stained to assess mature

organ expression specificity of the transgene. Both in whole mount and sections, a pattern of β-gal activity consistent with islet-specific expression in the pancreas was observed, preferentially in β cells (Figure 15). The two $Pdx1^{I-II-III}$lacZ lines showed differing levels of expression in the adult (Figure 15A, B), and the expression pattern within islets was sometimes variegated (Figure 15C), but both lines showed expression within the same cell types. Exocrine expression was not detectable in adult pancreata (Figure 15C, D). The expression pattern at this stage was similar to that seen in $Pdx1^{PB}$Cre; $R26R$ mice (Figure 14), and to that previously reported for the $Pdx1^{PB}$hsplacZ (Wu et al. 1997; Gannon et al. 2001) and $Pdx1^{PB}$Cre-ER^{TM};$R26R$ transgenic mice (Zhang et al. 2005).

$Pdx1^{I-II-III}$lacZ transgenic embryos were examined at E9.5, E10.5, and E11.5, the time during which pancreatic buds first become visible and outgrowth begins. $Pdx1^{I-II-III}$lacZ expression was undetectable at E9.5, but was detected broadly and robustly at both E10.5 and E11.5 within the dorsal and ventral pancreatic buds, but not in the adjoining gut tube (Figure 16D, E). Consistent with the lack of transgene expression in the antral stomach and rostral duodenum, X-gal staining co-localized with Pdx1 protein in the pancreatic region at E10.5, but was absent from the Pdx1-labeled stomach (Figure 16C). At E14.5 and E16.5, whole mount β-gal expression was observed throughout the pancreas at relatively equal levels (Figure 16F). In contrast, whole mount analysis at E18.5 and P1 revealed an expression pattern more consistent with forming islet clusters (Figure 16G, H). Examination of sections at these time points showed β-gal expression in both exocrine and endocrine cells, however, the level of β-gal

expression observed in endocrine cells was more intense than that observed in exocrine cells, and expression in exocrine cells was variegated (Figure 16I, J). To verify our findings, we collected two additional independent F_0 transgenic $Pdx1^{I\text{-}II\text{-}III}$lacZ mice at late embryonic stages, which had patterns of expression consistent with our propagated $Pdx1^{I\text{-}II\text{-}III}$lacZ transgenic lines. Thus, the expression of $Pdx1^{I\text{-}II\text{-}III}$lacZ gradually increases within endocrine cells and diminishes from exocrine cells between E14.5 and P1, eventually becoming restricted to β cells in adult tissue, similar to endogenous $Pdx1$ expression within the pancreas.
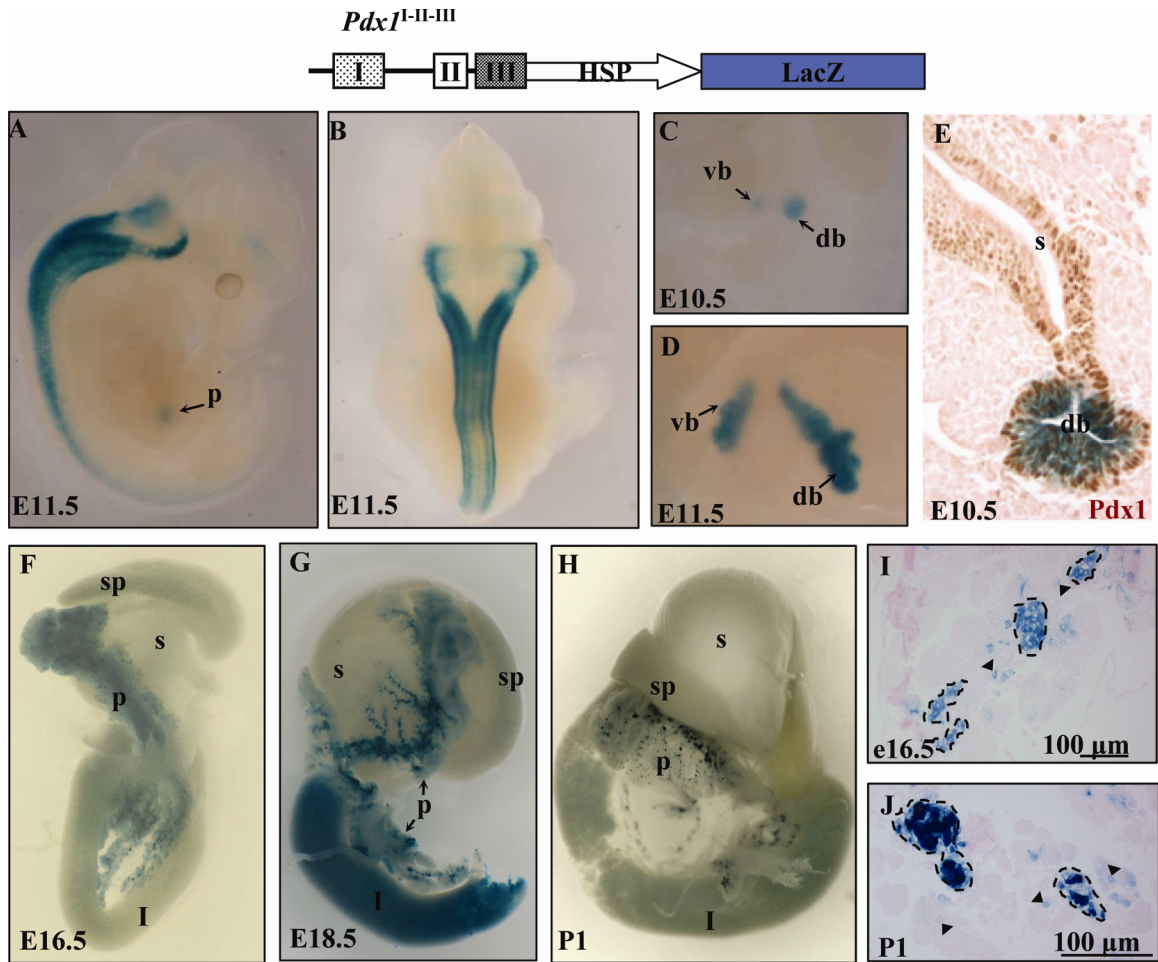
Figure 16. *Pdx1*[I-II-III]lacZ is expressed in both endocrine and acinar cells during development. X-gal stained (blue) embryos at E11.5 (A-B) showing expression in pancreas and ectopic expression in the neural tube. X-gal staining in the developing pancreas at E10.5 (C) and E11.5 (D). Section from an X-gal stained E10.5 (E) embryo labeled for Pdx1 protein (brown). Whole mount pictures of X-gal stained dissected digestive organs at E16.5 (F), E18.5 (G), and P1 (H). Section from an X-gal stained E16.5 (I) and P1 (J) embryo. Triangles point to blue acinar tissue. Pictures are representative of expression observed in four of four independent transgenic mice. Top diagram refers to transgene used to create the mice analyzed in this figure (not to scale). Luminal X-gal staining was observed in the duodenum of some transgenic and non-transgenic littermates at late gestation and in neonates (G). Pancreas (p), spleen (sp), stomach (s), rostral duodenal portion of the intestine (I), pancreatic dorsal bud (db) and ventral bud (vb), embryonic day (E), postnatal day (P).

Pdx1[I-II-III]lacZ mice exhibited β-gal activity beginning at E10.5 in the dorsal neural tube caudal to the midbrain/hindbrain junction (Figure 16A, B), which was observed in all four transgenic animals generated, suggesting that this ectopic expression was inherent to the transgene. This expression in the neural tube was broader than previously described transgenes containing only area I and II, which was found in the dorsal root ganglia (Gannon et al. 2000; Gannon et al. 2001; Van Velkinburgh et al. 2005; Zhang et al. 2005). In the two lines characterized at adult stages *Pdx1*[I-II-III]lacZ mice also had low levels of ectopic expression in the kidneys and hair follicles, similar to *Pdx1*[PB]Cre-ER[TM];R26R bigenic mice (Zhang et al. 2005). Expression was not observed in other sites of endogenous Pdx1 expression, such as the antral stomach or rostral duodenum (Offield et al. 1996), at any time point analyzed.

The Area III-containing XhoI-BglII fragment drives expression early throughout the *Pdx1* domain

The *Pdx1*[XB] fragment drives reporter gene expression transiently in β cells, which was observed at E14.5 and P1, but not in the adult (Gannon et al. 2001). Earlier embryonic stages were not examined in these transgenic lines. To trace the cumulative effect of activity of this fragment throughout development, we generated four independent *Pdx1*[XB]Cre transgenic mice lines. All four lines shared the same overall pattern of recombination (Figure 17), although in one line recombination was not as robust. We identified cells and their progeny that expressed Cre at high enough levels to cause recombination of the *R26R*

reporter (Soriano 1999), even after $Pdx1^{XB}$ activity is reported to have ceased (Gannon et al. 2001). In all $Pdx1^{XB}$Cre;*R26R* transgenic mice, we observed recombination throughout the endogenous *Pdx1* domain at both E14.5 and P1 (Figure 17F, G). The pattern of recombination observed in whole mounts included pancreas, duodenum, antral stomach, and bile duct, which are all tissues that arise from cells expressing endogenous *Pdx1* (Offield et al. 1996). Analysis of sections from P1 $Pdx1^{XB}$Cre;*R26R* animals revealed complete recombination throughout both the endocrine and exocrine pancreas (Figure 17C), suggesting an early recombination event. Indeed, examination of earlier embryonic stages revealed that the $Pdx1^{XB}$Cre transgene is active in the posterior foregut region as early as E9.5. At E10.5, *R26R* recombination was observed in the entire dorsal and ventral pancreatic buds of the dissected digestive organs, and was also detectable in the adjoining gut tube (Figure 17A, B). By E11.5, *R26R* recombination was also apparent in the common bile duct (Figure 17D), consistent with later stages. Occasionally, whole mice underwent ubiquitous recombination, otherwise no ectopic expression was observed; recombination was consistently restricted to the endogenous *Pdx1* expression domain.
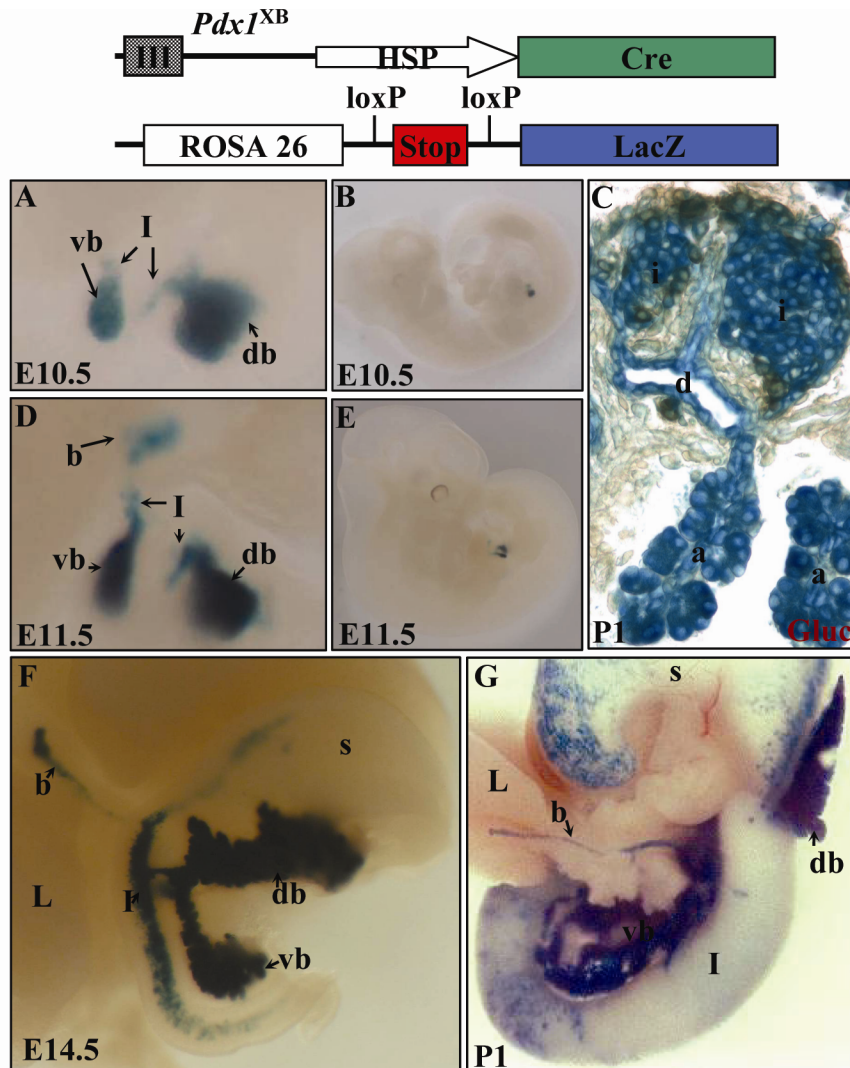
Figure 17. Lineage tracing of Area III-containing, $Pdx1^{XB}$Cre-mediated recombination throughout the endogenous $Pdx1$ domain. Whole mount pictures of X-gal stained E10.5 (B) and E11.5 (E) embryos, and dissected digestive organs at E10.5 (A), E11.5 (D), E14.5 (F), and P1 (G). (C) Cross-section of X-gal stained P1 pancreas labeled with antibodies to glucagon (Gluc; brown). Pictures are representative of expression observed in four of four independent transgenic mice. Top diagram refers to transgene used to create the mice analyzed in this figure and R26R reporter construct for lineage tracing of Cre expressing cells (not to scale). Dorsal pancreas (d), ventral pancreas (v), liver (L), stomach (s), rostral duodenal portion of the intestine (I), bile duct (b), embryonic day (E), postnatal day (P), pancreatic islet (i), acinus (a), duct (d), dorsal bud (db), ventral bud (vb).

Ptf1a binds to Area III *in vitro*

Collectively, the data demonstrate that Area III is involved in directing early, pancreas-wide *Pdx1* expression. Area III sequences were thus analyzed *in silico* using Tfsearch and the TRANSFAC database to identify a potential transcription factor(s) responsible for controlling this expression. A PTF1-like binding site was found at the 3' end (nucleotides -1730 to -1709) of Area III, which was highly relevant since *Ptf1a* is co-expressed with *Pdx1* throughout the pancreatic buds at E10.5 (Kawaguchi et al. 2002). This site had high identity within both the E and TC boxes that define Ptf1a-HEB-RBP-Jκ/L (PTF1) activator binding (Figure 18C)(Cockell et al. 1989; Rose et al. 2001; Beres et al. 2006), whereas a PTF1-like site was not found in Area I or II.

Gel shift analyses were performed using the PTF1-like binding site from Area III and the well-characterized PTF1-binding site from the *elastase 1* gene (*Ela1*; Figure 18)(Rose et al. 2001). Nuclear extracts enriched in PTF1 from adult mouse pancreata illustrated that the Area III site formed a complex of similar mobility to the *Ela1* PTF1 element (Figure 18A, lanes 9 and 12), suggesting that the putative Area III PTF1 site bound to the same protein component(s) as the *Ela1* PTF1 element. Furthermore, the Ptf1a antibody completely retarded the mobility of the co-migrating complex of both Area III and *Ela1* PTF1 probes (Figure 18A, lanes 10 and 13). In contrast, a complex of quite different mobility, and insensitive to the Ptf1a antibody, was formed with extracts from the MIN-6 mouse β cell line, which does not endogenously produce Ptf1a (Krapp et al. 1996)(Figure 18A, lane 14 and 15). These results suggested that a complex

containing PTF1a was capable of binding to the -1730 to -1709 bp element of

Area III.

