HOX6 FUNCTION IS NECESSARY FOR ENDOCRINE PANCREAS DEVELOPMENT *IN VIVO* AND *IN VITRO*

by

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To my family for all of their support throughout my life and through school and to my fiancé Nicole for all of her encouragement, support, and advice. Thank you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Diabetes affects millions of Americans and is caused by a disruption in β-cell abundance or function. β-cells are one of the five types of endocrine cells found in the pancreas. A promising potential cellular therapy for the treatment of diabetes is the differentiation of human embryonic stem cells to insulin-producing β-cells. The study of endocrine pancreas development has been critical for the design of *in vitro* protocols for the differentiation of insulin-producing cells. The mesoderm is critical for the differentiation of endocrine cells and despite significant advances in our understanding of endocrine cell development, the function of the pancreatic mesodermal niche in this process is poorly understood. Fully elucidating the role of the mesoderm in endocrine cell development can greatly contribute to the design of protocols for the successful generation of functional β-cells *in vitro.*

We have discovered a novel role for mesodermally expressed *Hox6* genes in endocrine pancreas development. Inactivation of all three *Hox6* paralogs leads to a dramatic loss of endocrine cells, including β-cells, as well as mild delays and disruptions in branching and exocrine cell differentiation. Loss of *Hox6* function in the mesenchyme of the pancreas results in reduced expression of *Wnt5a* in the

pancreatic mesenchyme. This leads to subsequent loss of expression of Wnt inhibitors *Sfrp3* and *Dkk1* in endocrine progenitor cells.

In addition to a critical role in endocrine pancreas differentiation *in vivo*, we have also shown that *Hox6* function is critical for the differentiation of insulin-producing cells *in vitro.* Utilizing a differentiation protocol that directs endoderm to insulin-producing cells, we show that mesoderm with a *Hox* expression profile appropriate for the pancreatic region is also generated. Loss-of-function of *Hox6* impairs the ability of mESCs to differentiate to insulin-producing cells *in vitro,* similar to what we observe *in vivo*. Further elucidation of the mechanisms involved in mesodermal support of pancreatic endoderm and insulin-producing cells will contribute to protocols to generate replacement β-cells for the treatment of diabetes.

CHAPTER 1

INTRODUCTION

Hox genes

Hox genes are critically important, evolutionarily conserved, transcription factors that are necessary for both anteroposterior patterning of the body axis, and were first discovered in the fruit fly, *Drosophila melanogaster* (Lewis, 1978). *Hox* genes are typically expressed in a collinear manner coinciding with their location along the chromosome. Expression of the 3' *Hox* genes occurs earlier, with a more anterior limit in the embryo while the 5' genes are expressed later in development in the more posterior regions of the embryo (Dressler and Gruss, 1989; Duboule and Dolle, 1989; Graham et al., 1989; Gaunt, 1991; Izpisua-Belmonte et al., 1991; Gaunt and Strachan, 1996; Deschamps and van Nes, 2005; Iimura and Pourquie, 2006). This interesting pattern of expression led to the postulation of the *Hox* code in which the combination of *Hox* genes functioning at any given location along the anteroposterior (AP) axis result in specific morphologies (Kessel and Gruss, 1990).

Hox gene function in AP patterning has been studied extensively utilizing both loss-of-function (LOF) and gain-of-function (GOF) experiments. A very interesting trend emerged in which LOF mutations result in anterior homeotic transformations while GOF mutations result in posterior homeotic transformations (Gehring, 1987; Gehring, 1993).

For example, in *Drosophila,* loss of *Ubx* function results in an anterior homeotic transformation of the T3 segment into the T2 segment (Lewis, 1978). In contrast, GOF of *Antp* results in a posterior homeotic transformation in which legs grow in place of antennae (Schneuwly et al., 1987).

Hox genes are evolutionarily conserved and through a series of genome duplications, four complexes (*HoxA, B, C,* and *D*) of *Hox* genes have arisen in mammals (Krumlauf, 1994; Zakany and Duboule, 1999). There are 39 *Hox* genes subdivided into 13 paralogous groups based on their position along the chromosome and sequence similarity (Fig. 1.1)(Scott, 1992; Krumlauf, 1994). Due to the functional redundancy of *Hox* genes in a given paralogous group, many single *Hox* mutant mice exhibit little to no noticeable phenotype, however, when all genes of the same paralogous group are disrupted, dramatic phenotypes are revealed (Condie and Capecchi, 1994; Davis et al., 1995; Horan et al., 1995a; Horan et al., 1995b; Maconochie et al., 1996; St-Jacques and McMahon, 1996; Zakany et al., 1997; Chen et al., 1998; Studer et al., 1998; Chen and Capecchi, 1999; Rossel and Capecchi, 1999; Wellik et al., 2002; Wellik and Capecchi, 2003). Consistent with studies performed in *Drosophila,* examples of anterior homeotic transformation in LOF mutants and posterior homeotic transformations in GOF mutants have been shown in the vertebral column of the mouse. In *Hox10* GOF animals there is a complete loss of ribs resulting from a posterior homeotic transformation of the thoracic region to the lumbar region (Fig. 1.1B, C)(Carapuco et al., 2005). Conversely, LOF of all three genes of the *Hox10* paralogous group results in anterior homeotic transformation of the lumbar region to thoracic with more posterior rib formation (Fig. 1.1B, D)(Wellik and Capecchi, 2003). In contrast to

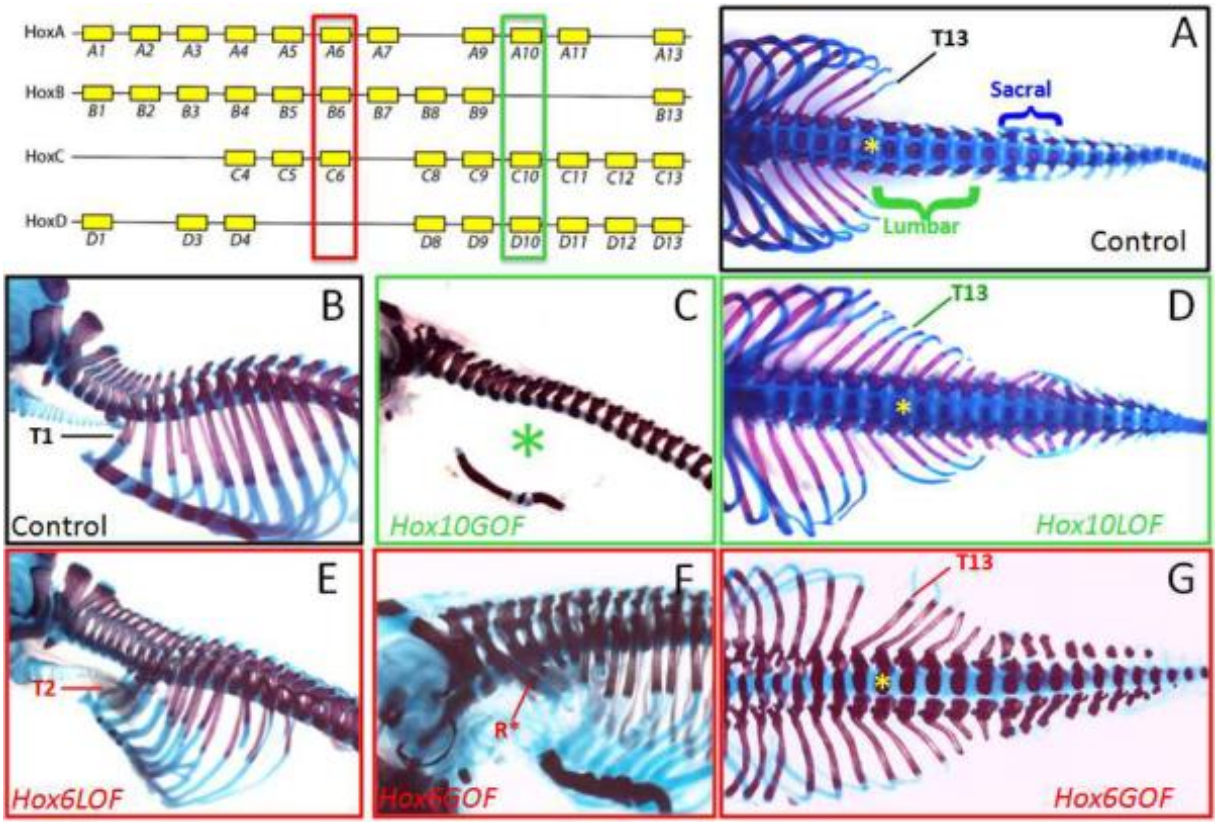

Figure 1.1. Homeotic transformations in *Hox6* and *Hox10* mutant mice. Skeletal preparations of global LOF or GOF in the *Hox6* (red boxes) and *Hox10* (green boxes) paralogous groups show both anterior and posterior homeotic transformations respectively. (A-B) Skeletons of WT embryos show the axial skeleton (A) and the rib cage (B). (C) *Hox10* GOF shows loss of the rib cage (green asterisk) in a posterior homeotic transformation. (D) Ectopic rib formation in *Hox10* LOF animals. (E) *Hox6* LOF animals have a smaller rib cage and fusion of the first rib and the second thoracic segment. (F-G) *Hox6* GOF shows animals with ectopic ribs at the cervical (F) and lumbar (G) levels. The first thoracic (T1), last thoracic (T13) and first lumbar (yellow asterisk) are labeled for reference. Figure adapted from (Mallo et al., 2010).