A

Competitor: AIII PTF1   *Ela1* PTF1   Bl

Ptf1a Ab | | | | | | | | | | + | | + | | + |
AIII PTF1 probe | + | + | + | + | + | + | + | | | + | + | + | + | + |
Ela1 PTF1 probe | | | | | | | | + | + | + | | | | |
Panc nuc extract | + | + | + | + | + | + | + | | + | + | + | + | | |
Min6 nuc extract | | | | | | | | | | | | | + | + |

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B   Competitor: - AIII PTF1   E1   E2   Dup   TC E3

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

C

```
                   E box              TC box
Ela1 PTF1 gtCAccTGtgct------TTTCCCtgc
AIII PTF1 caCAggTGgctct-----TTTCCCtg
      E1  ..A...........-----........
      E2  .....t........-----........
     Dup  .............gctct........
      TC  ..............-----....AA..
      E3  ..TG..CA.....-----........
      Bl  ..AGtaGT.....-----GCGATA..
```
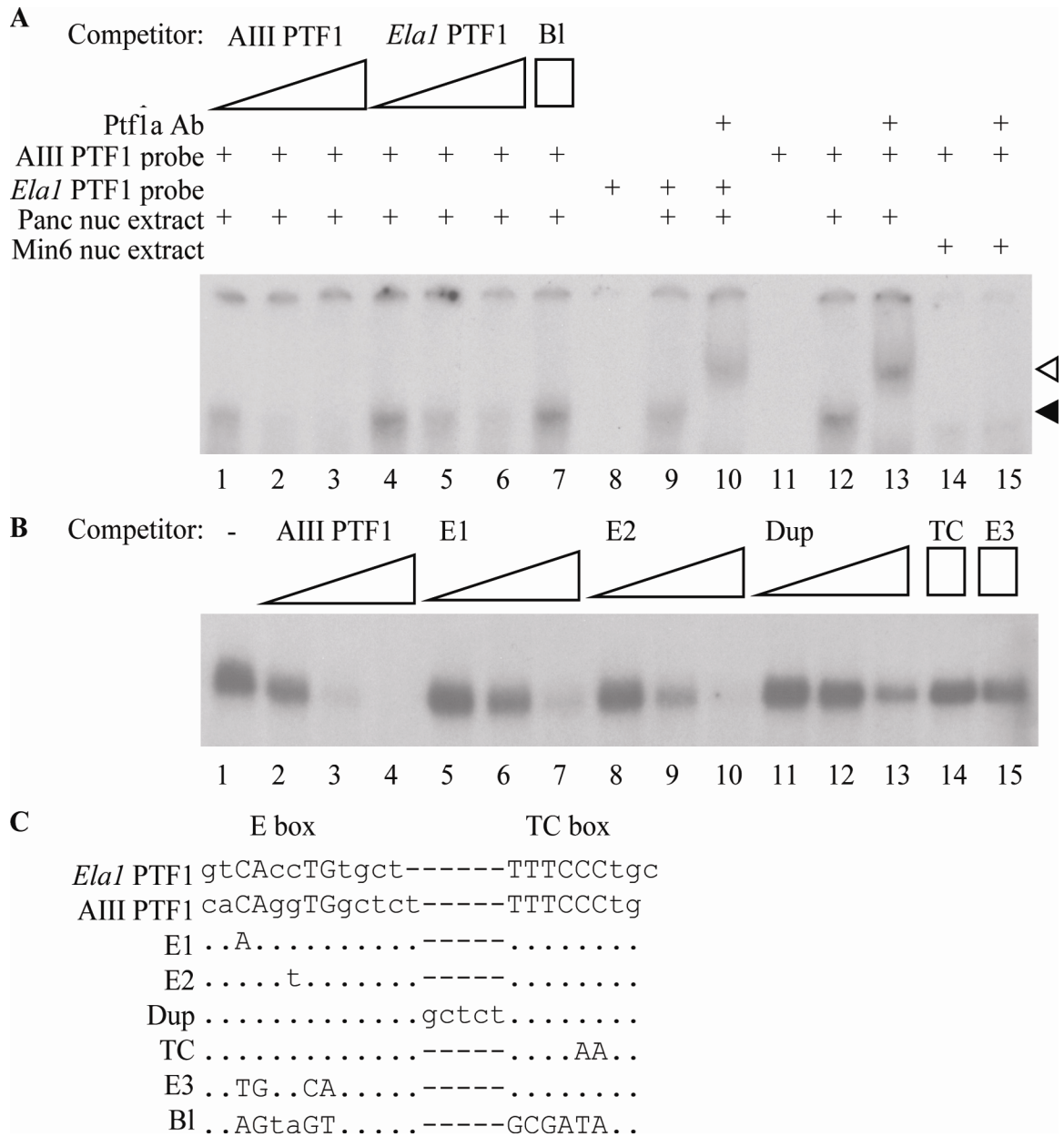
Figure 18. Ptf1a-containing complex binds Area III and requires both the E and TC box. (A) Gel shift using the putative PTF1 binding site from Area III or the PTF1 binding site from the elastase (*Ela1*) promoter (closed arrowhead refers to specific complex). Addition of Ptf1a antibody (Ab) results in a complete supershift of the PTF1 complex bound to both the AIII probe and *Ela1* PTF1 probe (open arrowhead). Right triangles depict increasing amounts of cold competitor: 10x, 50x, and 100x. Rectangle represents 100x competitor. (B) Gel shift competition using indicated E box or TC box mutant cold competitors. Right triangle depicts increasing amounts of cold competitor: 10x, 100x, and 1000x. Rectangles represent 1000x cold competitor. (C) Sequences of oligonucleotides used in the gel shift experiments. Dashes (-) indicate gaps, and periods (.) indicate homology to AIII PTF1 probe. Capital letters indicate conserved nucleotides.

Binding of the Ptf1a-containing complex to the Area III element requires both the E box and TC box

A PTF1 complex binding site contains both a bHLH E box (<u>CA</u>NN<u>TG</u>; the invariant nucleotides are underlined) for Ptf1a-HEB binding and a contiguous TC box (<u>TTTCCC</u>) for binding of RBP-J or RBP-L (Figure 18C). The PTF1 site was characterized to determine if both the E box and the TC box were necessary for PTF1 complex binding in the context of the Area III element. Increasing concentrations of mutant oligonucleotides were used in gel shift competition experiments to determine the requirement for each of these sites in binding of the complex. The PTF1 E box within Area III was modified in several different ways predicted to affect bHLH binding. Compared with unlabeled wild type probe, the E2 mutant, in which a guanine at position four in the E box was replaced with a thymine, was able to efficiently compete for the PTF1 complex only at high concentrations (Figure 18B, lanes 8-10). The E1 oligonucleotide contains a more severe mutation (the highly conserved cytosine at position one was converted to an adenine), and was less able to compete for the PTF1 complex (Figure 18B, lanes 5-7). In the E3 mutant, all of the conserved E box nucleotides were transposed, resulting in a virtual inability to compete even at the highest level of competitor (Figure 18B, lane 15). These data indicate that the Area III E box is essential for PTF1 complex formation, and other nucleotides within the element may also contribute to PTF1 complex binding. To this end, we generated a duplication (Dup) mutant oligonucleotide in which the sequence between the E box and the TC box was duplicated, increasing the spacing between the two sites a half a DNA helical turn, and thus placing the sites on opposite faces of the

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double helix (Figure 18C)(Cockell et al. 1989; Rose et al. 2001; Beres et al. 2006). The spacing between these elements is critical for PTF1 binding. The Dup oligonucleotide competed poorly for the PTF1 complex (Figure 18B, lanes 11-13), consistent with the notion that both the E box and TC box are important for PTF1 complex formation, and that transcription factors binding these sites likely interact for optimal complex formation. The TC mutant oligonucleotide was based on a previous mutation used to eliminate transcriptional activation of the RBP-J site (Rose et al. 2001). This mutant oligonucleotide was unable to compete for the PTF1 complex, despite having an intact E box, reiterating the importance of both the position and presence of an E box and TC box for proper PTF1 complex formation in the context of the Area III element (Figure 18B, lane 14). The competition patterns demonstrated that Ptf1a-HEB-RBP-Jκ/L are present in the Area III Ptf1a-containing binding complex.

PTF1 is important for Area III-mediated activation

To determine if the PTF1 site of Area III was involved in *Pdx1* promoter activation, the activity of a binding defective mutant constructed in the context of either a *Pdx1*[III] or *Pdx1*[I-II-III] reporter was compared to its wild type version in transfected AR42j cells. This pancreatic acinar cell line produces both PTF1 (Cockell et al. 1989; Yasuda et al. 2002) and *Pdx1* (Yamagata et al. 2002). The PTF1 site mutation significantly reduced *Pdx1*[I-II-III] TK-Luc and *Pdx1*[III] TK-Luc activity (Figure 19A, Figure 20). Moreover, *Pdx1*[III] activity was stimulated in a PTF1 site-dependent manner upon co-transfection of Ptf1a, HEB, and RBP-Jκ, L

in HEK 293 cells, a non-pancreatic cell line that does not endogenously produce PTF1 (Figure 19B, Figure 20)(Beres et al. 2006). Experiments performed with a reporter driven by six copies of the *elastase 1* PTF1 binding site verified the PTF1-dependent activity in both AR42J and HEK 293 cell lines (Figure 20). Collectively, these results demonstrate that PTF1 is capable of specifically binding to and stimulating activity through Area III.
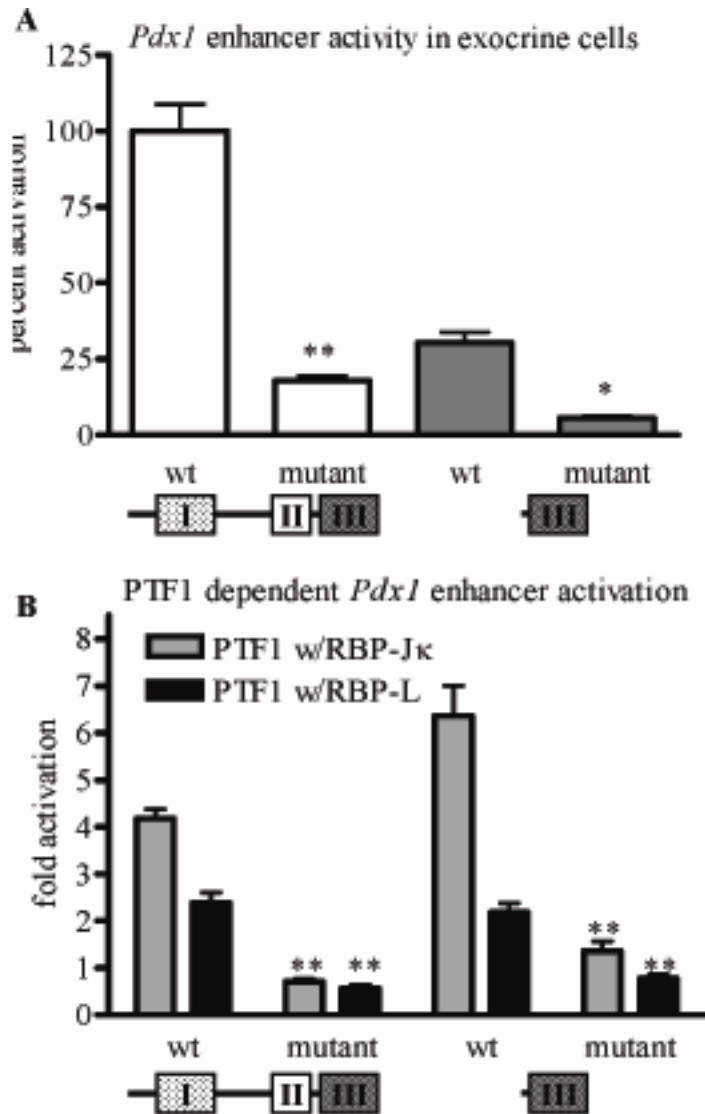
Figure 19. *Pdx1* Area III activity is dependent on PTF1. (A) Wild type (wt) and mutated (mutant) PTF1 binding site TK-Luc reporters were transfected into AR42j cells. Activity is expressed as percent *Pdx1*[I-II-III] ± SEM. (B) Expression plasmids of Ptf1a, HEB, and RBP-Jκ or -L were cotransfected into HEK 293 cells with the Area III-containing TK-Luc plasmids. Data is expressed as fold activation ± SEM over the respective Area III-containing TK-Luc cotransfected with insertless expression plasmids. Asterisk (*) indicates significance upon comparing mutant to wild type: * $p < 0.01$, ** $p < 0.001$.
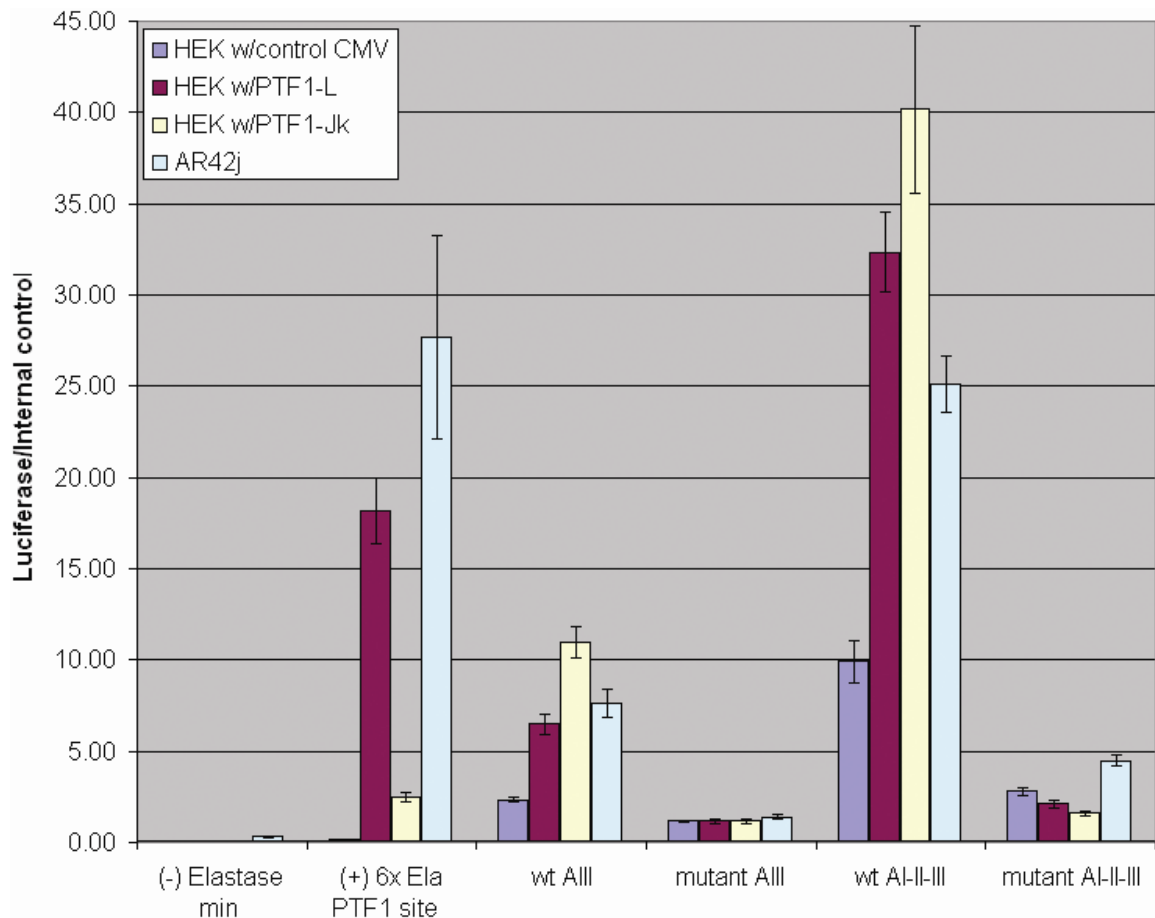
Figure 20. PTF1 dependent expression of luciferase reporter plasmids transfected into HEK293 (HEK) and AR42j cells. A subset of this raw data was used to generate the previous figure (Figure 19). The Elastase min and 6x Ela PTF1 site are identical except for the addition of six copies of the Elastase PTF1 binding site in the latter. Activity mediated by PTF1-Jκ relative to PTF1-L is attenuated in the context of the *Ela1* PTF1 site, whereas this relative activity is equivalent if not greater in the context of the *Pdx1* PTF1 site. Empty control plasmid or expression plasmids of Ptf1a, HEB, and RBP-Jκ or -L were cotransfected into HEK 293 cells with the Area III-containing TK-Luc plasmids as indicated. Activity is expressed as firefly/*Renilla* luciferase activity ± SEM.

Endogenous Ptf1a binds the *Pdx1* promoter *in vivo*

To investigate whether Ptf1a bound *Pdx1* upstream regulatory sequences during pancreatic organogenesis, we developed conditions for performing chromatin immunoprecipitation (ChIP) from tissues that were microdissected from E11.5 mouse embryos. Similarly staged embryos from *Pdx1*[lacZ/+] knock-in

mice (Offield et al. 1996) stained for β-gal served as controls for developing a protocol to dissect wild type dorsal pancreatic buds from the gut tube (Figure 21, panels A-E). Real-time RT-PCR analysis of RNA revealed that the microdissected buds expressed the *Ptf1a* gene, whereas RNA from the gut tube, heart, and liver bud were negative, as expected (Figure 21, panel G). Conversely, the *Ptf1a*-positive buds were negative for the liver gene, *albumin1* (*alb1*; Figure 21, panel G), also as expected. We pooled 163 such pancreatic buds, crosslinked the chromatin in the tissues, and performed ChIP with a Ptf1a-specific antibody. Pooled gut tubes served as negative controls. The scaled-down ChIP reactions required analysis of the PCR products on a microfluidics workstation; the data depict two analytical runs per PCR reaction. PCR analysis of the ChIP products with primers to the *elastase 1* gene promoter yielded markedly higher signals from the dorsal pancreatic buds than from the gut tube control (Figure 21, panel H). These data confirm the validity and specificity of the embryonic ChIP assay for Ptf1a.

Figure 21. Ptf1a protein is specifically enriched at Area III and Area IV of the *Pdx1* promoter. A–F: The midgut region (A) and (B) isolated from E11.5 day embryos. The region containing the dorsal pancreas was removed (C) and (D) and separated from contaminating tissue (E). Pancreatic buds pooled and processed for chromatin cross linking and isolation (F). (G) Tissue purity was assayed by Ptf1a or alb1 expression. mRNA isolated from dissected organs was subjected to real time RT-PCR and normalized to HPRT mRNA. (H) Ptf1a chromatin immunoprecipitation was performed on chromatin isolated from E11.5 dorsal pancreas and gut tube. The data represent the average of two Bioanalyzer runs per primer pair set amplified in parallel from single ChIP reactions (IgG or Ptf1a) from designated embryonic tissues.

Using primers specific for amplification of each of the previously identified highly conserved regions within the *Pdx1* promoter (Areas I-IV), we next analyzed whether Ptf1a interacted specifically with Area III or any of the other known regulatory regions. Strikingly, both Areas IV and III exhibited strong binding by Ptf1a in E11.5 embryonic pancreatic buds, compared to the gut tube controls. By contrast, the islet-specific Areas I and II did not exhibit binding to Ptf1a. These data further support a role PTF1 in expression of *Pdx1* in early exocrine and endocrine progenitors.

Discussion

The present study provides further insight into the dynamics of *Pdx1* regulation during pancreatic development by illustrating that the early pancreatic developmental regulator, PTF1, serves in Area III-mediated activation. Prior to the current study, a role for Area III in *Pdx1* gene regulation had not been established, despite the high degree of sequence conservation within this region among vertebrates (human, mouse, and chicken). In cell lines, Area III, and the larger XhoI-BglII fragment (*Pdx1*[XB]) in which it is located, were incapable of directing β cell-selective reporter gene activation (Wu et al. 1997; Gerrish et al. 2000), suggesting that these sequences might participate in other aspects of *Pdx1* gene expression. In transgenic analyses, however, *Pdx1*[XB] drove *lacZ* expression exclusively to β cells at E14.5 and P1, leading us to conclude that sequences within this fragment did indeed participate in β cell-specific regulation of *Pdx1*, although this activity was transient (Gannon et al. 2001). Here we provide evidence for Area III facilitating the early and broad expression of *Pdx1* in pancreatic buds.

Sequences within *Pdx1*[I-II-III] direct dynamic *Pdx1* expression in the pancreas

The *Pdx1*[I-II-III]lacZ transgene differs from the previously published endocrine-specific *Pdx1*[PB]hsplacZ only in the addition of Area III. *Pdx1*[I-II-III]lacZ was expressed throughout the pancreatic buds in precursors to all cell types at E10.5; as development proceeded expression was gradually diminished in acinar cells and intensified in endocrine cells. Thus, sequences within Area III likely

confer early pancreas-wide expression to the islet endocrine-specific $Pdx1^{PB}$ fragment. $Pdx1^{I-II-III}$ was unable to drive expression within the $Pdx1$ domain outside the pancreas (the antral stomach, rostral duodenum, or common bile duct). These expression data are consistent with the phenotype of mice containing a global deletion of $Pdx1^{I-II-III}$ ($Pdx1^{\Delta I-II-III}$) in which $Pdx1$ expression is dramatically reduced in the pancreatic buds, but is essentially normal in stomach and duodenum(Fujitani et al. 2006). Consequently, $Pdx1^{I-II-III}$ deletion resulted in limited pancreatic bud outgrowth and differentiation with little effect on stomach and duodenum, in contrast to the global $Pdx1$ deletion (Larsson et al. 1996; Offield et al. 1996).

Sequences in *Pdx1* Area III mediate embryonic pancreas-wide expression

Similar to $Pdx1^{I-II-III}$ reporter expression, the $Pdx1^{XB}$ fragment drove Cre recombinase activity throughout the pancreatic buds early in pancreatogenesis. The extent of recombination observed in $Pdx1^{XB}$Cre;*R26R* mice is reminiscent of that mediated by *Pdx1*Cre (Gu et al. 2002), in which Cre is driven by a 5.5 kb *Pdx1* promoter fragment, which contains sufficient sequence to recapitulate the endogenous *Pdx1* expression pattern (Stoffers 1999; Gannon et al. 2001). In contrast to the expression driven by $Pdx1^{I-II-III}$, $Pdx1^{XB}$Cre-mediated recombination was also detected at high levels in other areas of endogenous *Pdx1* expression (antral stomach, common bile duct and rostral duodenum). The conserved region in common between the $Pdx1^{I-II-III}$ and $Pdx1^{XB}$ fragments is Area III (Figure 13), suggesting that sequences within this region are responsible

for the early, broad expression of *Pdx1* within the pancreas. The region 3' to Area

III within the *Pdx1*[XB] fragment shows no significant sequence conservation

between species and is incapable of driving robust *lacZ* expression to *Pdx1*-

producing cell types *in vivo* (Gerrish et al. 2000; Gannon et al. 2001). It is

possible that antral stomach and duodenal expression are normally driven by

Area III *in vivo*, but repressed by sequences within the *Pdx1*[PB] fragment in the

Pdx1[I-II-III] transgene. This seems unlikely, however, given the phenotype of the

Pdx1[ΔI-II-III] mice, where in the stomach and duodenum only the enteroendocrine

cell are affected, specifically a 50% reduction in stomach gastrin cells and

duodenal GIP cells. Alternatively, sequences 3' to Area III within the *Pdx1*[XB]

fragment may mediate extra-pancreatic expression of *Pdx1*, but require

interaction with factors binding Area III to stabilize, or enhance their activity. To

date, a duodenal or gastric regulatory element has not been identified in the

Pdx1 gene. Our *in vivo* transgenic analysis supports a role for Area III in

regulation of early, broad expression of *Pdx1* throughout the pancreatic bud

epithelium.

Area III mediates acinar expression of *Pdx1*

Acinar expression of β-gal has not been observed in any mouse line

carrying transgenes driven by the Areas I and II-containing *Pdx1*[PB] fragment

[*Pdx1*[PB]hsplacZ (Wu et al. 1997; Gannon et al. 2001; Van Velkinburgh et al.

2005), *Pdx1*[PB]Cre (this study), or *Pdx1*[PB]Cre-ER[TM] (Zhang et al. 2005)],

suggesting that elements within Area III are responsible for endogenous *Pdx1*

acinar expression. Since Ptf1a expression is initially expressed broadly throughout the pancreatic buds but becomes restricted to acinar tissue, and is capable of binding to sequences upon which Area III activity is dependent, we hypothesize that the dynamic changes in *Pdx1* expression within acinar tissue are mediated, at least in part, by PTF1. Recent studies indicate that expression of another component of the PTF1 complex, the mammalian Suppressor of Hairless orthologue RBP-Jκ/L, is also developmentally regulated. RBP-Jκ is more highly expressed during embryonic pancreas development, while RBP-L is exclusively found in adult pancreata (Beres et al. 2006). As RBP-Jκ and RBP-L differ in their transcriptional activity (Beres et al. 2006), this developmental switch in the TC box binding factor may explain the change in acinar *Pdx1* expression.

PTF1 activates Area III and binds to this control region in embryonic pancreas

PTF1 was a likely candidate for early, broad regulation of *Pdx1*, since its pancreas-specific component, Ptf1a, is expressed pancreas-wide as early as E9.5 (Obata et al. 2001; Kawaguchi et al. 2002), correlating well with the early pancreatic pattern of β-gal expression we observed in the *Pdx1*[I-II-II]lacZ (Figure 16D, E) and the *Pdx1*[XB]Cre;*R26R* mice (Figure 14A, B). Also, the ectopic β-gal activity exhibited by the *Pdx1*[I-II-III]lacZ mice in the dorsal neural tube at E10.5 is identical to β-gal expression in the *Ptf1a*[Cre/+];*R26R* mouse in those regions (Kawaguchi et al. 2002; Glasgow et al. 2005). Analysis of Area III *in silico* revealed a putative PTF1 site: a conserved CANNTG (E box) and TTTCCC (TC box) separated center to center by one turn of DNA (Rose et al. 2001). Ptf1a

present in nuclear extracts from adult mouse pancreata bound the putative PTF1 site in Area III with relatively high affinity (Figure 18), and activity mediated by Area III was dependent on this site (Figure 19). *In vivo* relevance of the PTF1 site within Area III is demonstrated by the binding of endogenous Ptf1a to Area III within the endogenous *Pdx1* promoter in developing pancreatic buds at E11.5 (Figure 21). Our data do not exclude the possibility that other transcription factors affect transcription of *Pdx1* during early pancreatic bud formation and outgrowth (Boyer et al. 2006; Poll et al. 2006), but suggest that PTF1 activates, at least in part, early pancreas-wide expression of *Pdx1* through Area III.

The significance of Ptf1a binding to Area IV remains to be determined. The role of Area IV in regulation of *Pdx1* gene expression is only now beginning to be addressed. *Pdx1* upstream regulatory sequences lacking Area IV are capable of driving reporter transgene expression in a pattern temporally and spatially indistinguishable from endogenous Pdx1 (Stoffers 1999; Gannon et al. 2001). Transgene-based complementation experiments on *Pdx1* null mice, suggest however, that Area IV is required for appropriate levels of Pdx1 expression in the postnatal stomach and duodenum, although it is not required to rescue the pancreatic defects caused by *Pdx1* deficiency (Gannon 2001; Boyer et al. 2006).

In vivo relevance of Ptf1a binding Area III

In vivo deletion of Areas I-II-III from the *Pdx1* promoter results in a dramatic arrest in pancreas development, similar to that observed in the global

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Pdx1 null mice (Offield et al. 1996; Fujitani et al. 2006). Since we show that

Areas I and II are only active in endocrine tissue within the pancreas (Figure 14),

the failure of the pancreas to develop in the *Pdx1*$^{\Delta I-II-III}$ mice is most likely due to

the loss of Area III, suggesting that sequences within Area III, in particular the

PTF1 site, are important for expression in the developing pancreas. *Pdx1*

expression precedes and is in a broader domain within the posterior foregut than

Ptf1a expression. In Ptf1a null mice, *Pdx1* expression is maintained in the dorsal

pancreas (Kawaguchi et al. 2002). Taken together, these data suggest Ptf1a is

not necessary to initiate *Pdx1* expression in the pancreas, but is involved in

maintaining or augmenting *Pdx1* expression within the developing pancreatic

buds. We would predict that removal of Area III containing the PTF1 binding site

in vivo would result in an apancreatic phenotype without affecting the

development of other tissues and cell types in which *Pdx1* is expressed, similar

to the phenotype observed with deletion of Areas I, II and III.

CHAPTER III

CHARACTERIZATION OF INDUCTION SYSTEMS FOR THE TEMPORAL EXPRESSION OF VEGF

Introduction

The pancreas derives from dorsal and ventral outgrowths of the endodermal epithelium of the posterior foregut. During mid-gestation in the mouse, from embryonic day E13.5-16.5 the numbers of endocrine cells increase greatly but remain as small clusters of cells closely associated with the ducts. The signals stimulating the increase in endocrine differentiation at this time have not yet been determined. At E18.5, endocrine clusters begin to lose their proximity to the ductal epithelium and coalesce to form islets. In the mouse, these islets are composed of a core of β cells surrounded by a mantle of α, δ, ε, and PP cells. They express insulin, glucagon, somatostatin, ghrelin, and pancreatic polypeptide, respectively.

Vascular endothelial cells play an integral role in pancreatic endocrine cell development. Indeed, in embryos lacking endothelial cells, insulin-expressing cells are absent (Lammert et al. 2001; Yoshitomi and Zaret 2004). Conversely, if endothelial cells recruited to the pancreas during development are exposed to excessive signaling by overexpression of VEGF in the pancreas, then the number of endothelial cells increases resulting in a significant increase in insulin positive cells (Lammert et al. 2001). It is thought that in the developing pancreas a reciprocal inductive event exists whereby endothelial cells secrete a factor that

induces pancreatic endocrine cell formation, and the endocrine cells in turn secrete factors that actively recruit vascular endothelial cells (Figure 12)(Lammert et al. 2003).

Pancreatic endocrine cells normally express elevated levels of VEGF during embryogenesis, while vascular endothelial cells express VEGFR1 and VEGFR2 (Christofori et al. 1995). Elevated VEGF ligand and its receptors, VEGFR1 and VEGFR2, expression continues throughout islet formation and are maintained during adulthood (Christofori et al. 1995). Studies show that VEGF signaling is important in maintaining islet vasculature. Two independent groups examined conditional VEGF-A knockout mice, one examining VEGF loss of function in the pancreatic β cells using the RIP-Cre transgene and the other examining VEGF loss of function in the entire pancreas using the *Pdx1*-Cre transgene. Both of these groups found that if VEGF expression is reduced, then the islet vasculature is significantly reduced (Inoue et al. 2002; Lammert et al. 2003). Although the average islet size was smaller in these conditional knockouts, neither of these manipulations resulted in a profound pancreatic endocrine cell differentiation defect that is expected if endothelial cells are critical to endocrine development. This mild phenotype may be due to compensation by other VEGF genes expressed in the pancreas (Inoue et al. 2002) and compensation by VEGF-A expression derived from the mesenchymal cells in the pancreas, since only half of the VEGF-A mRNA expressed in the conditional knock-out pancreas originates from a recombined allele (Lammert et al. 2003)

Since pancreatic overexpression of VEGF has been shown to increase the total pancreatic endocrine mass (Lammert et al. 2001), we sought to determine whether this was the result of increased specification of pancreatic endocrine progenitors, proliferation of pancreatic endocrine progenitors, proliferation of differentiated pancreatic endocrine cells, or a combination of these mechanisms. More specifically, we attempted to employ an inducible expression system to control temporal expression of VEGF in the pancreas during different developmental stages. By controlling VEGF overexpression during discrete periods of pancreatic development, the window of competence of the endothelial cells and the pancreas to respond to these signals would be elucidated, and the genes and signals sufficient to induce pancreatic endocrine cell differentiation *in vivo* may be uncovered. This may aid in improving the recapitulation of islet formation *in vitro* from fetal pancreatic ducts or stem cells, experimental sources of islets for transplantation (Lechner and Habener 2003). This chapter chronicles the cassettes and transgenic animals created and characterized in an attempt implement exogenous temporal control over pancreas-wide VEGF transgene expression.

Materials and Methods

Transgene generation

Pdx1[PB]lacOLacZ: The LacZ responder transgene was generated by replacing the HNF6 cDNA from the Pdx[PB]lacOHNF6 vector with the LacZ coding region (Elizabeth Tweedie, Vanderbilt University). This vector contained three tandem copies of the lactose operator (lacO) sequence 220bp downstream of the transcription start site.

Pdx1[PB]lacOVEGF: The vector containing the coding sequence for human VEGF-165 with the internal myc tag (VEGF-myc) was previously characterized (Chavand et al. 2001). An HA tag IRESII eYFP (Clonetech) was subcloned to replaced the HNF6 coding region in the Pdx[PB]lacOHNF6 plasmid. The resulting vector was digested with BclI and EcoRI to remove the HA tag coding region and replaced with VEGF-myc digested with BamHI and EcoRI. Although, this vector was generated, it was only used to create subsequent transgenes.

Pdx1[4.3]hsppolyA: A 9kb XbaI-XbaI *Pdx1* genomic fragment was digested with XbaI, blunted with T4 DNA polymerase, then digested with SacI to generate the 4.3kb *Pdx1* fragment (Gannon et al. 2001). This fragment was subcloned into the SmaI/SacI digested phsppolyABSSKII plasmid provided by the Hogan Lab (Sasaki and Hogan 1996) to generate an empty expression cassette driven by the 4.3kb *Pdx1* promoter. Although, this vector was generated, it was only used to create subsequent transgenes.

Pdx1[4.3]lacOVEGF: A SpeI digestion fragment of the Pdx1[4.3]hsppolyA vector was replaced with a SpeI digested fragment of the VEGF-myc cassette from Pdx1[PB]lacOVEGF, resulting in a dicistronic VEGF-myc, YFP expression cassette driven by the 4.3kb *Pdx1* promoter under the control of three tandem lacO sequences. The vector served as a control representing our original design strategy.

Pdx1[4.3]I3VEGF: To create this cassette, the *Pdx1*[4.3]lacOVEGF was modified such that the three tandem lacO sequences were removed and the intron was replaced with a commercially available intron containing three operator sequences (Lacswitch, Stratagene).

Pdx1[4.3]RSVVEGF: To create this cassette, the *Pdx1*[4.3]lacOVEGF was modified such that the HSP68 minimal promoter, β-globin intron, and the lactose operator sequences were replaced with the commercially available RSV minimal promoter and intron containing strategically positioned lactose operator sequences (Lacswitch, Stratagene).