the apparent rib-inhibiting function of the *Hox10* paralogous group is the rib-promoting function of the *Hox6* paralogous group. In *Hoxb6* GOF animals there is ectopic rib formation into the posterior region of the embryo (Fig. 1.1A, B, F, and G)(Vinagre et al., 2010). LOF of the *Hox6* paralogous group does not result in complete loss of the rib cage however; the rib cage was smaller and exhibited abnormal rib phenotypes, including the loss of the first rib and distal fusions of ribs (Fig. 1.1B and E)(McIntyre et al., 2007). These data have shown that similar to *Drosophila*, the collinear expression and functional properties of *Hox* genes is conserved in mammals.

Consistent with collinear expression and function in the vertebral column, *Hox* genes function in organ development coinciding with their level of expression along the AP axis. When *Hox3* function is disrupted, the thyroid is hypoplastic and both the thymus and parathyroid are absent (Manley and Capecchi, 1998). When all three *Hox5* genes are disrupted, the mutant lung is significantly smaller than control lungs and exhibits severe proximal-distal patterning defects (Hrycaj et al., 2015). When *Hoxa10* function is disrupted, there is a homeotic transformation of the proximal 25% of the uterus into oviduct (Benson et al., 1996). *Hox10* triple mutants also have kidney defects in which the kidneys lack a proper kidney capsule, and as a result, exhibit decreased nephrogenesis and aberrant ureter branching (Yallowitz et al., 2011). Disruption of all three *Hox11* paralogs results in a complete loss of metanephric kidney induction (Wellik et al., 2002). The *Hox13* group is important for the proper development prostate (Economides and Capecchi, 2003). Consistent with organogenesis defects at the corresponding AP level of expression, we have found pancreas defects in mouse embryos with complete disruption of the *Hox6* paralogous group.

Pancreas development *in vivo*

The pancreas is derived from the endodermal gut tube and initiates as a bulging of the epithelium just posterior to the stomach at E8.5 (Slack, 1995). Both a dorsal and ventral pancreas bud from the endodermal gut tube into the surrounding mesoderm, and by E12.5 the separate buds fuse to form the single organ (Fig 1.2). The endodermally derived tissue of the pancreas consists of two major functional components, an endocrine and an exocrine component. The exocrine component is composed of both acinar cells and ductal cells (Fig.1.2, yellow cells). Acinar cells are responsible for secreting digestive enzymes that are transported through the ducts and into the duodenum to assist in nutrient metabolism. The exocrine component makes up approximately 90% of the overall mass of the pancreas (Puri and Hebrok, 2010).

The smaller, but equally important, endocrine compartment contains five distinct cell types that each secretes a specific hormone (Fig. 1.2, green cells). The five cells are termed α-, β-, δ-, ε-, and PP cells and secrete glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide respectively. All five types of endocrine cells are derived from progenitor cells that express *neurogenin 3* (*Ngn3*), and arise from the ductal epithelium (Gu et al., 2002). Endocrine progenitor cells migrate out of the epithelium into the surrounding mesenchyme and form clusters known as the islets of Langerhans (Rukstalis and Habener, 2009).

Islets are highly vascularized and endocrine cells secrete their enzymes into the blood stream in response to signals from the body (Puri and Hebrok, 2010). In the case of β-cells, insulin is secreted in response to increased blood sugar levels after a meal. Diabetes results when the pancreas cannot produce enough insulin in response to

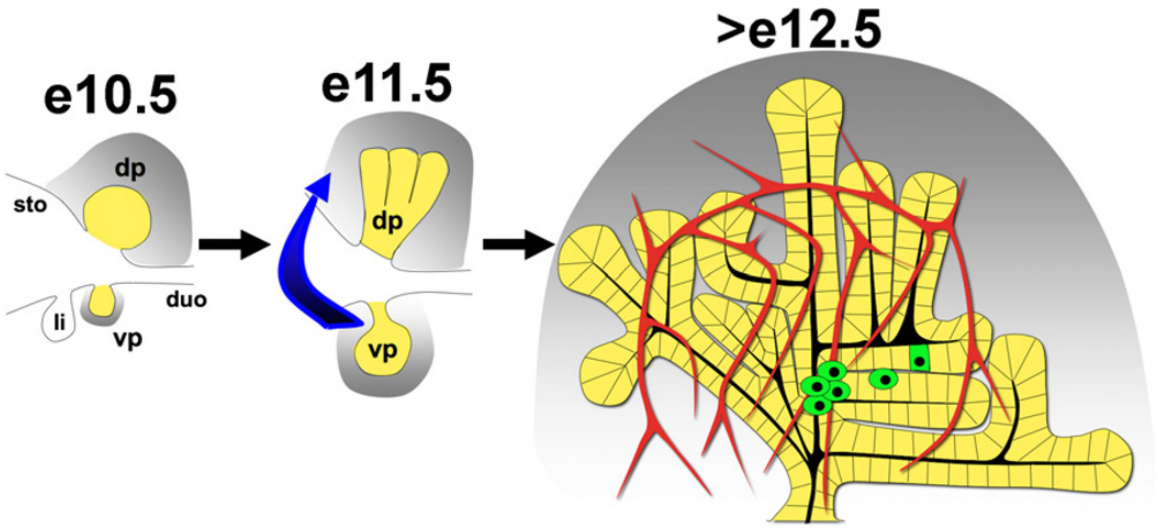

Figure 1.2 Model of early pancreas development
The pancreas epithelium (yellow) initiates at two separate buds, the dorsal pancreas (dp) and the ventral pancreas (vp). These buds grow from the gut tube into the surrounding mesenchyme (gray). The pancreas undergoes extensive branching morphogenesis at E12.5 and later developing a complex ductal network (black). Endocrine cells (green) derive from the epithelium and migrate into the surrounding mesenchyme. The pancreas is highly vascularized (red). Figure adapted from (Puri and Hebrok, 2010)

increased blood glucose, either due to an autoimmune attack on the β-cells (Type I diabetes), or impaired β-cell function (Type II diabetes) (Pagliuca and Melton, 2013).

The key pancreatic transcription factor and early marker of pancreatic specification is *Pdx1.* All epithelial cells and all endocrine cells (which are derived from the epithelium) of the pancreas arise from Pdx1-positive cells (Gannon et al., 2000; Gu et al., 2002). In order for *Pdx1* expression to be induced, Sonic Hedgehog (Shh) signaling must be inhibited in mouse and chick by notochord-derived signals that antagonize Shh signaling (Hebrok et al., 1998; Hebrok, 2003). A host of transcription factors characterize the premature pancreas epithelium and include *Foxa2, Gata4, Hnf1b, Nkx2.2, Nkx6.1, Ptf1a,* and *Sox9* (Zhou et al., 2007).

By E11.5 the pancreas bud resembles a teardrop-shaped structure of epithelial cells (Villasenor et al., 2010). At this point, the pancreatic epithelium contains a complex network of lumens termed the luminal “plexus” (Fig 1.3). The plexus undergoes significant remodeling and the multi-lumen network matures into a single lumen network (Fig. 1.3)(Villasenor et al., 2010). Isolated lumens fuse together to form longer ducts, while epithelial cells begin to express polarity markers such as ZO-1 on the apical, lumen facing side of the cell (Villasenor et al., 2010). Before epithelial branching is readily observed, the pancreas bud undergoes a complex series of stratification, plexus formation and remodeling, and cell shape and polarity changes (Villasenor et al., 2010). These processes eventually result in a simple cuboidal epithelium forming a complex single lumen branching network (Hick et al., 2009; Kesavan et al., 2009).