Vector constructs were assembled using standard molecular biology techniques (Russell 2001), and were verified by restriction digestion and partial sequencing. The transgenes cassettes were maxiprepped and purified by CsCl ultracentrifugation (Russell 2001). The cassettes were released from the vector backbone by NotI restriction digestion, isolated by gel electrophoresis, and purified by gelase extraction (Russell 2001). The DNA was measured via spectroscopy and provided to the Vanderbilt Transgenic Mouse / ES Cell Shared Resource for transgenic mouse generation. Pronuclei of one-cell embryos from

B6D2F1 (Charles River) females were injected with 1–5 pl DNA (3 ng/ml) and embryos implanted into pseudopregnant CD-1 females (Charles River)(Hogan 1994).

Transfections

Transient transfections were performed in HEK293 (a gift from Roger Colbran, Vanderbilt University) and βTC3 (a gift from Al Powers, Vanderbilt University) cells using 3 µL of Fugene (Roche) per µg of plasmid DNA according to manufacturer's specifications. Culture media containing 1 mM IPTG was used to derepress expression in cotransfections (Cronin et al. 2001). Culture media was collected and stored at -80°C. Protein was measured in these fractions using a Bio-Rad Protein Assay immediately prior to their use in either ELISA's or western blots.

Western blot and ELISA

Protein fractions isolated from transiently transfected cells or from pancreata were resolved by SDS PAGE under reducing conditions according to manufacturer's specifications (Bio-Rad). Blots were probed with either mouse anti-myc (1:100, 9E10, Abcam) or Rabbit anti-hVEGF (1:50, Lab Vision, Ab-8). VEGF expression was quantified by using an ELISA specific for human VEGF (Quantikine) according to the manufacturer's specifications.

Mice

An inbred hybrid line (B6D2; Jackson Labs) was used for all expression studies. Mice ubiquitously overexpressing the lactose repressor (lacI) under the control of the β-actin promoter were obtained from Heidi Scrable (University of Virginia) and have been previously described (Cronin et al. 2001). Transgenic mouse lines (PTA), which express the tetracycline activator (rtTA) and suppressor (tTS) under the control of the *Pdx1* promoter, were obtained from Guoqiang Gu (Vanderbilt University). Transgenic LacZ reporter tetracycline-responsive mice were provided by Al Powers (Vanderbilt University), and have been described previously (Ward et al. 2004; Ward et al. 2004).

When indicated, doxycycline (Sigma) in 10% sucrose was administered in the drinking water of animals throughout the duration of pregnancy. Doxycycline solution was freshly prepared from doxycycline salt every 2-3 days and kept in amber containers to protect it from degradation. Deprivation caps were employed to eliminate other water sources during administration.

For embryonic analyses, the morning of the vaginal plug was considered E0.5. Mice were maintained on mouse chow 5015 (24% fat, 57% carbohydrates, 19% protein; TestDiet) *ad libitum*. All mouse studies were performed in accordance with the Vanderbilt Institutional Animal Care and Use Committee guidelines under the supervision of the Division of Animal Care.

DNA extraction and genotyping

Brain (embryos and early perinatal mice), tail (post-natal mice), or ear-punch (post-natal mice) samples were used to prepare DNA for PCR genotyping. Samples were digested overnight at 55°C in nuclei lysis buffer (brain and tail samples; Promega) or Lab Buffer 3A (ear-punch samples; 20 mM Tris pH 8.0, 100 mM KCl, 0.2 mM EDTA pH. 8.0, 1% NP-40, 1% Tween-20) with 30 µg/mL Proteinase K (Fisher). For brain and tail samples, DNA was extracted using organic solvents by standard methods (Hogan 1994). Ear-punch samples were prepared for PCR by further digesting samples with 30 µg/mL RNAase A at 37°C for 1 hour, then inactivating at 95°C for 10 min. Following centrifugation of undigested material (10 min at 13,000 rpm), DNA was stored at 4°C.

To determine genotypes by Southern blotting we used standard methods (Russell 2001). Transgenes containing the YPF cDNA were genotyped using the PstI/MscI 550bp YFP fragment to screen for the 3.4kb fragment from the transgene in SpeI digested genomic DNA. Transgenes containing the LacZ cDNA were genotyped using the PvuI 500bp LacZ fragment to screen for the 3.2kb fragment from the EcoRI digested genomic DNA.

To determine genotypes by PCR of putative transgenic mice encoding VEGF we used the following oligos:

HomMusKdrFltupper: GGA GAG ATG AGC TTC CTA CAG C

HomKdrFltlower: TTG CTC TAT CTT TCT TTG GTC TGC.

These oligos strategically flank the myc tag within the transgene producing a ~100 bp product in the presence of the transgene. They also flank the intron in

the endogenous allele, but not with 100% homology, producing a larger ~450 bp

product. The touchdown strategy favors the production of the transgene product

if present, since oligos preferentially bind there due to their homology, but

ultimately the conditions also allow the endogenous band to be produced. The

presence of the band representing the endogenous allele verifies that DNA

samples from animals not carrying the transgene are able to produce a PCR

band can truly be labeled negative instead of a null result. To achieve both bands

we use a touchdown PCR as follows: 95°C 5 minutes; 95°C 20 seconds, 55°C 20

seconds (-1°C per cycle), 72°C 30 seconds, 15 cycles; 95°C 20 seconds, 40°C

20 seconds, 72°C 30 seconds, 21 cycles; 72°C 5 minutes, 4°C.

The LacI repressor mice were genotyped with the following oligos,

laclfor: CGA TGT CGC AGA GTA TGC CGG T and

laclrev: CAC CAG TGA GAC TGG CAA CAG CTG. PCR was performed as

follows: 94°C 2 minutes; 94°C 20 seconds, 60°C 20 seconds , 72°C 60 seconds,

30 cycles; 72°C 5 minutes, 4°C. The ~1kb band indicated a positive genotype.

To determine the genotypes of the putative PTA mice the following oligos

were used, tts1: GAG GTC GGA ATC GAA GGT TTA AC and

tts2: GCT CCA TCA CGA TGG ACC AGT AA. PCR was performed as follows:

94°C 2 minutes; 94°C 20 seconds, 54°C 20 seconds, 72°C 30 seconds, 30

cycles; 72°C 5 minutes, 4°C. The ~250bp band indicated a positive genotype.

The LacZ responder mice were genotyped with the following oligos,

tetLZfor: ATA GGA ACC AGC CTC CTC TCT and

tetLZrev: AAG GAC ACT GTT GTT GGT GGT A. PCR was performed as follows: 94°C 2 minutes; 94°C 20 seconds, 51°C 20 seconds , 72°C 30 seconds, 30 cycles; 72°C 5 minutes, 4°C. The ~420bp band indicated a positive genotype.

Tissue preparation and histology

Digestive organs or isolated pancreata from varying embryonic and adult stages were dissected in phosphate-buffered saline (PBS; 2.7 mM KCl, 14.7 mM KH_2PO_4, 136.9 mM NaCl, 8.1 mM Na_2HPO_4*$7H_2O$, pH 7.4) and fixed immediately in 4% paraformaldehyde (pH 7.4) in PBS at 4°C for either two-three hours (paraffin embedding) or overnight (frozen embedding). For paraffin embedding, tissues were dehydrated in an increasing ethanol concentration series followed by two xylene washes, infiltrated with xylene:paraffin (1:1, v/v) and two changes of paraffin at 56°C, and embedded for sectioning. For frozen embedding, tissues were partially dehydrated in 30% sucrose in PBS overnight at 4°C, passed once through 15% sucrose PBS/ 50% OCT media (VWR), embedded in OCT media, and stored at -80°C until cryosectioned.

To detect LacZ reporter expression, β-galactosidase (β-gal) activity was detected using X-gal as previously described (Wu et al. 1997) with minor modifications. Following fixation, pancreata were washed two times for 30 minutes each in permeabilization solution (2.0 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 in PBS). Samples were then incubated overnight at room temperature in X-gal staining solution (1 mg/mL X-gal, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris, pH 7.4), post-

fixed in 4% paraformaldehyde in PBS at 4°C for one hour, and dehydrated for embedding as above, substituting isopropanol for xylene to minimize leaching of the blue precipitate. Sections were counter-stained with eosin (Surgipath) for contrast.

Immunohistochemistry

Paraffin sections (7 μm) were deparaffinized in xylene and rehydrated in a decreasing ethanol series to distilled water. Frozen sections (7 μm) were thawed to room temperature. Sections were permeabilized in 0.1% Triton in PBS for 15 minutes at room temperature, then incubated with blocking solution: PBS with 1% bovine serum albumin (BSA; New England Biolabs) and 5% normal donkey serum (NDS; Jackson Immunoresearch). Primary antibodies were used at the following dilutions in blocking solution: guinea pig anti-insulin (1:1000, Linco; recognizes all forms of insulin), rabbit anti-glucagon (1:1000, Linco), rat anti-PECAM (1:200, BD Pharmingen), mouse anti-myc (1:25-1:500, 9E10, Abcam), and rabbit anti-hVEGF (1:50, Lab Vision, Ab-8). All primary antibodies were incubated overnight at 4°C in a humidified chamber.

For fluorescent detection of protein, primary antibodies were detected by species-specific donkey secondary antibodies conjugated with Cy2, Cy3, or Cy5 fluorophores (1:500, Jackson Immunoresearch). Fluorophores were excited at the appropriate wavelength using either an Olympus BX41 research microscope or a Zeiss LSM 510 confocal microscope. For non-fluorescent detection of protein, anti-mouse secondary antibodies conjugated with horseradish

peroxidase (HRP; 1:500, Promega) were used to detect primary antibody, and colorimetric detection of HRP was facilitated by the addition of diaminobenzidine (DAB) peroxidase substrate with nickel chloride to produce a gray-black precipitate, per manufacturer's recommendations (Vector Laboratories). Tiff images were captured using either MagnaFire software (Optronics) or LSM Viewer (Zeiss), and image tonal range and brightness were minimally adjusted using Adobe Photoshop.

Pancreatic extracts and measurement of insulin content

Pancreata from perinatal and adult mice were dissected, weighed, and homogenized in acid alcohol for extraction of insulin (Brissova et al. 2002). Insulin content from acid alcohol-extracted pancreas was measured by solid-phase radioimmunoassay (^{125}I-insulin, Diagnostic Products) for mouse anti-insulin (MP Biomedical). For pancreatic extracts, average insulin concentration was calculated as a function of total protein from pancreatic extracts.

Statistical analyses

All statistical analyses were performed by two-tailed Student's t-test using Excel software (Microsoft).

Results

The goal of the investigation was to determine the window of competence during pancreas development that is responsive to VEGF signaling. Specifically, the changes in pancreatic endocrine cell development were to be examined. The strategy was to overexpress VEGF in the pancreas during different stages of pancreatic development. Initially, to have exogenous temporal control of transgene expression, the lactose inducible system was employed (Figure 22). It was necessary to create a responder allele for this system to be used in the study.
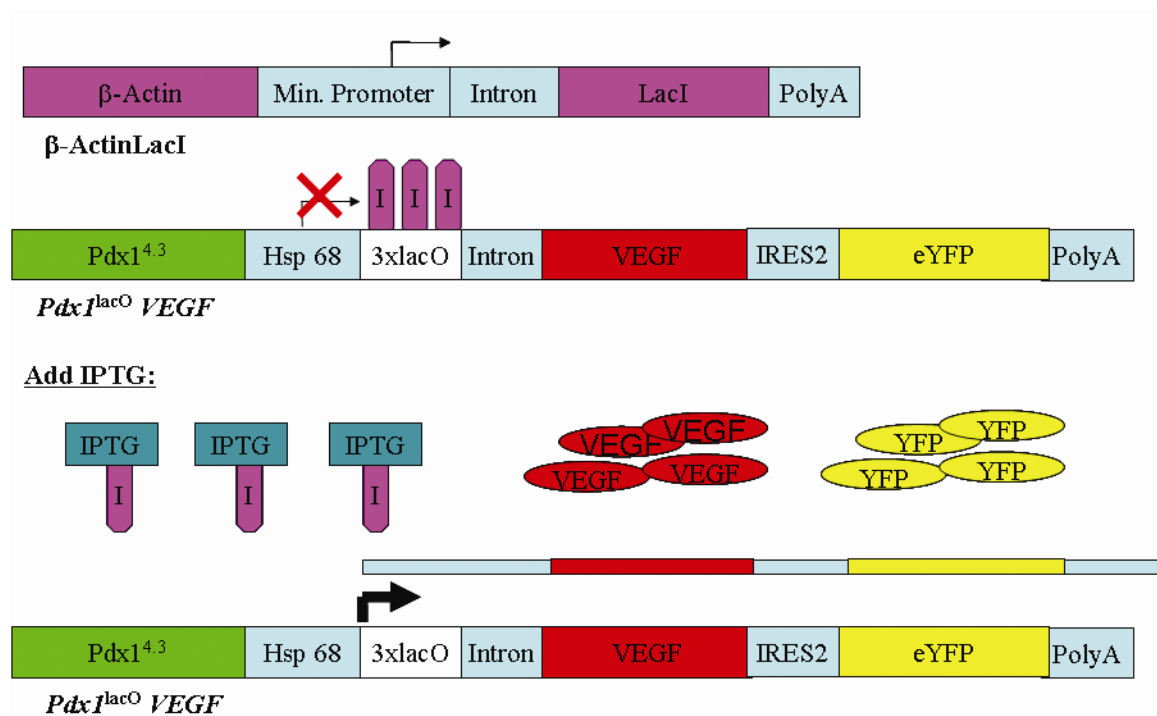
Figure 22. Lactose inducible system diagram depicting predicted expression outcome and construct design. In the absence of the lactose repressor (LacI) the transgene's expression is mediated by the *Pdx1* 4.3kb fragment, which spatially restricts expression to the endogenous Pdx1 expression domain. In the presence of a transgene expressing LacI, expression should be repressed since binding of these proteins to their target will physically interfere with transcription machinery. Upon addition of IPTG, the LacI protein will preferentially bind to IPTG and not to its DNA binding site, thus allowing for transcription.

Lactose Inducible System

To create and validate the responder allele initial design, we utilized a well-characterized expression cassette (Sasaki and Hogan 1996; Gannon et al. 2000; Zhang et al. 2005), inserted the cDNA for the β-galactosidase reporter cDNA (LacZ), and inserted three copies of the lactose operator sequence (LacO) in tandem 220bp downstream of the transcription start site (Figure 23A). The design was created and approved in collaboration with Heidi Scrable, a leader in the field of *in vivo* lactose inducible systems (Cronin et al. 2001; Scrable 2002; Cronin et al. 2003). Three founders were generated and their offspring were characterized for LacZ reporter expression. In this system, transgenic responder animals are both temporally and spatially controlled by cis-regulatory elements within the transgene expression cassette in the absence of a transgenic driver allele. Therefore, transgenic offspring should express LacZ temporally and spatially under the control of the *Pdx1*[PB] fragment, which should be detectable in the β cells of the pancreas after birth (Gannon et al. 2000; Gannon et al. 2001; Van Velkinburgh et al. 2005; Zhang et al. 2005; Wiebe et al. 2007). Reporter expression in all three lines generated was undetectable.

Figure 23. Diagram depicting expression cassettes constructed for the testing and creation of a transgene that can be controlled by the lactose inducible system. A. VEGF expression cassette without lactose repressor binding sites (lacO) referred to as "no operator". B. VEGF expression cassette with lacO sites in tandem (courtesy of Elizabeth Tweedie) referred to as "old vector". C. VEGF expression cassette with a commercially designed intron sequence containing three strategically positioned lacO sites (Stratagene) referred to as "I3". D. VEGF expression cassette with a rous sarcoma virus (RSV) minimal promoter and intron wherein the transcription start site is optimally flanked by lacO sites to control expression referred to as "RSV".

This original design for temporal control had limited success *in vivo* perhaps due to inadequate spacing between operators, and attempts at creating an improved cassette were unsuccessful. Instead, commercially available lactose inducible cassettes (Stratagene) were obtained, and the repressible elements within these commercial vectors were inserted into the existing vector. These elements included an intron sequence with three optimally positioned LacO sequences (I3) and a rous sarcoma virus minimal promoter and intron

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combination with three optimally positioned LacO sequences (RSV). Both of these elements were empirically shown to function within their expression cassette (Stratagene). These elements were cloned into the VEGF expression vector to replace their functionally equivalent sequence (Figure 23). The l3 sequence was subcloned to replace the β-globin intron in the expression cassette, generating the *Pdx1*l3VEGF expression vector (Figure 23C). The HSP68 promoter and beta-globin intron were replaced with the RSV sequence, generating the *Pdx1*RSVVEGF expression vector (Figure 23D). To control spatial expression of these transgenes, the 4.3kb *Pdx1* enhancer was subcloned upstream of the promoter. This enhancer fragment directs early pancreas-wide expression consistent with the previously described VEGF overexpression studies in the pancreas (Lammert et al. 2001).

The expression cassettes were screened via transient transfections in HEK293 and βTC3 cells lines. VEGF expression from either the *Pdx1*l3VEGF or *Pdx1*lacOVEGF expression cassette as measure by ELISA was similar in the HEK293 cells (Figure 24A). Cotransfections of the *Pdx1*l3VEGF or *Pdx1*lacOVEGF expression cassette with the equimolar amounts of the LacI expressing vector did not significantly repress either expression cassette in the HEK293 cell line. Derepression could not be interpreted in this experiment since repression was not achieved. Repeating this experiment in βTC3 cells with threefold higher amounts of LacI expressing vector revealed the ability of this expression system to function *in vitro* (Figure 24B). The *Pdx1*l3VEGF expression cassette was repressed when cotransfected with the LacI expressing vector and

this repression was alleviated upon addition of IPTG to the media. Repression was also observed for the *Pdx1*lacOVEGF upon cotransfection with LacI expressing vector, but not as greatly as *Pdx1*I3VEGF (Figure 24B). The *Pdx1*RSVVEGF expression cassette had low expression in these cells and repression/derepression was insignificant (Figure 24B). Since testing VEGF expression revealed that the *Pdx1*I3VEGF responder expression cassette expresses, and can effectively be repressed and derepressed (Figure 24), this construct was used to prepare transgenic mice, from which we received three founders (Figure 25). One founder never produced offspring, and could not be included in the investigation.
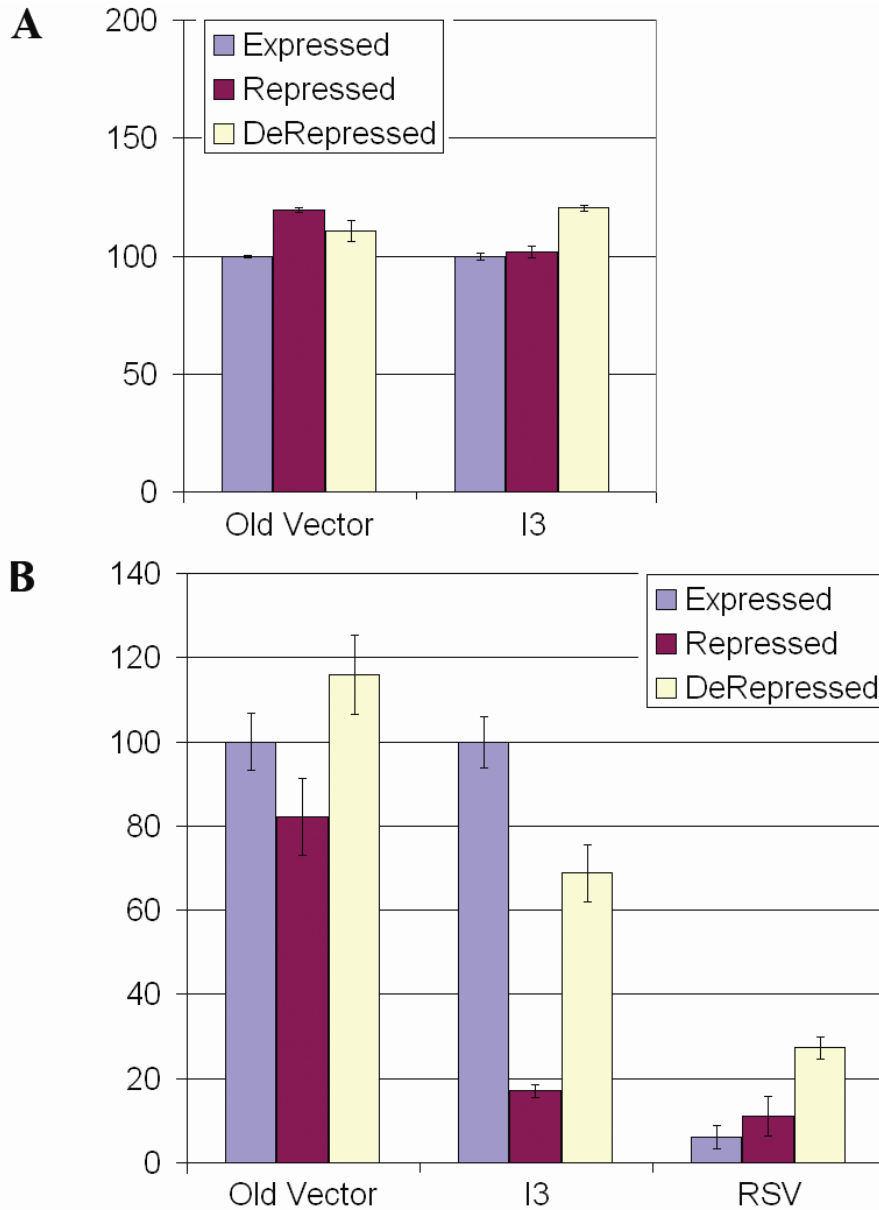
Figure 24. VEGF Expression detected in the media of transfected cells. Cells transfected with VEGF expression constructs (Expressed), cotransfected with a Lac repressor expressing vector (Repressed) and with the addition of IPTG (DeRepressed). A. VEGF expression in HEK293 cells cotransfected with equimolar amounts of Lac repressor. B. VEGF expression in βTC3 cells cotransfected with three-fold higher amount of Lac repressor.

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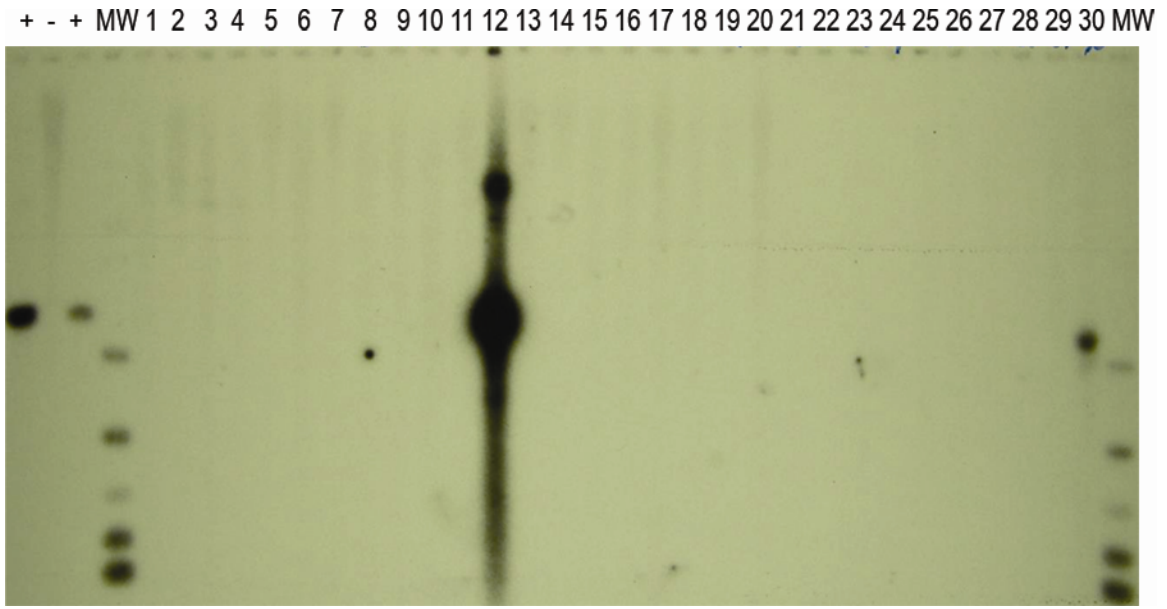
Figure 25. Southern blot of genomic DNA isolated from putative transgenic founder mice harboring the *Pdx1* "I3" VEGF transgene. Predicted 3.2kb fragment is consistent with positive controls in the first and third lanes. Both founder number 12 and number 30 contain the predicted band size and were kept for characterization.

Previously, transgenic mice overexpressing VEGF in the pancreas via the *Pdx1* enhancer/promoter fragment were shown to have islet hyperplasia and increased β cell mass in two month old mice (Lammert et al. 2001). To compare this phenotype to the *Pdx1*I3VEGF transgenic mice, similar ages were examined. All founders obtained were bred with wild type (B6D2) mice and with transgenic mice expressing the lactose repressor (LacI) under the control of the β-actin promoter (Cronin et al. 2001). The pancreata of these mice were assayed for total insulin content (Figure 26). Successful expression should be characterized by an increase in total insulin content, consistent with the previously described increase in β cell mass (Lammert et al. 2001). There was no change in total pancreatic insulin content as compared with the littermate controls (Figure 26).
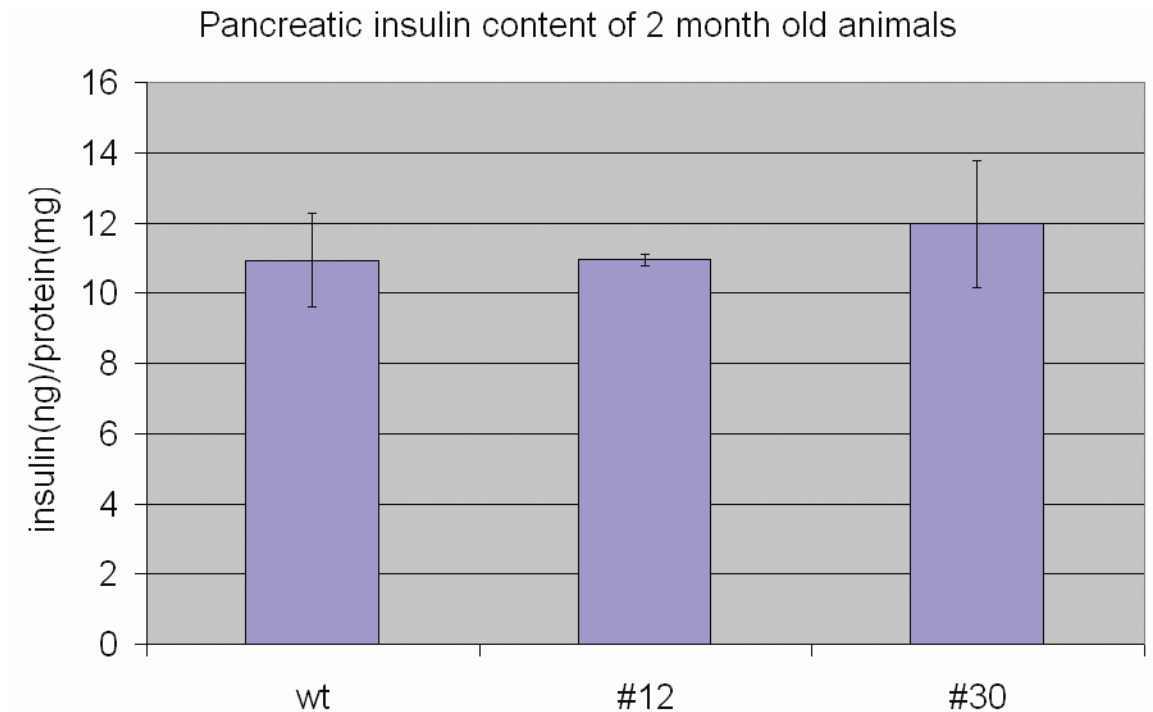
Figure 26. Pancreatic insulin content of transgenic mice compared to wild type littermate controls. No change in pancreatic insulin content was observed. Student T-tests were used to measure significant changes of transgenic animals compared to their littermate controls. p=.98 and p=.68 for line #12 and #30 respectively.

It was possible that the endocrine mass may have changed without a concomitant change in total insulin content. As such, the islets within pancreatic sections were examined from transgenic animals and their littermate controls. There was no change in islet size or islet vasculature in either line. Consistent with this result, transgene expression was not detected in the pancreas sections of these animals via immunofluorescence. Pancreatic protein extracts were collected from these animals and analyzed via western blot with two different antibodies that target the VEGF produced by the transgene. Since the VEGF

produced is the human isoform and contains a myc tag, the anti-human VEGF (hVEGF) and the anti-myc antibodies were used. The anti-hVEGF antibody is polyclonal and cross-reacts with at least three endogenous isoforms of mouse VEGF (Figure 27). The VEGF produced from the transgene is 21kd, slightly larger due to the myc tag than the 19kd endogenously produced mouse VEGF homolog. The protein extract from one adult pancreas carrying both the driver LacI transgene and the responder *Pdx1*I3VEGF transgene produced a band of consistent size on the western blots with the anti-myc and the anti-hVEGF antibody (Figure 27, lane1). This result was not reproducible, however. In addition, expression of the responder VEGF transgene in this mouse should be repressed due to the presence of the driver LacI allele, since IPTG is absent (Figure 22). Taken together, the absence of an islet phenotype and the fact that transgene expression is detectable, despite the presence of lacI, strongly suggested that the responder transgenic animals were not going to be useful for the proposed experiments although the LacO sequences were more optimally positioned.
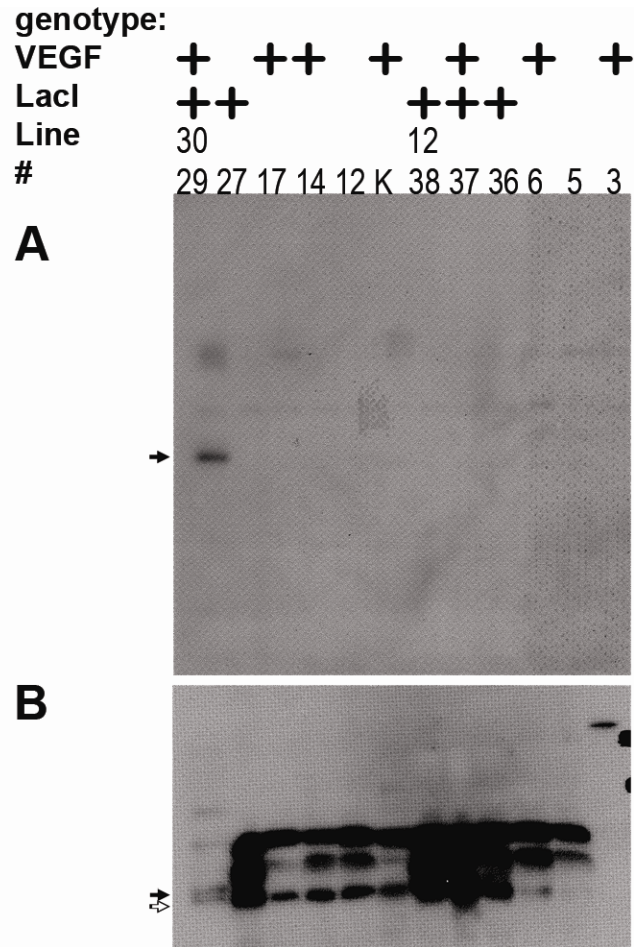
Figure 27. Western blot of pancreatic extracts from two month old mice. Genotypes of mice are demarked as "+" if they carry the given transgene: VEGF for *Pdx1*I3VEGF and LacI for Beta-Actin LacI (Scrable lab). A. Mouse anti-myc (9E10). B. Rabbit anti-hVEGF (Neomarkers). Solid arrows correspond with band size consistent with slightly larger transgene generated hVEGF-myc. White arrow corresponds with band size consistent with cross reaction with endogenous mouse VEGF 165. Larger bands are consistent with other endogenous mouse VEGF isoforms.