Concomitant with branching, the pancreas epithelium begins to differentiate into separate “tip” and “trunk” domains by E12.5 (Zhou et al., 2007). The tip domain is

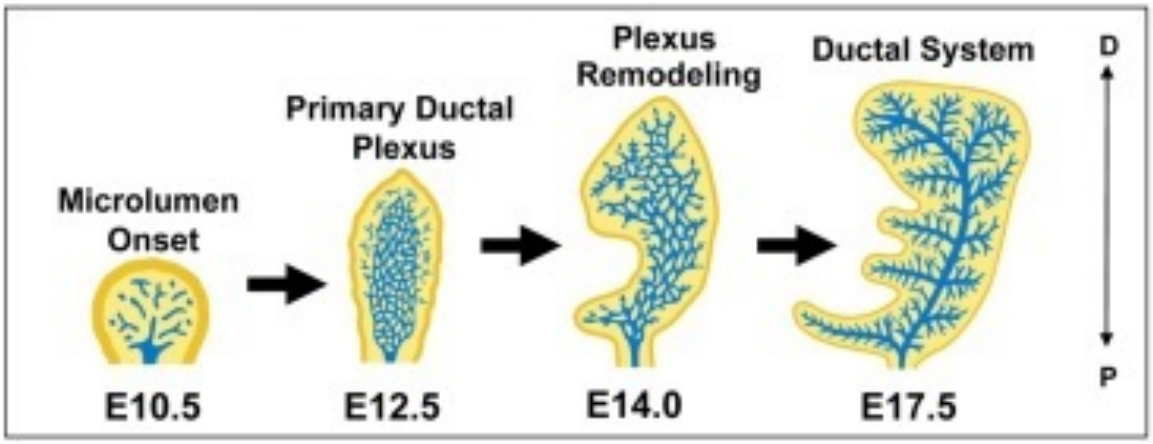

Figure 1.3 Pancreas duct development
Pancreatic branching begins at E10.5 with the onset of microlumen formation. The microlumens then fuse together to form a ductal plexus, which further remodels into the single lumen ductal network of the mature pancreas. Figure adapted from (Villasenor et al., 2010).

initially composed of multipotent progenitor cells (MPCs) capable of producing cells of the acinar, ductal and endocrine lineages (Zhou et al., 2007). The tip (acinar) and trunk (ductal) domain are established through the dynamic expression of several critical transcription factors. The tip domain is characterized by the expression of *Cpa1, c-Myc*, and *Ptf1a* (*P48*) while the trunk domain expresses *Hes1, Hnf1β, Sox9, Nkx6.1/6.2 Prox1* and *Onecut-1* (Jacquemin et al., 2003; Wang et al., 2005; Vanhorenbeeck et al., 2007; Zhou et al., 2007; Solar et al., 2009; Schaffer et al., 2010; Klinck et al., 2011; Kopinke et al., 2011; Kopp et al., 2011). Nkx6.1 (in the trunk cells) and Ptf1a (in the tip cells) have an antagonistic relationship that is thought to establish the separation of the tip and trunk domains (Schaffer et al., 2010). After E13.5, during the secondary transition, tip MPCs lose their multipotency and from then on only produce acinar cells (Zhou et al., 2007). Trunk cells retain multipotency capabilities and continue to produce both ductal cells and all five lineages of endocrine cells (Zhou et al., 2007; Solar et al., 2009; Kopinke et al., 2011; Kopp et al., 2011).

In mouse, endocrine cell differentiation occurs in three waves through development termed “transitions” (Rukstalis and Habener, 2009). The primary transition occurs from E8.5-E12.5 and during this step mostly glucagon-producing α-cells are made, but it is unclear how many of these early endocrine cells actually persist into the adult organ (Herrera et al., 1994; Herrera, 2000). The majority of endocrine cell differentiation occurs during the secondary transition, which is between E13.5-E15.5 in the mouse embryo. This is when the bulk of insulin-producing β-cells are generated (Rukstalis and Habener, 2009). This is slightly different in humans where β-cells are actually the first endocrine cells detected, followed by α-, δ-, and then PP-cells (Piper et

al., 2004; Jeon et al., 2009). During the tertiary transition, the mature endocrine cells migrate away from the ductal epithelium and consolidate into clusters of endocrine cells throughout the pancreas called the islets of Langerhans (Rukstalis and Habener, 2009). In the adult, endocrine cell mass is maintained by slow, steady rates of proliferation. It is presently unclear if an endocrine stem cell exists that can contribute significantly to the mass of endocrine cells (Teta et al., 2005; Teta et al., 2007).

All endocrine cells arise from the ductal epithelium of the pancreas. Intermittent ductal cells begin to express *Ngn3*, delaminate from the epithelium into the mesenchyme, and mature into hormone-producing endocrine cells (Gu et al., 2002). *Ngn3* is necessary for the differentiation of endocrine cells as shown by a failure to generate any cells of the five endocrine lineages in mice deficient for *Ngn3* function (Gradwohl et al., 2000). While all five endocrine cell types arise from *Ngn3*-expressing precursors, it is not completely clear how precursor cells are instructed to form the five specific endocrine lineages. A current hypothesis is that not all Ngn3-positive cells have the same developmental potential and that spatio-temporal cues influence the lineage decision of each endocrine sub-type (Johansson et al., 2007; Desgraz and Herrera, 2009; Mastracci et al., 2013). The use of tamoxifen inducible expression of *Ngn3* in Pdx1+ epithelium, in an *Ngn3* -/- background, has shown that at different time points, different endocrine cell lineages are induced to differentiate (Johansson et al., 2007). At E8.5, primarily α-cells are formed (Schwitzgebel et al., 2000; Johansson et al., 2007). Between E10.5 and E12.5 β- and PP-cells begin to differentiate (Johansson et al., 2007). Lastly, δ-cells only differentiate at E14.5 and later (Johansson et al., 2007). The molecular mechanisms involved in this temporal differentiation scheme are still not fully

understood although it has been shown that the mesenchyme plays a role in the timing of β-cell differentiation (Duvillie et al., 2006; Attali et al., 2007).

The role of the mesenchyme in pancreas development

The mesenchyme is critical for the growth and development of both the exocrine and endocrine pancreas. The importance of the mesenchyme has been appreciated since the 1960's when initial studies found that in culture, the epithelium of the pancreas failed to grow when separated from the mesenchyme (Golosow and Grobstein, 1962; Rutter et al., 1964; Wessells and Cohen, 1967). Culture experiments like these have shown critical roles for the pancreas mesenchyme in both exocrine and endocrine cell growth and differentiation (Gittes et al., 1996; Miralles et al., 1998; Miralles et al., 1999b; Li et al., 2004; Duvillie et al., 2006). Cultures in which there was a close proximity of the epithelium to the mesenchyme promoted exocrine, but not endocrine, development (Li et al., 2004). When epithelium devoid of mesenchyme was cultured in Collagen I gel with mesenchyme on the opposite side of a diffusible filter, endocrine cell differentiation was promoted (Li et al., 2004). Further studies have shown that the endocrine-inhibiting signals induced by epithelial-mesenchymal cell-cell contact are mediated by Notch signaling through the expression of *Hes1* and the inhibition of *Ngn3* (Shih et al., 2012).

The pancreas epithelium branches into the mesenchyme at E12.5 making it extremely difficult to physically separate the mesenchyme from the epithelium. Genetic tools are therefore necessary to further explore the mechanisms of mesenchymal-epithelial crosstalk later in development. Recently, genetic ablation of the mesenchyme with diphtheria toxin was demonstrated using *Nkx3.2*-Cre to drive expression of

diphtheria toxin receptor specifically in the mesenchyme of the embryonic pancreas (Landsman et al., 2011). When the mesenchyme is ablated early in development, there is a drastic reduction in overall pancreas mass and endocrine cell mass (Landsman et al., 2011). Even when the mesenchyme is ablated as late as E16.5 there is a significant reduction in pancreas mass at E18.5 (Landsman et al., 2011). While it has been known for over 60 years that the presence of mesenchyme is necessary for pancreas development, the specific signaling mechanisms involved are largely unknown.

Signaling in pancreas development

Fgf10 is produced by mesenchymal cells and activates Fgfr2 in the epithelial cells (Bhushan et al., 2001; Dichmann et al., 2003; Seymour et al., 2012). Fgf signaling is critical for the proliferation and expansion of the exocrine pancreas, but appears to have little to no effect on the development of the endocrine pancreas (Miralles et al., 1999a). When cultured in the absence of mesenchyme, treatment of the epithelium with Fgf1, Fgf7 or Fgf10 protein could rescue exocrine cell expansion, but there was no effect on the development of the endocrine compartment (Miralles et al., 1999a). While Fgf plays a critical role in pancreas development, the overall level of Fgf10 is barely detectable by E13.5 (Bhushan et al., 2001; Elghazi et al., 2002). Further studies have shown important roles for Retinoic Acid (RA), Wnt, Bmp, Notch, Tgfβ, and Egf signaling as critical mesenchymal regulators of epithelial pancreas development however, little is known about the function of individual members of these pathways (Stafford et al., 2006; Tulachan et al., 2006; Jonckheere et al., 2008; Ahnfelt-Ronne et al., 2010).