Tetracycline inducible system

The next approach was to use the tetracycline inducible system. One

deciding factor was that the transgenic animals needed for this study already

existed and were readily available. Three *Pdx1* tet driver (PTA) mice lines were

obtained from Guoqiang Gu (Vanderbilt University). The lines were not yet

completely characterized, therefore it was critical to determine which line induced

the best expression in the presence of doxycycline and permitted the least

expression in the absence of doxycycline (Figure 28).
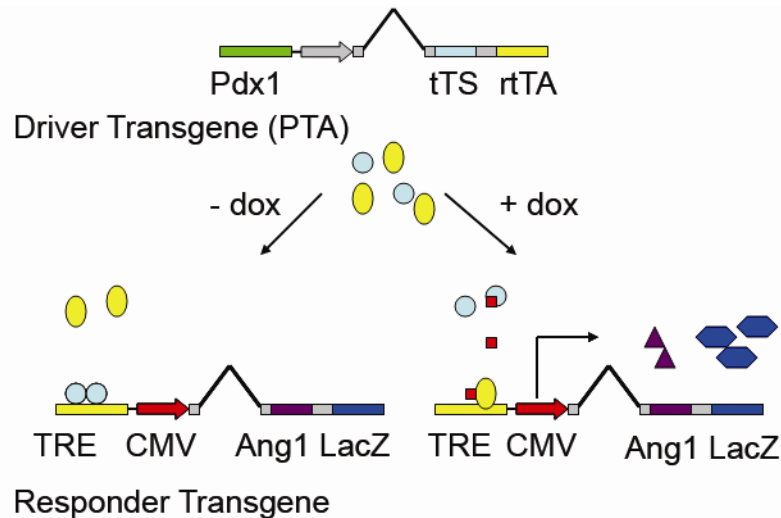
Figure 28. Transgenic mice for the temporal control of expression within the Pdx1 expression domain. Driver transgenic mice (PTA) express the tetracycline suppressor protein (tTS) and the tetracycline dependent activator protein (rtTA) under the control of the *Pdx1* promoter. They are expressed in a dicistronic message utilizing an internal ribosomal entry site. In the absence exogenous doxycycline tTS binds to the tetracycline response element (TRE) of the responder transgene, suppressing transcription. In the presence of doxycycline, rtTA binds to the TRE, displacing tTS and activating transcription. The expression through responder transgene is mediated by the cytomegalovirus promoter (CMV) and expresses Angiopoietin 1 (Ang1) and Beta-galactosidase (LacZ), the reporter, in a dicistronic message.

All three lines were crossed with the previously characterized transgenic

LacZ responder mice (Ward et al. 2004; Ward et al. 2004), which were provided

by Al Powers (Vanderbilt University). Control of responder allele expression in

the absence or constant presence of the doxycycline inducer was assessed at

P1. All three PTA lines produced offspring that included mice that were double transgenic (bigenic) for the PTA driver and LacZ responder allele. In the absence of exogenous inducer, in all the offspring, LacZ expression was never observed (Table 1A). This was consistent with the anticipated result, suggesting that the driver PTA transgene repressed responder allele expression in the absence of the inducer (Figure 28).

Table 1. Analysis of the tetracycline inducible system using the transgenic lines expressing rtTA and tTS under the control of the *Pdx1* promoter. Offspring were collected after (A) no doxycycline treatment and no offspring had detectable reporter staining, (B) 2 mg/mL doxycycline treatment and no offspring had detectable reporter staining, and (C) 10 mg/mL doxycycline treatment and no offspring had detectable reporter staining.

A

No Dox Line	WT, PTA, or tetoLacZ	Detectable Staining	Bigenic PTA: tetoLacZ	Detectable Staining
3 (n=9)	8	0	1	0
6 (n=5)	1	0	4	0
7 (n=8)	4	0	4	0

B

2mg/mL Dox Line	WT, PTA, or tetoLacZ	Detectable Staining	Bigenic PTA: tetoLacZ	Detectable Staining
3 (n=8)	5	0	3	0
6 (n=1)	1	0	0	0
7 (n=9)	6	0	3	0

C

10mg/mL Dox Line	WT, PTA, or tetoLacZ	Detectable Staining	Bigenic PTA: tetoLacZ	Detectable Staining
3 (n=6)	4	0	2	0
6 (n=7)	4	0	3	0
7 (n=7)	5	0	2	0

Administration of 2mg/mL doxycycline in the drinking water of the pregnant females throughout the duration of their pregnancy did not induce LacZ expression in the offspring (Table 2B). Although, PTA line #6 did not produce bigenic offspring in this experiment, PTA line #3 and #7 did, and yet there was no detectable LacZ expression. This was not consistent with the anticipated result. In the presence of the doxycycline, the bigenic animals at P1 should have had detectable LacZ expression within the *Pdx1* domain (Figure 28). The experiment was repeated with an elevated dose of doxycycline (10mg/mL) administered in the drinking water throughout the duration of pregnancy. All three PTA lines produced offspring including bigenic animals. In all cases, no detectable LacZ expression was observed (Table 1C). Again, this was inconsistent with our anticipated result. Bigenic animals in the presence of the inducer should have had detectable levels of LacZ responder expression (Figure 28). Induction from these lines was undetectable, despite the elevated amount of doxycycline. These experiments suggested that the PTA driver transgenic mice were not able to induce expression during embryonic stages. As such, they were not useful to the investigation of VEGF overexpression in the developing embryonic pancreas.

Discussion

Investigations using genetically modified organisms can be a very powerful means to elucidate a genetic pathway. Historically, these investigations have been performed with gain or loss of gene function. Gain of function rudimentarily consists of gene overexpression controlled both spatially and temporally by a specific enhancer and/or promoter region. Typically, in these systems the investigator has no exogenous control over expression once the genetically modified animal has been generated.

With the advent and implementation of recombinase technology, gain and loss of function analysis in whole animals has improved, since in this system spatial and temporal expression is controlled by both the recombinase expressing allele and the targeted allele. Therefore, by having different combinations of recombinase expressing alleles and targeted alleles a greater number of spatial expression studies can be investigated. However, once a mouse is generated, the investigator has little exogenous control of spatial or temporal expression within the animal using this methodology. This shortcoming was overcome by the creation of the Cre recombinase and estrogen receptor fusion protein (Cre-ER)(Garcia and Mills 2002). This recombinase is sequestered to the cytoplasm until tamoxifen is present, wherein the tamoxifen binds to the fusion protein, allowing it move to the nucleus where it can act on the target allele. This modification allows similar spatial and temporal control to the standard Cre recombinase system except that temporal control of recombinase activity is controlled exogenously by the investigator by means of the addition of

tamoxifen. The shortcoming that remains in this system is the inability to revert the system back to its original state once modification occurs. For example, if the recombination event causes gene loss of function within the target allele then the allele function cannot be restored.

Both the lactose inducible and the tetracycline inducible system have the potential to overcome this shortcoming. All three systems share similar design properties in that one driver allele expresses at least one protein that controls expression of a second target responder allele. In addition, the proteins that arise from the driver allele can be temporally controlled exogenously by the investigator upon addition of the inducer molecule. The potential advantage of the lactose inducible and the tetracycline inducible system is that upon removal of the inducer molecule the driver allele reverts to its uninduced state.

Lactose inducible system

The advantage of the lactose inducible system compared to the tetracycline inducible system is that temporal expression can be more tightly controlled. Induction of the targeted allele in animals with this system can be observed within four hours after application of the inducer, IPTG, to the drinking water of mice (Cronin et al. 2001). Expression can be reverted 24 hours after removal of the inducer, which may be attributed to the physical nature of IPTG in that it is not fat-soluble (Cronin et al. 2001). In addition to this tight temporal control, the inducer crosses the placenta in mice, allowing for embryonic

investigations (Cronin et al. 2001). Together, these properties are advantageous when investigating narrow time spans during mouse development.

At the outset of the current investigation, the lactose inducible system seemed the most advantageous, the system had been better characterized in mice, and the transgenic mice expressing the driver allele were readily available. The only tool necessary to utilize this system was to create a responder mouse specific to the investigation. The initial design and creation of a responder allele was unsuccessful, as expression was undetectable, and was perhaps due to construct design (Figure 23).

Upon reevaluation of the transgene expression cassette design, it was determined that the position of the lactose operator sequences relative to each other was critical to their function. The 23 bp operator sequences should be optimally placed either 70.5, 92.5 or 115.5 bp apart, center to center for ideal lactose repressor protein (LacI) binding (Muller et al. 1996). These optimal distances are separated by 12 bp increments, which is consistent with one full turn of a DNA double helix. These distances allow the LacI dimers that bind the individual operators to optimally form a tetrameric complex, since they are close enough to be in physical contact with each other, but far enough for the DNA to bend allowing for tetramer formation (Lewis et al. 1996). A third operator site, placed either 5' or 3' to the other two, also participates in repression of expression of the DNA sequence, but spacing does not appear to be as critical for this site (Reznikoff et al. 1974; Pfahl et al. 1979). LacO placement relative to the transcription start site was also critical to functionally critical, with optimal

placement at or immediately flanking the transcription start site (Brown et al. 1987).

The suboptimal placement of the LacO sites in our expression cassette did not necessarily explain the inability of our transgenic lines to express the LacZ reporter. In fact, suboptimal placement of LacO sites in the transgene cassette more likely causes an inability to repress this responder transgene in the presence of the LacI driver transgene. Since expression was not detected in any of these responder LacZ transgenic lines independent of the LacI driver transgene, repression was a moot point. In addition, the absence of expression from these responder LacZ transgenic lines had to be interpreted as a null result. The absence of expression could not necessarily be attributed to the LacO position alone. For example, although unlikely, the absence of expression could also be due to position affect variegation.

With the criteria regarding the LacO placement, the expression cassette was redesigned and constructed. Instead of the LacO being placed suboptimally in tandem 220 bp downstream of the transcription start site, they were to be inserted flanking the transcription start site of the HSP68 promoter of our expression cassette using a PCR strategy (Young and Dong 2004). Ultimately, this strategy failed to yield the desired sequence and was substituted. Instead, plasmids from the LacSwitch (Stratagene) inducible system were obtained, which contained elements within the expression cassettes that had optimally placed lactose operator sequences, consistent with the criteria necessary for inducible expression. These expression cassettes were assayed with an ELISA for VEGF

expression, repression, and derepression via transient transfections in βTC3 and HEK293 cell lines. The *Pdx1*I3VEGF expression vector was the only vector that had significant expression, repression, and derepression (Figure 24). This vector was therefore used to generate responder transgenic mice.

Three transgenic founders were identified, but only two generated offspring (Figure 25). Neither transgene expression nor anticipated phenotypic characteristics were observed (Figure 26, Figure 27). As with the initial strategy, it may be possible that these effects were due to position effect variegation. Ultimately, it seems more likely that introduction of LacO sequences intrinsically negatively affected expression via interference with transcription or translation, and overcoming this obstacle may be specific to an individual responder expression cassette.

Tetracycline inducible system

In parallel to the attempt to create a tightly regulated inducible VEGF expression system, investigators from other institutions had advanced the tools and technology associated with the tetracycline inducible system in pancreata (Holland et al. 2002). Initially it was decided this system was inadequate for this investigation, due to its lack of technological development and because of its inability to tightly control expression. The inducing agent in this system, doxycycline, is fat-soluble and takes over two days to leave the system. The driver mouse in this system can either express the tetracycline-regulated transactivator protein (tTA), which activates responder expression only in the

absence of doxycycline, or the tetracycline activator (rtTA) and suppressor (tTS) protein, which activates responder expression in the presence of doxycycline, but suppress expression in the absence of doxycycline. Therefore, either expression can be initiated rather quickly and not rapidly "turned off", or expression can be stopped quickly and not "turned on" rapidly, depending on the driver allele utilized.

The available genetically modified driver mouse line developed and characterized was a knock-in of the tetracycline-regulated transactivator in the *Pdx1* locus, which replaced the Pdx1 coding region in this allele (Holland et al. 2002). There were two specific problems associated with using this mouse for our investigation. First, as previously mentioned the timing of expression could not be tightly controlled due to practical limitations of doxycycline administration. Second, animals harboring the driver tTA would intrinsically be heterozygous for *Pdx1*. This was of particular importance to the study, since insulin-producing β cell function is compromised in *Pdx1* heterozygous animals (Brissova et al. 2002). The changes in specification, differentiation, and proliferation of β cells due to changes in VEGF signaling are the desired measurements to be made during this investigation, and at the outset, these properties might be influenced by a decrease in Pdx1. It is possible to control for the partial loss of Pdx1 by comparing results to *Pdx1* heterozygous animals, except if Pdx1 itself were involved in the signaling pathway, then a potential phenotype might be masked. For example, if expression of the VEGF responder transgene was verified and appropriate, but in all cases there was no detectable change in β cell

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phenotypes, then it would be impossible to exclude the *Pdx1* heterozygous genotype as contributing to the apparent lack of a VEGF response.

These shortcomings were potentially overcome by the generation of PTA transgenic lines designed to express the tetracycline activator and tetracycline suppressor as a dicistronic message under the control of the *Pdx1* promoter (Guoqiang Gu, Vanderbilt University). These PTA mice made it possible to perform the investigation without directly compromising endogenous *Pdx1* expression. Three PTA transgenic mouse lines were crossed with mice carrying the LacZ responder allele (Ward et al. 2004; Ward et al. 2004). Induction of reporter expression was never observed, despite even elevated levels of doxycycline (10 mg/mL; Table 1)

The LacZ responder allele used in this investigation also carried the Angiopoietin1 (Ang1) cDNA as part of the dicistronic message (Figure 28). The effect of Ang1 expression was of potential interest, since it signals to vascular endothelial cells, but was not the principle goal of this investigation. Although it is possible that the Ang1 expression from the responder allele could have indirectly compromised the characterization of the PTA driver mice, this is unlikely since the whole-mount vasculature morphology was unchanged, which is a target for Ang1. In addition, the driver allele and the responder allele expression should arise from the endodermally derived pancreatic tissue, which was unchanged and not known to be directly responsive the Ang1.

Since with all three PTA driver lines, LacZ reporter expression was undetectable under any condition in P1 bigenic animals, we concluded that for

exogenous temporal control of VEGF during embryological investigations, the appropriate genetic tools were not available. It may be possible to use another existing transgenic driver line under the control of the insulin promoter, RIP-rtTA (Efrat et al. 1995). Although in this case, the goals of this investigation would be compromised in at least two ways. One, the expression would only occur after cells had been specified to the endocrine lineage. Two, the expression pattern would be within a smaller subset of cells and tend to occur much later in development. Due in part to the aforementioned constraints, the committee agreed that pursuit of this aim should be discontinued.

CHAPTER IV

NEITHER ACTIVATION OF VEGF RECEPTOR 1 NOR 2 IS SUFFICIENT TO INCREASE β-CELL MASS IN THE DEVELOPING PANCREAS

Introduction

The previous chapter detailed the efforts to create a transgenic mouse line with which the VEGF overexpression in the pancreas could be temporally controlled exogenously. In this chapter, VEGF signaling was examined to elucidate the contribution of the VEGF receptors and their corresponding downstream signaling pathways to the induction of endocrine cells in the pancreas.

To determine through which receptor VEGF stimulates pancreatic endothelial cells to secrete an endocrine inducing factor(s), we obtained receptor-selective VEGF variants from Genentech (South San Francisco, CA). These variants were designed based on the crystal structure of the receptor binding domain of VEGF interacting with its receptors and contain alterations in residues thought to be important for mediating binding to each specific receptor (Li et al. 2000). The fltsel variant shows a 470-fold reduction in binding to VEGFR2 while retaining wild-type affinity to VEGFR1, while KDRsel shows a 2000-fold decrease in binding to VEGFR1 while retaining wild-type affinity to VEGFR2 (Table 2). Each variant has been shown to specifically activate the appropriate downstream signaling pathways (Li et al. 2000; Gille et al. 2001). These distinct pathways are useful to determine whether VEGF stimulation of

VEGFR1 or VEGFR2 (or both) is responsible for the increase in VEC-mediated endocrine induction seen in animals with increased VEGF expression (Lammert et al. 2001).

Table 2. The K_d values and relative binding affinities of wild type VEGF and receptor-selective VEGF mutants (Gille et al. 2001).