Notch signaling is important for maintaining progenitor cells of the pancreas in an undifferentiated state, and can be activated by mesenchymal Fgf signals (Hart et al., 2003; Norgaard et al., 2003). Inactivation of Notch components leads to the accelerated differentiation of endocrine cells, indicating a critical role for Notch signaling in determining the decision between endocrine and progenitor/exocrine cell fates (Apelqvist et al., 1999). Conversely, sustained activation of Notch signaling results in a loss of differentiated acinar and endocrine cells because the progenitor cell population remains locked in an undifferentiated state (Hald et al., 2003; Murtaugh et al., 2003).

Tgfβ is also critical for regulating the exocrine/endocrine fate decision (Miralles et al., 1998). Pancreatic rudiments cultured without mesenchyme show an increase in exocrine cell differentiation and a decrease in endocrine cell differentiation when treated with the Tgfβ inhibitor follistatin (Miralles et al., 1998). Tgfβ signaling is also functional in human β-cells as shown by a study in which cultured human islets were treated with Nodal and a modest increase in β-cell proliferation was observed (Boerner et al., 2013). Egf signaling has roles in both exocrine and β-cell growth and abundance. Betacellulin is an Egf family member expressed in the pancreas epithelium at E11.5 (Thowfeequ et al., 2007). Betacellulin has been shown to induce proliferation of undifferentiated epithelial cells in human fetal pancreas and can induce proliferation of Pdx1-positive epithelium in mouse pancreas cultures (Demeterco et al., 2000; Thowfeequ et al., 2007). Egf ligand is an important regulator of epithelial-mesenchymal interactions and can induce duct development in cultured embryonic pancreas (Warburton et al., 1992; Sanvito et al., 1994).

The mesoderm also produces signals to induce pancreas specification by activating the expression of *Pdx1*. RA and Bmp/activin signals are produced in the mesoderm and are sufficient to induce *Pdx1* expression in otherwise non-pancreatic endoderm (Kumar et al., 2003). RA from the mesoderm also helps to establish the AP position of the liver and the pancreas (Kumar et al., 2003).

Wnt signaling is another important developmental signaling pathway with many components expressed in the pancreatic mesenchyme (Heller et al., 2002). Early studies have shown the expression of Wnt ligands *Wnt2b, Wnt4, Wnt5a,* and *Wnt7b,* and many Frizzled receptors and inhibitors throughout the epithelium and mesenchyme of the developing pancreas (Heller et al., 2002). To date, *Wnt5a* and *Wnt7b* knockout mice are the only Wnt ligand specific knockouts to exhibit a pancreas phenotype (Kim et al., 2005; Afelik et al., 2015). *Wnt5a* null embryos exhibit normal overall morphology and exocrine cell development (Kim et al., 2005). However, endocrine cells exhibit aberrant islet organization and impaired migration (Kim et al., 2005). *Wnt7b* knockout mice exhibit pancreatic hypoplasia due to a lack of proliferation of pancreatic progenitor cells (Afelik et al., 2015).

More studies have been done that manipulate the Wnt signaling pathway in a broader sense by activating or inactivating the necessary canonical Wnt component, β-catenin. These studies have produced sometimes directly conflicting results demonstrating the complexity of the canonical Wnt signaling pathway in pancreas development. Three separate groups have investigated the effects of an early, epithelial deletion of β-catenin using two different conditional deletion alleles of β-catenin and three different *Pdx1*-Cre deletor mice (Murtaugh, 2008). A study from the Melton lab

reported a hypoplastic pancreas with no apparent endocrine cell defects (Murtaugh et al., 2005). A separate study demonstrated endocrine cell loss with some exocrine cell defects and perinatal pancreatitis when β-catenin was deleted in the pancreas epithelium by a different *Pdx1-Cre* mouse (Dessimoz et al., 2005). Lastly, another group reported pancreas hypoplasia similar to the Melton group with acinar to ductal metaplasia and no apparent endocrine cell defects (Wells et al., 2007). This discrepancy in phenotype was resolved by a direct comparison of two different *Pdx1-Cre* drivers which determined that the two Cre drivers induced recombination at slightly different time points and with varying efficiency (Heiser et al., 2006).

These studies have shown a clear requirement for β-catenin in acinar cell formation early in development, but the role of Wnt signaling specifically in endocrine cell development is still unclear. β-catenin appears to encourage endocrine cell development only in an indirect fashion. In addition to the study previously mentioned by Dessimoz et al., a more recent study has shown that deletion of β-catenin function in the pancreas epithelium results in decreased β-cell mass as a result of lost MPCs (Baumgartner et al., 2014). This study also rules out a direct contribution of β-catenin to endocrine cell development by specifically deleting β-catenin in Ngn3+ endocrine progenitor cells and reporting no change in β-cell mass (Baumgartner et al., 2014). Canonical Wnt activity may in fact be inhibitory for endocrine cell differentiation. A surprising number of Wnt inhibitors are expressed in endocrine cells, and excess canonical Wnt activity results in reduced endocrine cell differentiation (Heller et al., 2002; Pedersen and Heller, 2005). GOF of canonical Wnt ligand *Wnt7b* in the pancreas also results in failed differentiation of endocrine cells (Afelik et al., 2015).

Multiple Wnt pathway receptors including *Fzd2, Fzd3,* and *Fzd7* are expressed in endocrine cells of the pancreas, which suggests that Wnt ligands can act directly on endocrine cells (Heller et al., 2002). In zebrafish, noncanonical Wnt ligand *Wnt5a* activates *Fz-2* to promote proper endocrine cell development (Kim et al., 2005). Other members of the noncanonical Wnt planar cell polarity (PCP) pathway, *Celsr2* and *Celsr3*, are necessary for the differentiation of all five types of endocrine cells (Cortijo et al., 2012). This growing body of evidence suggests that the proper combination of canonical Wnt inhibition and noncanonical PCP activation is necessary for pancreatic endocrine cell formation during pancreas organogenesis. The multitude of Wnt components expressed in the pancreas during development and the extreme phenotypes observed with manipulation of Wnt/ β-catenin signaling suggests an extremely complicated signaling network of which we are only beginning to understand.

Differentiation of ESCs to insulin-producing cells *in vitro*

The study of pancreas development, and especially the signals critical to inform proper endocrine pancreas development, is necessary to design efficient protocols of embryonic stem cell (ESC) or induced pluripotent stem cell (iPSC) differentiation to glucose responsive insulin-producing cells for the treatment of diabetes. Diabetes is a metabolic disease that can occur in multiple different ways. Type I diabetes is caused by the autoimmune destruction of pancreatic β-cells. Type II diabetes occurs when consistently high blood sugar creates an excessive demand for insulin, which in turn leads to β-cell malfunction, de-differentiation and apoptosis (Ashcroft and Rorsman, 2012; Talchai et al., 2012). Diabetes leads to devastating side effects including

cardiovascular disease, retinopathy and blindness, amputations caused by neuropathy, kidney disease and eventual renal failure (Pagliuca and Melton, 2013). One of the most common treatments for diabetes is the administration of exogenous insulin. Insulin injections are necessary for the treatment of Type I diabetes especially, and requires several blood glucose measurements and insulin injections per day. This is a taxing treatment option for the patient, and if not monitored properly, can lead to devastating side effects including ketosis and coma (Nathan et al., 2009).

The prospect of ESC- or iPSC-derived β-cells for the treatment of diabetes is appealing because it is only a single cell type that needs to be replaced and if successful, could eliminate the diabetic patient's requirement for exogenous insulin (Pagliuca and Melton, 2013). There is precedent for the concept of using ESC-derived mature cell types for the treatment of disease as techniques have been utilized for other organ systems as well. ESC-derived cardiomyocytes have been used to engraft onto injured hearts and prevent arrhythmias (Shiba et al., 2012). Another example is ESC-derived oligodendrocytes that have been able to restore mobility to rats with spinal cord injuries (Keirstead et al., 2005).