Mutants	VEGFR2 binding		VEGFR1 binding	
	K_d	Relative to VEGF$_{165}$	K_d	Relative to VEGF$_{165}$
	pM		*pM*	
VEGF$_{165}$	97	1	37	1
KDR-sel (VEGFR2^{sel})	100	1	87,000	2,000
Flt-sel (VEGFR1^{sel})	12,416	128	98	2.6

We anticipated that, similar to other systems, selective activation of VEGFR2 in the pancreas would result in increased endothelial cell proliferation and thus increased vasculature, while selective activation of VEGFR1 would not (Figure 29). Increased vasculature should in turn result in increased production of a putative endocrine inducer. Therefore selective activation of VEGFR2 in the pancreata of mice should have a similar phenotype to the overexpression of VEGF in the pancreas in that there will be an increase in islet vascular density. In contrast, overexpression of wild type VEGF also activates VEGFR1, which can cause secretion of growth factors from VECs (Figure 29)(LeCouter et al. 2003). Unlike VEGF overexpression in the pancreas, selective overactivation of VEGFR2 would not directly result in VEC growth factor secretion. Therefore,

selective activation of VEGFR2 in the pancreata may not result in as large an

increase of endocrine cells as seen in the VEGF overexpression in the pancreas.
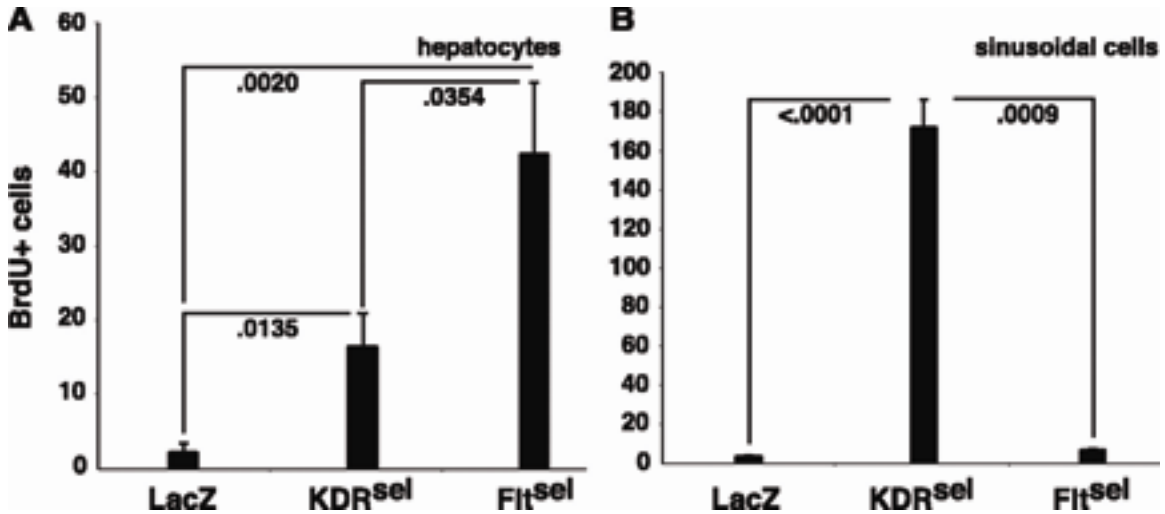
Figure 29. Proliferation of hepatocytes versus sinusoidal cells in response to selective VEGFR activation. Quantitative analysis of proliferating hepatocytes (A) and sinusoidal cells (B) was performed after BrdU immunohistochemistry of liver sections from animals treated with Av-LacZ, Av-KDRsel, or Av-Fltsel 10 days after Av administration. The VEGFR-1 selective agonist Fltsel results in the greatest hepatocyte proliferation but fails to induce sinusoidal endothelial cell proliferation. KDRsel is a potent mitogen for sinusoidal cells and also results in increased hepatocyte proliferation. Values are means ± SEM. Level of significance was assessed by unpaired t tests; P values are indicated (LeCouter et al. 2003).

In contrast, selective overactivation of VEGFR1 does not cause an

increased proliferation of VECs (Figure 29)(LeCouter et al. 2003). Therefore

selective overactivation of VEGFR1 in the pancreas should contain a similar

density of vasculature as wild-type littermates. However, since endogenous

VEGF stimulation of VEGFR1 results in production of certain growth factors

independent of VEC proliferation, we predicted that there would be an increase in

total endocrine cell mass despite the lack of increase in vasculature. This chapter

describes the generation and phenotype of transgenic mice overexpressing

VEGF variants in the pancreas that selectively activate either VEGFR1 or 2.

Materials and Methods

Transgene generation

Vectors containing the coding sequence for either KDR-sel or FLT-sel were obtained as a kind gift from Genentech (Li et al. 2000). Vector containing the coding sequence for human VEGF-165 with the internal myc tag (VEGF-myc) was previously characterized (Chavand et al. 2001). The coding sequence for VEGF-myc was cloned into the pBluescript vector (Stratagene) with BamHI and EcoRI. Both the coding region for KDR-sel and FLT-sel were partially digested with Nco1 and completely digested with BsmI. The coding fragments were cloned into the VEGF-myc vector digested with NcoI and BsmI. The resulting coding sequence generated a human VEGF-165 isoform, with an internal myc tag, specific for either KDR or FLT, which are referred to as VEGFR2^{sel} or VEGFR1^{sel}, respectively. Coding regions were verified by restriction digestion and sequencing (Vanderbilt Sequencing Core).

Pdx^{PB}lacOHNF6 vector was generated in the Gannon lab and kindly provided by Elizabeth Tweedie Ables. An HA tag, IRESII eYFP (Clonetech) was used to replace the HNF6 coding region. This vector was digested with XbaI and ligated to remove the 3xLacO sequence from the 5' region. The resulting vector was digested with BclI and EcoRI to remove the HA tag coding region and replace with either the coding region VEGFR2^{sel} or VEGFR1^{sel} digested with BamHI and EcoRI. A 9kb XbaI-XbaI *Pdx1* genomic fragment was digested with XbaI, blunted, and SacI to generate the 4.3kb *Pdx1* fragment. This fragment was

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subcloned into the SmaI/SacI digested phsppolyABSSKII provided by the Hogan

Lab to generate an insertless expression cassette driven by the 4.3kb *Pdx1*

promoter. The SpeI digested VEGFR2^{sel} or VEGFR1^{sel} was subcloned into the

resulting SpeI digested vector creating the final expression vectors. These

constructs were verified by restriction digestion.

The transgene cassettes were maxiprepped and purified by CsCl

ultracentrifugation. The cassettes were released from the vector backbone by

NotI restriction digestion, isolated by gel electrophoresis, and purified by gelase

extraction. The DNA was measured and provided to the Vanderbilt Transgenic

Mouse / ES Cell Shared Resource for transgenic mouse generation.

Mice

An inbred hybrid line (B6D2; Jackson Labs) was used for endogenous

VEGF protein expression studies. For embryonic analyses, the morning of the

vaginal plug was considered E0.5. Mice were maintained on mouse chow 5015

(24% fat, 57% carbohydrates, 19% protein; TestDiet) *ad libitum*. All mouse

studies were performed in accordance with the Vanderbilt Institutional Animal

Care and Use Committee guidelines under the supervision of the Division of

Animal Care.

DNA extraction and genotyping

Mice were genotyped using the same techniques and procedures described in the previous chapter since the transgene construction was almost identical and shared the region detected in the genotyping technique.

RNA extraction and analysis

Dorsal pancreata from P1 mice were dissected and immediately placed into 5 volumes (~100 µL) of RNAlater (Ambion) and stored at -20°C until genotyped. RNA was extracted from tissue using RNAqueous (Ambion), DNA contamination was reduced using TURBO DNA-free (Ambion), and cDNA was prepared with or without reverse transcriptase using SuperScript (Invitrogen) all according to manufacturers' specifications.

To assay for the presence of the transcript, the following oligos were used:
Hspfor: GCG GAG CGC AGC CTT CCA GA
Bglobinrev: CAA CCA GCA CGT TGC CCA GGA.
These oligos flank the β-globin intron of the transgene. One oligo anneals in the heat shock minimal promoter (hsp68) and the other anneals 3' of the β-globin intron RNA splice site. The PCR product arising from the transgene mRNA that has been properly spliced will be ~100bp, whereas the PCR product arising from the genomic DNA or unspliced RNA will be ~350bp. There is no PCR product arising from an endogenous locus.

In addition, to assay for the presence of the transcript, the genotyping oligos described in the previous chapter were used on the cDNA prepared from

P1 pancreata. In this case an additional PCR product can be formed that is ~75bp in length representing the endogenous spliced mRNA. The shortcoming of this approach is that the PCR product arising from the transgene transcript is identical in size to the PCR product arising from the transgene itself. Therefore DNAase treated RNA was used as a control to verify that PCR products did not arise from the genomic DNA.

Tissue preparation, histology, and immunohistochemistry

Digestive organs or isolated pancreata from varying embryonic and adult stages were dissected and processed as described in the previous chapter.

Morphometric analysis

Sections of perinatal pancreata were stained at 250 μm intervals along the length of the pancreas (at least eight sections per pancreas) for insulin and PECAM. Total islet insulin area was quantitated by a threshold of pixel intensity using Metamorph morphometric software (Molecular Devices). Total islet PECAM area was then determined by threshold of pixel intensity within the insulin+ area. Average vascular density was expressed as a percentage of total islet area measured.

Physiological assessment of glucose homeostasis

Intraperitoneal glucose tolerance tests (IP-GTT) were performed as previously described (Gannon et al. 2000). Briefly, eight week old mice fasted for

16 hours were given an intraperitoneal injection of filter-sterilized glucose (2.0 mg dextrose/g body weight) in PBS. Glucose concentration was measured in tail vein blood using the Freestyle glucose meter and test strips (Therasense) before injection (time 0) and 15, 30, 60, 90, and 120 min after injection. Blood glucose concentrations at time 0 and time 120 were used to classify mice as either physiologically normal (fasting < 140 mg/dL; ending < 200 mg/dL), glucose intolerant (fasting < 140 mg/dL; ending ≥ 200 mg/dL), or diabetic (fasting ≥ 140 mg/dL; ending ≥ 200 mg/dL).

Pancreatic extracts and measurement of insulin content

Pancreata were dissected and processed as described in the previous chapter.

Statistical analyses

All statistical analyses were performed by two-tailed Student's t-test using Excel software (Microsoft).

Results

Our strategy for this aim was to overexpress either VEGFR1 or VEGFR2 selective agonist under the control of the *Pdx1* 4.3 kb enhancer/promoter and assay their effects at postnatal day (P)1 and 2 months of age. Transgene expression was predicted to be throughout the pancreatic epithelium from early bud stages and gradually become enriched only in β cells before birth, similar to endogenous Pdx1 expression. These agonists have been developed, characterized, and published by a group at Genentech (Li et al. 2000; Gille et al. 2001). Using partial digestions, a portion of each coding sequence, which contained coding variation, was subcloned into the coding sequence for VEGF contained in the pBluescript vector. The coding region was verified by sequencing, and these constructs were used to create transgenic mice.

Three transient transgenic founders were generated with the *Pdx1*VEGFR2^sel cassette, and two from the *Pdx1*VEGFR1^sel cassette. Pancreata from P1 transient transgenic founder mice were collected and their pancreata were morphometrically characterized. Mice expressing the VEGFR2 agonist had a 49% increase in endothelial to endocrine ratio, but islet size was similar to wild type (Figure 30). This robust increase in vascular endothelial cells is exemplified in the transient transgenic mouse labelled "K1.7" (<u>K</u>dr agonist at P<u>1</u>, sample <u>7</u>), where PECAM staining is much denser than in the wild type control (Figure 30). There was significant variation in the vascular phenotype observed between lines, and this is likely due to variation in expression of the transgene, although protein levels were not determined.
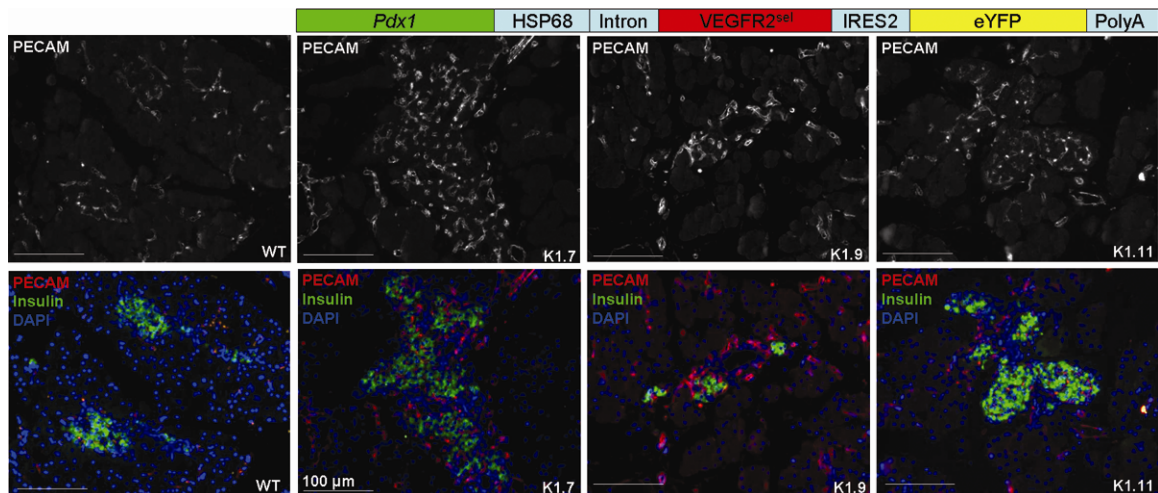
Figure 30. Representative sections of P1 wild type and *Pdx1*VEGFR2[sel] transient transgenic pancreata labeled with antibodies for PECAM, Insulin, and DAPI. The expression cassette is depicted at the top of the figure and is not to scale.

In contrast, pancreatic VEGFR1 agonist expression resulted in a decreased vascular density, and the islet size in these transient transgenics was not significantly different from the wild type littermate controls (Figure 31). As predicted, the endocrine to endothelial cell ratio was increased in these animals. This phenomenon is not due to an increase in endocrine cell mass, but instead an unexpected decrease in the endothelial cell mass. Since endocrine mass is insignificantly changed in these animals, there is likely an alternative mechanism mediating the change in endocrine to endothelial cell ratio.
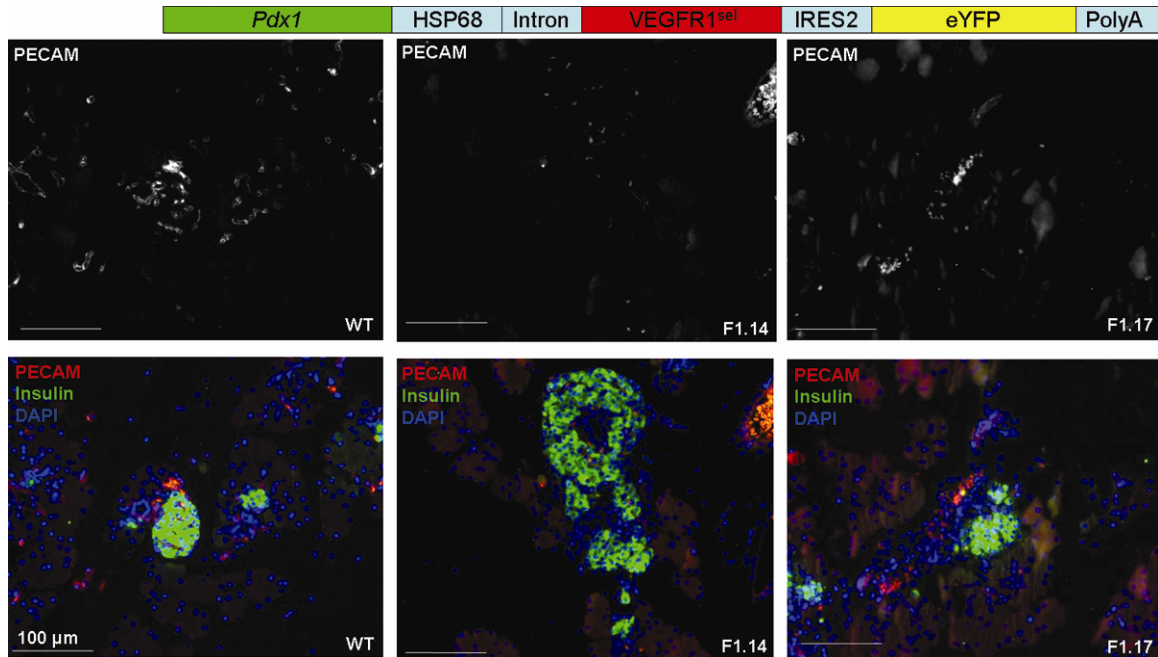
Figure 31. Representative section of P1 wild type and *Pdx1*-VEGFR1^{sel} transient transgenic pancreata labeled with antibodies for PECAM, Insulin, and DAPI. The expression cassette is depicted at the top of the figure and is not to scale.