While replacement β-cells would be of great benefit to those with Type II diabetes, there is still the problem of an autoimmune attack on replacement β-cells in patients with Type I diabetes. The recipient's immune system would also attack replacement β-cells from a donor. Fortunately, replacement β-cells could be transplanted to non-endogenous sites allowing those cells to be enclosed in immune-protective devices (Pagliuca and Melton, 2013). In fact, β-cells donated by human cadavers have been transplanted into the hepatic portal vein of the recipient with

successful results (Mullen et al., 1977; Lacy and Scharp, 1986; Shapiro et al., 2000; Shapiro et al., 2006; Bellin et al., 2012). Although the transplantation of human cadaveric β-cells has been a successful treatment for diabetes, the overwhelming demand for these cells versus their availability creates a necessity for alternate treatments (Pagliuca and Melton, 2013).

The first major step in the generation of β-cells *in vitro* was the identification of human ESCs (hESCs) (Thomson et al., 1998). This, however, caused a significant amount of controversy and even led to a ban of NIH funding on research involving newly generated hESCs (Murugan, 2009). Research on a restricted number of hESC lines is allowed, but this makes it difficult to study specific disease mutations that are not already present in accepted cell lines, and also limits the genetic diversity available for study (Murugan, 2009). A critical breakthrough in this area of research was the discovery of a method to generate iPSCs from murine fibroblasts (Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007). The generation of iPSCs from human cells followed shortly thereafter (Takahashi et al., 2007; Yu et al., 2007; Lowry et al., 2008; Nakagawa et al., 2008). Critically, iPSCs are not generated from human blastocysts, circumventing controversy, and these cells have the ability to generate all cell types (Wernig et al., 2007; Okita et al., 2010). This allows for greater funding flexibility and significantly expands the scientific questions that can be answered regarding specific genetic backgrounds/mutations. iPSCs also allow patient-specific cell generation to circumvent complications such as graft-versus-host disease.

Forced expression of genes such as *Pdx1* and *Ptf1a* are capable of converting non-pancreas cells into pancreas cells however, manipulating the genome of cells that

are to be used for transplantation into diabetic patients can lead to unintended mutations and consequences (Fig. 1.4B)(Afelik et al., 2006). Investigators have instead utilized various methods of ESC to insulin producing cell (IPC) differentiation through the treatment of cells with combinations of growth factors found in normal pancreas development *in vivo* (D'Amour et al., 2006). The most successful methods for the differentiation of IPCs *in vitro* mimic the sequential steps that a developing β-cell undergoes *in vivo* (Fig. 1.4A) (Pagliuca and Melton, 2013).

All endocrine cells of the pancreas are derived from the epithelium of the pancreas, which in turn is derived from endoderm (Gittes, 2009). The first step in differentiation protocols is then to produce an abundant population of endodermal cells from pluripotent stem cells. The first efficient protocol for the generation of definitive endoderm from hESCs used Activin A, a member of the TGF-β family, to induce endoderm differentiation (D'Amour et al., 2005). The basis for using Activin A as a director of endodermal differentiation derived from studies that have shown primitive streak defects caused by the disruption of either the Wnt or TGF-β signaling pathways (Conlon et al., 1994; Haegel et al., 1995; Liu et al., 1999; Brennan et al., 2001; Kelly et al., 2004). Activin A in combination with low serum conditions can produce a population of up to 80% endodermal cells as determined by the expression of transcription factors such as *FoxA2, Sox17,* and *Gsc* (D'Amour et al., 2005). The dose of Activin A is also critical as concentrations between 50-100 ng/mL lead to efficient differentiation of definitive endoderm (DE) whereas lower doses do not (Kubo et al., 2004; D'Amour et al., 2005). Several studies have shown that treating cells with factors including Wnt activators (Wnt3a and CHIR9902), GSK3β, sodium butyrate, PI3K pathway antagonists,

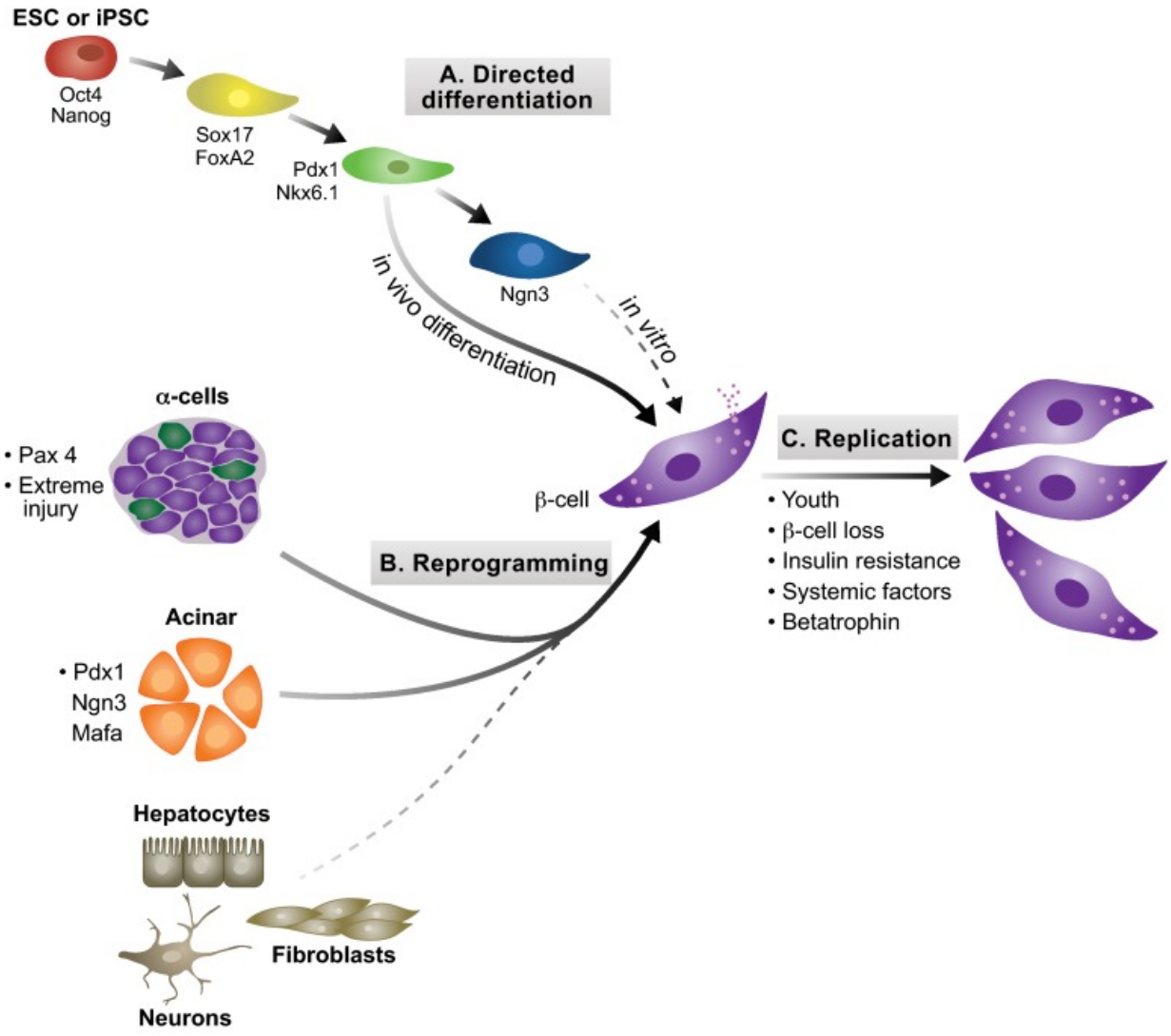

Figure 1.4 Strategies for β-cell generation *in vitro*
(A) Pluripotent stem cells can be directed to differentiate through sequential cell types to mimic normal *in vivo* β-cell development using a series of growth factors and small molecules. Until recently, an implantation *in vivo* differentiation step was required to differentiate endocrine progenitor cells in functional glucose-responsive β-cells. (B) Acinar or other endocrine cells can be directly reprogrammed to β-cells using overexpression techniques. Successful reprogramming of mature cell types from other organs has yet to be achieved. (C) Inducing replication in existing β-cells or β-cells generated *in vitro* is another possible way to produce a large population of β-cells for implantation into a diabetic patient. Figure adapted from (Pagliuca and Melton, 2013)

and GDF8 (a TGF-β family member) can improve the efficiency of endoderm differentiation (Jiang, W. et al., 2007; McLean et al., 2007; Zhang et al., 2009; Bone et al., 2011; Kunisada et al., 2012; Bruin et al., 2014).

After differentiation to endoderm, the cells must further mature to pancreatic endoderm. All epithelial cells and all endocrine cells (which are derived from the epithelium) of the pancreas arise from Pdx1-positive cells (Gannon et al., 2000; Gu et al., 2002). As previously discussed, Shh signaling must be inhibited for *Pdx1* expression to be induced. In addition to the repression of *Shh,* RA is required for pancreas induction and Fgf10 is required for the growth and expansion of the epithelium (Chen et al., 2004; Martin et al., 2005; Molotkov et al., 2005; Stafford and Prince, 2002; Bhushan et al., 2001). The combination of Shh inhibition, Fgf10 and RA is sufficient to differentiate definitive endoderm into pancreatic endoderm *in vitro* (D'Amour et al., 2006).

All pancreatic endocrine cells derive from ductal epithelium cells that begin to express *Ngn3,* and then subsequently delaminate from the epithelium, migrate into the surrounding mesenchyme, and mature into functional endocrine cells (Gu et al., 2002). The process of determining which cells along the duct begin to express *Ngn3* is regulated by Notch signaling (Shih et al., 2012). Notch signaling promotes the expression of *Sox9,* which can then cell-autonomously activate the expression of *Ngn3* (Shih et al., 2012). This signaling mechanism is dose dependent however, as too much Notch activity can actually inhibit *Ngn3* expression by promoting the expression of *Ngn3* repressor *Hes1* instead (Shih et al., 2012). Some groups have utilized this knowledge and include DAPT (a gamma secretase inhibitor) to help limit Notch activity and

therefore promote the induction of *Ngn3* expressing cells in culture (D'Amour et al., 2006; Chen et al., 2009; Thatava et al., 2011; Pagliuca and Melton, 2013).

A number of additional growth factors and small molecules have been used in an effort to induce endocrine cell differentiation from pancreatic endoderm *in vitro* including BMP inhibitors, PKC activators, EGF, KGF, and exendin-4 (D'Amour et al., 2006; Kroon et al., 2008; Rezania et al., 2012; Schulz et al., 2012). However, until recently, no combination of these factors was able to generate an abundant population of functional glucose-responsive insulin-producing cells. A number of groups have been able to generate IPCs, but there were problems with these cells such as the failure to secrete insulin in response to glucose, poly-hormonal expression, and lack of expression of *Nkx6.1* and *MafA* (D'Amour et al., 2006; Jiang, J. et al., 2007; Jiang, W. et al., 2007; Shim et al., 2007; Kroon et al., 2008; Tateishi et al., 2008; Zhang et al., 2009; Cai et al., 2010; Mfopou et al., 2010; Kelly et al., 2011; Nostro et al., 2011; Xu et al., 2011). Functional, glucose-responsive β-cells were only generated if the endocrine progenitor cells from culture were implanted into a mouse and left to further differentiate *in vivo* for four months (Kroon et al., 2008; Rezania et al., 2012).

There has been recent success in the field of IPC differentiation as multiple different groups have been successful in the generation of functional glucose-responsive insulin-producing cells *in vitro* (Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015). Timothy Kieffer's group developed a seven-step protocol that was able to generate glucose-responsive insulin-secreting cells capable of treating diabetes in the mouse (Rezania et al., 2014). Doug Melton's group generated a protocol for the differentiation of hESCs and hiPSCs into functional β-cells (termed SC-β cells) *in vitro*

by testing over 70 different compounds in more than 150 combinations (Pagliuca et al., 2014). This 4-5 week protocol utilizes a three-dimensional culture method and the manipulation of numerous pathways including Wnt, Activin, Shh, EGF, TGFβ, thyroid hormone, and RA (Pagliuca et al., 2014). The SC-β cells performed just as well as cadaveric β-cells in terms of glucose dependent insulin-secretion and the ability to treat diabetes in the mouse (Pagliuca et al., 2014). These studies both report the presence of a large population of C-peptide-positive cells that do not express *Nkx6.1* and may result in the rise of polyhormonal cells that can result from the early induction of endocrine cell differentiation in Pdx1+Nkx6.1- cells (Rezania et al., 2014; Russ et al., 2015). A study by Mattias Hebrok's lab modifies these previous protocols by omitting BMP inhibitors and focusing on a slightly later stage in the differentiation process with the addition of EGF and KGF to induce a greater population of *Nkx6.1* expressing cells (Russ et al., 2015). They report that their simplified protocol enables the generation of a more restricted endocrine population without polyhormonal cells and more closely resembles the mechanisms of *in vivo* endocrine cell differentiation (Russ et al., 2015). It is possible that the omission of BMP inhibitors in this protocol allows for some mesodermal cell differentiation to supply additional signals to the differentiating endodermal cells to aid in the differentiation of endocrine cells, a topic discussed in Chapter 3 of this dissertation. While these studies have shown extremely promising results, there still remains the presence of cells that have not fully differentiated to mature, functional β-cells (Rezania et al., 2014; Russ et al., 2015). These remaining immature cells may cause problems if implanted into a diabetic patient. It is possible that fully elucidating the molecular signals

from the mesenchyme critical for endocrine cell differentiation will lead to protocols capable of differentiating pure populations of functional β-cells.

Unanswered questions in the field

Many of the factors utilized in protocols of *in vitro* β-cell differentiation are produced by the pancreatic mesenchyme (Gittes, 2009; Puri and Hebrok, 2010). In order to generate a pure population of functional β-cells, critical signals from the mesenchyme will need to be replaced in culture. This will only happen when the role of the mesenchyme in pancreas development *in vivo* is fully understood. The complexity of signaling between the mesenchyme and epithelium is still not well understood despite the fact that the mesenchyme has been known to play critical roles in pancreas development for more than 50 years (Golosow and Grobstein, 1962; Wessells and Cohen, 1967). Studies like these in the next two chapters have a significant impact on our understanding of mesenchymal-epithelial signaling in pancreas development.

Signaling pathways such as Wnt have a multitude of ligands, receptors, and inhibitors expressed throughout pancreas development, but the function of only a few of these specific members has been reported (Heller et al., 2002; Kim et al., 2005; Afelik et al., 2015). It is likely that many of the members of this and other pathways have critical roles in pancreas development that will need to be elucidated. There is still the lack of a consensus phenotype for broader functional studies of Wnt in the pancreas that either block or promote overall canonical Wnt activity through the manipulation of β-catenin (Murtaugh, 2008). Despite the abundance of published articles on Wnt and other pathways there is still more to be investigated. Here we find a critical role for multiple

members of the Wnt signaling pathway that will ultimately contribute to our overall understanding in the role of Wnt signaling in endocrine pancreas development.

CHAPTER 2

MESENCHYMAL *HOX6* FUNCTION IS REQUIRED FOR PANCREATIC ENDOCRINE CELL DIFFERENTIATION

Summary

Despite significant advances in our understanding of pancreatic endocrine cell development, the function of the pancreatic mesodermal niche in this process is poorly understood. Here we report a novel role for *Hox6* genes in pancreatic organogenesis. *Hox6* genes are expressed exclusively in the mesoderm of the developing pancreas. Genetic loss of all three *Hox6* paralogs (*Hoxa6, Hoxb6, Hoxc6*) leads to a dramatic loss of endoderm-derived endocrine cells including insulin-secreting beta cells, as well as mild delays and disruptions in pancreas branching and exocrine differentiation. Ngn3-expressing pan-endocrine progenitor cells are specified normally in *Hox6* mutant pancreata, but fail to mature into hormone-producing cells. Reduced expression of *Wnt5a* is observed in mutant pancreatic mesenchyme, leading to subsequent loss of expression of critical Wnt inhibitors *Sfrp3* and *Dkk1* in endocrine progenitor cells. These results reveal a key role for *Hox6* genes in establishing *Wnt* mesenchymal/epithelial crosstalk in pancreatic development.

Introduction

Hox genes play a well-established role in axial and appendicular skeletal patterning, and knowledge of their importance in organogenesis is expanding. *Hox* genes have important roles in the development of organs that correspond to *Hox* expression along the AP axis. Examples include the *Hox3* paralogous group genes, which are critical for thymus, thyroid, and parathyroid development, *Hox5* genes in lung development, *Hox9, 10, 11* in the reproductive tract, *Hox10* and *Hox11* genes in kidney development, and *Hoxb13* for prostate development (Dolle et al., 1991; Benson et al., 1996; Gendron et al., 1997; Taylor et al., 1997; Manley and Capecchi, 1998; Podlasek et al., 1999; Economides and Capecchi, 2003; Schwab et al., 2006; Yallowitz et al., 2011; Boucherat et al., 2013; Raines et al., 2013; Hrycaj et al., 2015).