To resolve the phenotypes and characteristics observed in the transient transgenic animals, transgenic lines were generated from the *Pdx1*VEGFR2^{sel} and *Pdx1*VEGFR1^{sel} cassettes. There were two lines obtained using the *Pdx1*VEGFR2^{sel} cassette, and three lines obtained from the *Pdx1*VEGFR1^{sel} cassette. To examine expression pancreatic expression in these lines, RNA was extracted and cDNA created from P1 pancreata of transgenic mice and their wild type littermates. Two strategies were employed to detect transgene expression. Initially, oligonucleotides were used that flanked the region coding for the myc epitope within the VEGF sequence. Comparing DNAase treated and untreated samples of the extracted RNA revealed the expected product in the DNAase treated sample and not in the untreated samples (Figure 32). Genomic DNA

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contamination in the extracted RNA would result in the same size product. To resolve this, oligos were designed that flanked the transgene intron. The PCR product arising from the cDNA of this region is ~100 bp, whereas the PCR product from genomic DNA would be ~450 bp. Transgenic lines were screened for transgene mRNA expression at P1. All lines examined had detectable mRNA levels except *Pdx1*VEGFR2[sel] line #28, consistent with the previous detection strategy (Figure 32).
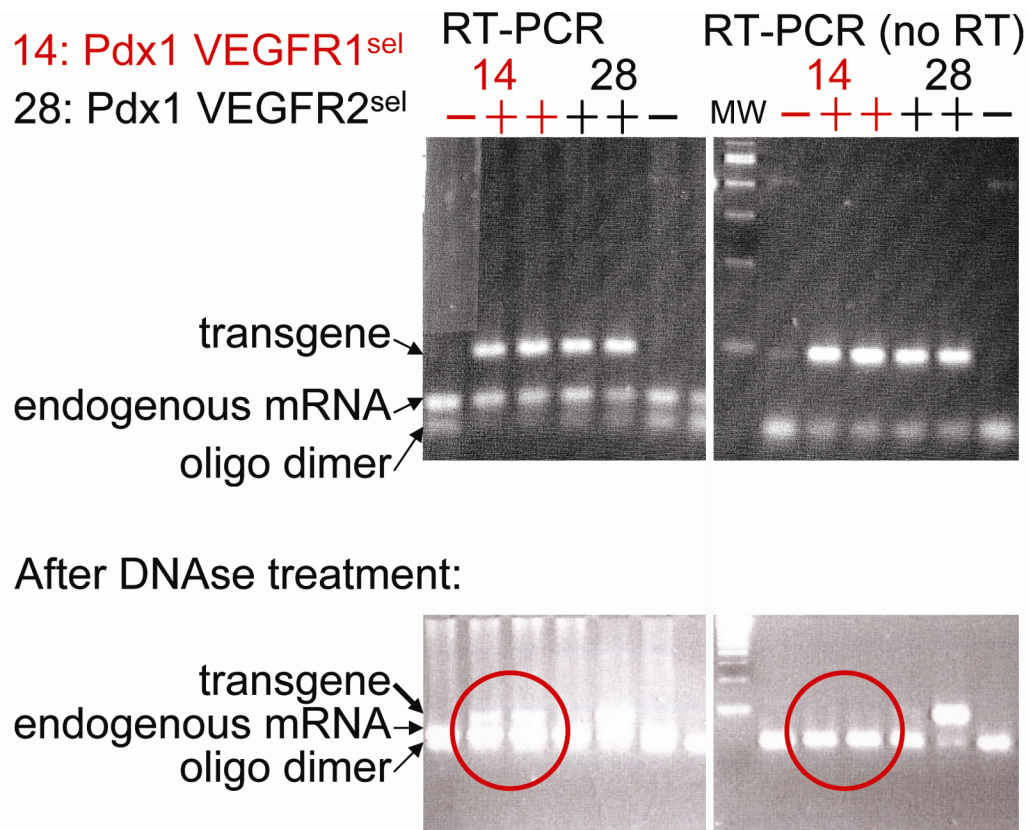
Figure 32. Detection of transgene mRNA in P1 pancreata of transgenic animals overexpressing VEGFR1^{sel} or VEGFR2^{sel}. The upper panel shows the presence of a band consistent with the transgene with or without reverse transcriptase (RT). Endogenous mRNA is only amplified in the presence of the RT. The lower panel represents the same samples after DNAse treatment of the extracted RNA. In this case there is selective amplification of line 14 transgene mRNA in the presence of RT, but not in its absence, indicating transgene expression. Line 28 amplification pattern is equivalent with or without RT, indicating no transgene expression. "-" indicates wild type littermate control RNA. "+" indicates transgenic littermate.

Pancreata were collected at P1, 1 month and 2 months of age from the three *Pdx1*VEGFR1^{sel} lines and the one *Pdx1*VEGFR2^{sel} line, which all had detectable transgene mRNA expression. These time points were chosen to enable the comparison of phenotypes between these mice to the mice

overexpressing VEGF under the control of the *Pdx1* enhancer/promoter (Lammert et al. 2001). VEGF is capable of activating both VEGFR1 and 2, therefore *Pdx1*VEGF animals are a control for the pancreatic phenotype of either VEGFR1 or 2 overactivation. Protein was extracted at P1, 1 month and 2 months of age from the three *Pdx1*VEGFR1^{sel} lines and the one *Pdx1*VEGFR2^{sel} line and insulin content was determined (Figure 33). At all time points examined, the insulin content was not significantly different from the wild type littermate controls. At one month of age, the mice overexpressing VEGFR1^{sel} tended to have an increase in the total insulin content, but this was not significantly different from their wild type littermates. Moreover, by two months of age the mice overexpressing VEGFR1^{sel} did not tend to have an increased insulin content. In fact, at this time point no transgenic line had an average total insulin content greater than the wild type control.

Taken together, VEGFR1 and VEGFR2 signaling have distinct roles in pancreatic islet development, and the expected expansion of endocrine mass mediated by increased VEGF may require increased signaling through both receptors, since overactivation of either VEGF receptor alone is not sufficient to increase islet size or pancreatic insulin content, despite changes in endothelial cell density, unlike overactivation of both receptors in the pancreas (Lammert et al. 2001).
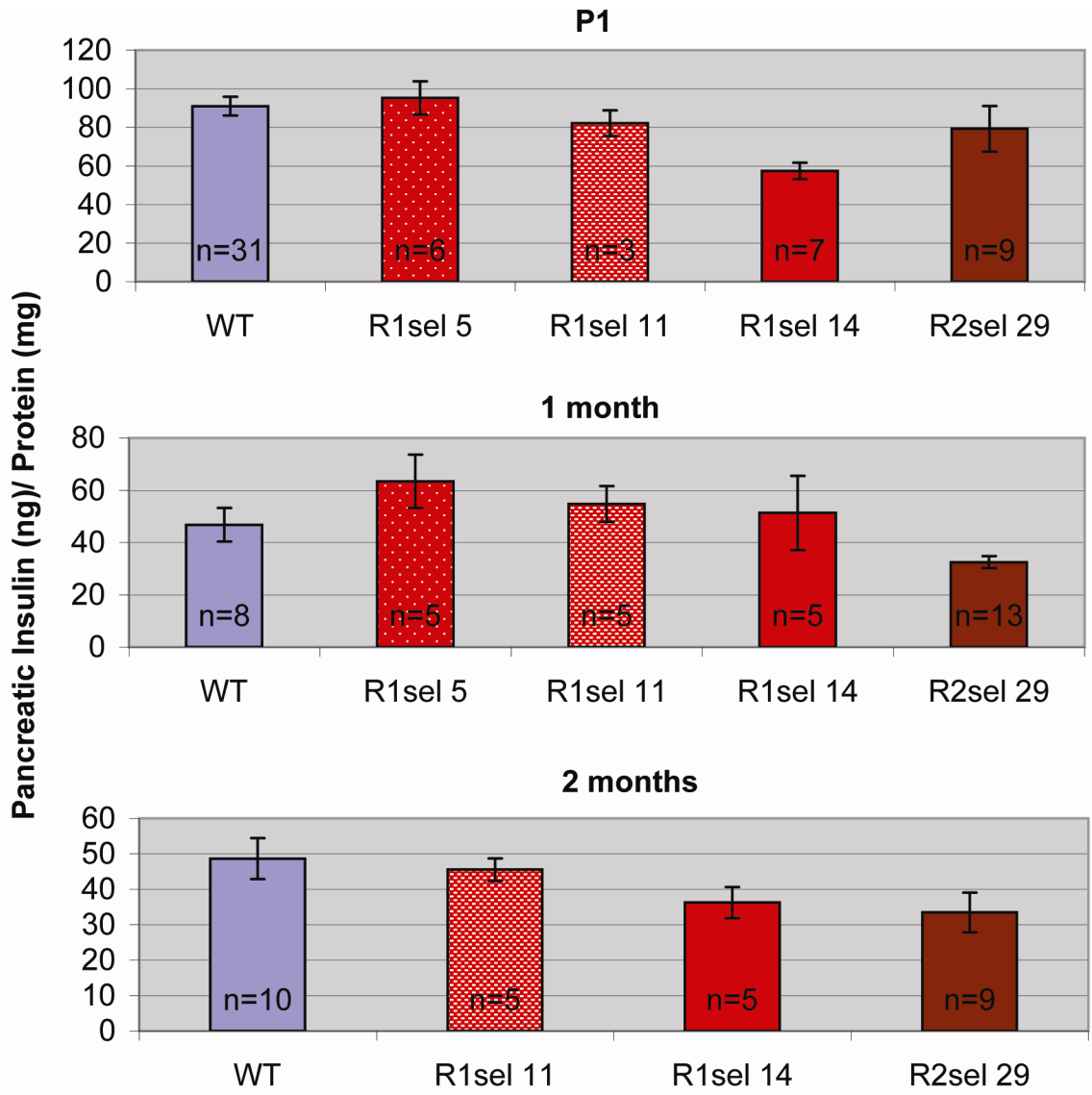
Figure 33. Total insulin content measured in mice overexpressing VEGF selective agonists under the control of the *Pdx1* enhancer. Total insulin content was not significantly different from wild type at P1, 1 month, or 2 months of age. Wild type (WT) littermates are blue, lines overexpressing VEGFR1[sel] (R1sel5, 11, 14) in textured red columns, the line overexpressing VEGFR2[sel] in brown (R2sel 29). Columns are average +/- SEM extracted insulin (ng) normalized to protein content (mg).

Discussion

The interaction between endothelial cells and endocrine cells during development remains a provocative relationship given the effect of VEGF overexpression in the pancreas (Lammert et al. 2001). Yet there is mounting evidence to suggest this interaction may not be as profound as originally suggested (Lammert et al. 2003). In fact, the expansion of endocrine cells in the *Pdx1*VEGF transgenic mouse is more likely the result of increased nutrition supplied by the increase in vascular density, than a direct reciprocal signaling mechanism between endothelial cells and endocrine cells. For example, investigators have overexpressed VEGF under the control of the rat insulin promoter (RIP) and observed no change in islet size (Gannon et al. 2002). The embryonic expression of VEGF in the RIP-VEGF mice pancreas may be at too low of a level or not early enough to significantly affect islet endocrine cell specification, but it is unlikely.

Unlike in mice, the endothelial cells are not required in zebrafish for the induction of endocrine cells in the pancreas (Field et al. 2003). It is possible this may be an evolutionary distinction, but the lack of conservation of this phenomenon across species undermines its significance. Deletion of the VEGF-A gene in β cells or pancreas-wide results in a slight reduction in islet size (Inoue et al. 2002; Lammert et al. 2003; Brissova et al. 2006). This can be easily explained by the reduction in vascular density which decreases the nutrient supply to the islet mass. Certainly VEGF secretion from the islet is necessary for islet vasculature function given the changes in glucose physiology and

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endothelial morphology observed in the pancreas tissue specific knockouts, but the role of VEGF in a reciprocal inductive signaling event between endocrine cells and endothelial cells is not supported by these investigations.

Islet VEGF secretion is indirectly important for endocrine cell survival. Several islet transplant studies have shown that VEGF secretion by the islet, or VEGF added to transplanted or cultured islets, improves the survival of this tissue due to its ability to recruit and maintain vasculature (Zhang et al. 2004; Lai et al. 2005; Mathe et al. 2006; Cross et al. 2007). The indirect β cell survival role for VEGF could also explain the reduction in islet size in the pancreas tissue specific VEGF knock out investigations (Inoue et al. 2002; Lammert et al. 2003; Brissova et al. 2006).

It is possible that VEGF is capable of causing an increase in endocrine cell mass by a reciprocal signaling event. In the current investigation we observed a significant increase in vascular density without a concomitant increase in endocrine cell mass (Figure 30). This is inconsistent with the phenotype of the VEGF overexpression throughout the pancreas, where both endocrine mass and vascular density was increased (Lammert et al. 2001), suggesting that an increase in vasculature alone is not sufficient to increase endocrine cell mass. If the role of the vasculature in the developing islet were to provide nutrition for growth and proliferation of the endocrine cell mass, then an increase in vasculature should increase endocrine mass, but this was not observed in mice overexpressing VEGFR2^{sel} in the pancreas at P1 (Figure 30); although it is likely this animal would have shown an increase in endocrine mass

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comparable to that seen in pancreata from animals overexpressing VEGF in the entire pancreas if we had collected transgenic pancreata at a later stage. An increased endocrine mass at a later stage may be a consequence of increased nutrition, due to the increased vascular density. Perhaps the increase in endothelial mass observed in mice overexpressing VEGFR2[sel] in the pancreas at P1 does not cause an increase in endocrine mass because the endocrine mass has not yet had the opportunity to benefit from increased nutrition.

The transgenic animals in this study are still being analyzed in this ongoing investigation. Thus far, the evidence in this chapter suggests that VEGF signaling in the pancreas requires signaling through both VEGFR1 and VEGFR2 for expansion of the endocrine cell mass. Alternatively, expansion of endocrine mass may be due to a nutritive or survival affect of increased VECs and not an inductive effect.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Diabetes is due to inadequate signaling through the insulin receptor of target tissues, preventing appropriate glucose uptake, but the source of this inadequacy can be much more complex. In both type 1 and 2 diabetes there is an ultimate loss of insulin producing cells in the pancreas, and monogenic forms of diabetes compromises insulin production from the pancreas. The inadequate amount of insulin producing cells in the pancreas has become a focal point for therapeutic strategies. In essence, the strategy would be to either cause existing cells to generate more or to transplant exogenous glucose-responsive insulin producing cells. For either of these strategies to succeed, elucidating the endogenous cascade of events that is necessary for a glucose-responsive insulin producing cell to normally differentiate may be vital. The intentions of the investigations described in this work were to uncover specific molecular events and cellular relationships involved in the differentiation of glucose-responsive insulin producing cells.

In Chapter II we uncovered a role for the PTF1 transcription factor complex in activating the transcription of the Pdx1 transcription factor. This molecular event occurs very early during development when the pancreas is just beginning to form. Although null mutations in either of these transcription factors share a similar gross pancreatic phenotype (Offield et al. 1996; Krapp et al. 1998;

Kawaguchi et al. 2002), their expression pattern and function In the mature pancreas diverges into separate tissue types, suggesting independent roles for these factors during pancreas development. What remains unclear is how elevated levels of PTF1 in the pancreatic exocrine tissue fail to activate Pdx1 expression at levels similar to that of Pdx1 during embryonic development. We suggest that this expression is modulated by the change in expression of the third isoform of the PTF1 complex from RBP-J to RBP-L, which is coincident with the changes in Pdx1 expression. However this proposed mechanism remains speculative, and requires continued investigation. Perhaps the next step would be to determine if by chromatin immunoprecipitation a change in occupancy can be observed at the PTF1 site of the Pdx1 enhancer.

Another phenomenon that remains to be elucidated why the Area III containing Pdx1^{XB} fragment had early broad expression throughout the early endogenous Pdx1 region, but the Area III containing Pdx1$^{I-II-III}$ fragment was restricted to the pancreas. In either case the expression in the pancreas can be explained by the activity of PTF1 through Area III, but the expanded expression of Pdx1^{XB} must be caused by some other factor. It has been demonstrated that Hnf6 can bind to Area III of the Pdx1 enhancer (Poll et al. 2006). Since Hnf6 precedes Pdx1 expression in the foregut endoderm and its expression pattern overlaps the early Pdx1 expression, it seems a likely candidate to control this early broad expression of Pdx1. However, the Pdx1$^{I-II-III}$ fragment also contains the Hnf6 site and does not have the early broad expression pattern outside of the

pancreas. To resolve this possibility, it would be prudent to determine if early broad expression of Pdx1 is also controlled by Hnf6.

In Chapter III, we describe the creation and characterization of several putatively inducible transgenic animals. Ultimately these attempts were unsuccessful, yet some of the questions to be addressed remain. For example, what are the critical qualities that an inducible transgene must possess and can an inducible transgene be more rigorously and accurately tested in vitro prior to its use in vivo, or is the production of a functional inducible transgene a matter of chance? These questions are outside of the scope of this investigation, but may be addressed or become moot with advancing technology.

The investigations in Chapter IV coupled with the findings in Chapter III, although consistent with each other, were not necessarily consistent with previous investigations (Lammert et al. 2001; LeCouter et al. 2003). Since an elevated amount of VEGF expression in the pancreas resulted in an increase in insulin producing cell mass, we wondered whether this was restricted to either a time window or specific step in the differentiation of insulin producing cells. As described in Chapter IV, there have been a couple published investigations that are inconsistent with this phenomenon (Gannon et al. 2002; Field et al. 2003). Our findings in Chapter III and IV are also inconsistent with this phenomenon, but fail to be definitive. Further investigation is necessary to determine if endothelial cells are indeed sufficient to increase pancreatic endocrine mass via a paracrine signaling event.

Taken together, our findings suggest that early pancreas cell differentiation requires PTF1 to enhance Pdx1 expression, and that signaling from endothelial cells may be necessary for Ptf1a expression, but are not sufficient. Therefore, if new glucose-responsive insulin producing cells are to be produced as a cellular therapy for diabetes, then Ptf1a expression may be necessary at early stages of differentiation to recapitulate the enhancement of Pdx1 expression that normally occurs, and signaling from endothelial cells may be critical for this process, but not capable of causing expansion of insulin producing cells.

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