The *Hox6* paralogous group includes three genes: *Hoxa6, Hoxb6* and *Hoxc6.* Paralogous *Hox* genes have been shown to exhibit a high degree of functional redundancy due to sequence similarity and significant overlap in expression. Loss of a single gene within a paralogous group often results in little to no observable phenotype, however, disruption of all members of a given paralogous group result in dramatic patterning phenotypes (Davis and Capecchi, 1994; Horan et al., 1995b; Fromental-Ramain et al., 1996b; Fromental-Ramain et al., 1996a; Manley and Capecchi, 1998; Rossel and Capecchi, 1999; van den Akker et al., 2001; Wellik et al., 2002; Wellik and Capecchi, 2003; McIntyre et al., 2007; Yallowitz et al., 2009; Xu and Wellik, 2011; Yallowitz et al., 2011; Xu et al., 2013). Single mutant animals for each of the *Hox6* paralogs have undetectable or very mild defects, but collectively this group has been demonstrated to play important roles in patterning the rib cage and in neuronal cell fate

determination (Kostic and Capecchi, 1994; Rancourt et al., 1995; McIntyre et al., 2007; Mallo et al., 2010; Lacombe et al., 2013). *Hox6* genes are also expressed in the pancreatic mesoderm suggesting a possible role for *Hox6* in pancreas organogenesis.

In mouse, the pancreas is specified at approximately E9.5 and expands as a dorsal and ventral bud from the endoderm-derived gut tube into the surrounding mesoderm (Wells and Melton, 2000; Gittes, 2009; Puri and Hebrok, 2010). At E11.5, these buds fuse to become the dorsal and ventral regions of the single pancreas. The pancreas has two main components: an exocrine and endocrine component. The exocrine component is composed of digestive enzyme-secreting acinar cells and ductal cells. Ductal cells form a complex branching network that transports the digestive enzymes into the small intestine. The endocrine component is composed of five distinct types of endocrine cells which each secrete a single hormone: insulin, glucagon, somatostatin, ghrelin, or pancreatic polypeptide. Endocrine cell differentiation is initiated in the ductal epithelium by the expression of *Ngn3* in a sub-population of the epithelial cells (Gu et al., 2002). These Ngn3-positive (Ngn3+) cells subsequently delaminate from the ductal epithelium into the surrounding mesenchyme and further differentiate to the specific endocrine lineages. The bulk of endocrine cell differentiation occurs from E12.5-E15.5, which is termed the secondary transition. While all of the major functional components of the pancreas are derived from the endoderm, the surrounding mesodermally-derived mesenchyme is critical for the growth and development of these cell types (Golosow and Grobstein, 1962; Gittes et al., 1996; Miralles et al., 1998; Bhushan et al., 2001; Attali et al., 2007; Gittes, 2009; Puri and Hebrok, 2010).

Explant studies were first used to demonstrate the importance of the mesenchyme. When pancreas epithelium was cultured in the absence of its surrounding mesenchyme, both endocrine and exocrine development arrested with defects in growth and differentiation. These defects were rescued by recombination with pancreatic mesenchyme (Golosow and Grobstein, 1962; Wessells and Cohen, 1967). A more recent study in which the pancreas mesenchyme was genetically ablated *in vivo* showed a similar failure of all components of the pancreas to develop (Landsman et al., 2011).

Wnt signaling is critical for multiple aspects of pancreas development. A multitude of Wnt ligands, receptors, modifiers, and inhibitors have reported expression in both the epithelium and mesenchyme of the pancreas, with gene expression highest early in development and declining with organ maturation (Heller et al., 2002). Wnt has largely been studied in the pancreas through manipulation of required canonical Wnt signaling components such as β-catenin. Others have shown specific roles for individual *Wnt* ligands, and taken together, there are significant roles for both Wnt/ β-catenin signaling and the non-canonical Wnt planar cell polarity pathway for the proper development of both endocrine and exocrine pancreas (Heller et al., 2002; Kim et al., 2005; Murtaugh et al., 2005; Heiser et al., 2006; Attali et al., 2007; Wells et al., 2007; Jonckheere et al., 2008; Murtaugh, 2008; Baumgartner et al., 2014; Afelik et al., 2015).

While *Hox* genes have been shown to be important for the development of many organs of endodermal/mesodermal origin, a role for *Hox* genes in pancreas development has not been reported. Herein, we report that *Hox6* genes function in pancreatic organogenesis. *Hox6* genes are expressed only in the mesoderm of the

developing pancreas and not in the endoderm. The *Hox6* mutant pancreas buds normally and Ngn3+ endocrine progenitors are specified, but there is a >90% reduction of mature endocrine cells in the *Hox6* mutant pancreas compared to controls. Loss of *Hox6* function results in a decrease in *Wnt5a* expression in the pancreatic mesenchyme (though epithelial *Wnt5a* expression is unperturbed). This leads to a subsequent loss of expression of two Wnt inhibitors, *Sfrp3* (*Frzb*) and *Dkk1,* in endocrine progenitor cells. The addition of recombinant Wnt5a protein to pancreas explant cultures is sufficient to rescue endocrine cell differentiation in *Hox6* mutant pancreata. Thus, regional mesodermal patterning factors are critical for establishing the mesenchymal/epithelial crosstalk required for proper endocrine cell differentiation in pancreas development, highlighting the potential importance of considering the mesodermal niche in *ex vivo* beta cell differentiation protocols.

Results

Pancreas specification occurs normally in Hox6 mutants with mild defects in morphology and acinar differentiation

Hox6 aabbcc (lower case letters represent null alleles) mice do not survive post-natally, however, mutant embryos are indistinguishable in appearance (Fig. 2.1A) and mass (Fig. 2.1B) from littermate controls. Examination of internal organ defects reveals somewhat abnormal pancreas morphology in *Hox6* mutants compared to controls (Fig. 2.2A). The ventral pancreas (black arrowheads) and trunk of the dorsal pancreas (yellow arrowheads) are more compact in *Hox6* mutant pancreata compared to controls.

Despite mildly perturbed pancreas morphology, a normal expression pattern of acinar cell marker amylase (Amy) is observed in the mutant pancreas (Fig. 2.2B).

Early stages of pancreas initiation were examined by immunofluorescent staining for pancreas epithelial marker Pdx1. Quantification of immunofluorescent staining of the entire early pancreas (dorsal and ventral bud) shows that both pancreatic buds initiate normally and the volume of Pdx1-positive epithelium is unchanged between the control and *Hox6* mutant at E10.5 (Fig. 2.3A,B). A small decrease in epithelial volume was observed at E11.5 (Fig. 2.3A,D). Minor decreases in both epithelial and mesenchymal proliferation were measured at E11.5 and E14.5 (Fig. 2.3C,G), however no overall differences in mesenchyme volume were measured (Fig. 2.3E), and there is no overall change in pancreas mass at E18.5 regardless of *Hox6* genotype (Fig. 2.3H). There were no differences in apoptosis measured by cleaved Caspase-3 staining (Fig. 2.3I).

Examination of morphological defects at E14.5 by staining with E-cadherin (Ecad) reveals typical loose, lobular branching epithelium in the control pancreas, but more compact clusters of epithelial cells with less branching in the mutant pancreas (Fig. 2.2C). Branch pattern was further analyzed at E14.5 using Muc-1 antibody staining in whole pancreata (Villasenor et al., 2010). *Hox6* null pancreata exhibit impaired branching and apparent defects in the remodeling of the early ductal plexus (Fig. 2.2D). Many thin, single lumens have resolved throughout the control pancreas while these lumens are less apparent in the mutant (Fig. 2.2D, red arrowheads). Bright, dense staining for Muc-1, indicating acinar clusters, is found readily throughout both control and *Hox6* null pancreata (Fig. 2.2D, white arrowheads).

Prior to E13.5, the “tip” acinar cells of the pancreas are multipotent progenitor cells (MPCs) and can produce exocrine, ductal, and endocrine cells. All epithelial cells at these early stages express *Sox9* and *Hnf1β*. After E13.5, as *Sox9* and *Hnf1*β are down regulated in tip cells, these cell types become unipotent and only give rise to acinar cells (Zhou et al., 2007; Kopp et al., 2011). In contrast to controls, *Hox6* mutant pancreata demonstrate continued Sox9 and Hnf1β in Cpa1-positive tip cells at E14.5 (Fig. 2.2E,F). This is resolved by E16.5, consistent with the eventual differentiation and maturation of MPCs into mature exocrine acinar cells. This defect may be related to the perturbed morphology and branch pattern observed in mutants.

Endocrine differentiation is inhibited in Hox6 mutant pancreata

The mild defects in branching and in exocrine differentiation are in contrast to a dramatic reduction of all five mature endocrine cell hormones in *Hox6* mutant pancreata (Fig. 2.4A,B). Antibody staining for insulin (Ins) and glucagon (Gcg) reveals a dramatic reduction of both endocrine protein and mRNA expression of all five endocrine hormones at E14.5 (Fig. 2.4A). This decrease is more pronounced by newborn stages with *Ins* and *Gcg* mRNA expression reduced to less than 5% of control values (Fig. 2.4B).

All five endocrine cell types derive from Ngn3+ cells that arise from the ductal epithelium. During pancreatic development, sporadic ductal cells express *Ngn3,* initiating a delamination process and allowing these cells to migrate into the surrounding mesenchyme where they mature into the five types of hormone-producing cells. We investigated the cellular defects leading to the dramatic decrease of endocrine cells in

the mutant by examining the initiation and differentiation of this cell type. There is no change in the amount of Ngn3+ immunofluorescent staining (Fig. 2.5A) or expression of *Ngn3* mRNA by qRT-PCR (Fig. 2.5D) in the mutant pancreas compared to controls. There were also no measured changes in expression of *Nkx6.1* or *Nkx2.2* (Fig. 2.5D), however, these genes are expressed more broadly in the epithelium (Sussel et al., 1998; Schaffer et al., 2010). There are significant reductions of pan-endocrine markers ChgA and Isl1 protein and mRNA at E14.5 (Fig. 2.5B-D). The mRNA expression of endocrine lineage genes *MafA, MafB,* and *NeuroD* are also significantly reduced (Fig. 2.5D). Despite the apparent loss of endocrine differentiation, Ngn3 staining in controls and mutants is comparable, suggesting that Ngn3+ progenitor cells do not accumulate in *Hox6* mutants through E18.5 (Fig. 2.6).

In the first phase of endocrine differentiation, sporadic Sox9-positive (Sox9+) ductal cells begin to additionally express *Ngn3,* allowing these cells to delaminate from the epithelium while concomitantly turning off *Sox9* expression (Gouzi et al., 2011; Shih et al., 2012). We examined this process by antibody staining control and *Hox6* mutant pancreata for Ngn3 and Sox9. Immunostaining reveals that about 25% of Ngn3+ cells are also Sox9+ in control pancreata at E14.5 (Fig. 2.5E, yellow arrows). This ratio is identical in the *Hox6* mutant pancreas, providing evidence that endocrine progenitor cells are able to adequately transition from Sox9+/Ngn3+ ductal cells into Sox9-/Ngn3+ early endocrine cells (Fig. 2.5E). Ngn3+ cells that have successfully migrated from the duct are observed throughout the control and in the *Hox6* mutant pancreas (Fig. 2.5E, white arrows), further supporting a differentiation defect post-specification and delamination.

Hox6 genes are expressed in the mesoderm of the pancreas during development

All three *Hox6* genes are expressed in the embryonic pancreas with expression highest early in development, persisting through E16.5 (Fig. 2.7A). Expression was below our limit of detection for all three *Hox6* genes at E18.5 (Fig. 2.7A). *In situ* hybridization analyses of *Hox6* mRNA reveal expression exclusively in the pancreas mesoderm at E11.5 and E12.5 (Fig. 2.7B). Mesodermally restricted expression was confirmed using a previously reported *Hoxb6*-inducible Cre reporter line (*Hoxb6CreER^{T};Rosa26-tdTomato*) (Nguyen et al., 2009; Madisen et al., 2010). Administration of tamoxifen at early stages (E9.5 and E10.5) reveals an anterior expression limit at the level of the budding dorsal and ventral pancreas, and also marks the majority of mesoderm posterior to this in the embryo (Fig. 2.7C).

Expression from the ROSA locus in these mice is observed throughout the pancreatic mesenchyme, but is completely excluded from early Ins-positive endocrine cells, Pdx1-positive pancreas endoderm, and PECAM-positive vasculature (Fig. 2.7C). As endocrine cells undergo epithelial to mesenchymal transition prior to differentiation, it was important to exclude possible initiation of *Hox6* expression in endocrine cells during the secondary transition. To test this, tamoxifen was administered to pregnant dams at E12.5, E13.5, and E14.5 and embryos were examined at E15.5. We found no Cre activity in Ngn3+ endocrine progenitors (Fig. 2.8A), with the pan-endocrine marker ChgA (Fig. 2.8B) or with epithelium (Fig. 2.8C). Similar results were obtained with tamoxifen administration daily from E10.5 to E17.5. There was no overlap of tdTomato with ChgA-positive endocrine cells, pancreatic epithelium, endothelial, neuronal or

smooth muscle cells (Fig. 2.7D and Fig. 2.8D, E). We observed extensive and complete co-labeling of tdTomato and Vmtn-positive mesenchyme (Fig. 2.7D), confirming *Hox6* expression is exclusive to the pancreatic mesoderm-derived mesenchyme.

Vasculature is normal in the Hox6 null pancreas

It has been previously reported that hyper-vascularization affects pancreas organogenesis by reducing branching and differentiation of epithelial cells, and signals from the vasculature are also important for the proper formation of endocrine cells of the pancreas (Lammert et al., 2001; Magenheim et al., 2011). Immunofluorescence staining for PECAM at E14.5 (Fig. 2.9A) and E18.5 (Fig. 2.9B) reveals no difference in vasculature between the control and mutant pancreas. There are also no differences in the association of endothelial and insulin-positive endocrine cells between control and *Hox6* null E18.5 pancreas (Fig. 2.9C).

Wnt signaling is disrupted in the mesoderm and differentiating endocrine cells in Hox6 mutant pancreata

Microarray analysis was performed on E12.5 control and *Hox6* mutant pancreata to probe for the molecular mechanism(s) responsible for the *Hox6* mutant phenotypes (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68390). ToppFun (https://toppgene.cchmc.org/enrichment.jsp) analysis identified changes in members of the Wnt receptor-signaling pathway as significantly enriched in the *Hox6* mutant pancreas, but no significant changes in other major signaling pathways.

To confirm our microarray results, we used qRT-PCR analysis to measure the expression levels of genes from major signaling pathways with reported or measured expression in the pancreas. No changes in the levels of Fgf, Bmp or Notch pathway members were measured in E12.5 mutant pancreata (Fig. 2.10A). There were also no differences in the measured expression of Wnt receptors between control and *Hox6* mutant pancreata (Fig. 2.10B). There are no differences in expression of *Wnt2b, Wnt4, Wnt7b,* or *Wnt11*, other Wnt ligands expressed in pancreas at E12.5, between mutant and control pancreata (Fig. 2.10B)(Heller et al., 2002), and we were unable to detect expression of *Wnt1* and *Wnt8b,* genes reported to be expressed in the mesenchyme later in development, in either the control or *Hox6* mutant pancreas at E12.5 (data not shown) (Heller et al., 2002). However, we observed significantly reduced expression levels of mesodermally expressed Wnt ligand, *Wnt5a,* at both E12.5 and E14.5 (Figs. 2.10B,C).

Hox6 genes are expressed solely in the mesoderm and therefore we examined mesodermally expressed Wnt genes as possible direct targets of *Hox6*. *Wnt5a* is expressed in both the pancreas epithelium and mesenchyme (Heller et al., 2002). *In situ* hybridization analyses of *Wnt5a* revealed much weaker expression in the *Hox6* mutant pancreas mesenchyme at E12.5 compared to controls (Fig. 2.10D). At E14.5, reduced *Wnt5a* expression is even more apparent in the *Hox6* mutant pancreas mesenchyme compared to controls, while expression in the epithelium appears identical (Fig. 2.10E). At E14.5 and beyond, we measure a significant reduction of more Wnt receptors and ligands as well, suggesting a secondary perturbation of Wnt signaling more globally in the *Hox6* mutant pancreas at later stages (Fig. 2.10C). *Wnt2b, Wnt4, Wnt7b* and *Wnt11*

were also examined by ISH however, no obvious changes in expression pattern were detected between the control and *Hox6* mutant pancreas (Fig. 2.11A-D).

We reasoned that this disruption in Wnt signaling in the mesoderm might lead directly to changes in the Ngn3-expressing endocrine precursors after delamination from the ductal epithelium. The expression of a number of Wnt inhibitors has been reported in the pancreas epithelium and in endocrine cells throughout development including *Sfrp1-4, Dkk1-3,* and *Wif1* (Heller et al., 2002; Gu et al., 2004). Of the Wnt inhibitors examined in the pancreas just two, *Sfrp3* and *Dkk1,* showed significantly reduced expression levels in the *Hox6* mutant at E12.5 (Fig. 2.12A). Using immunofluorescent staining with antibodies to Sfrp3 and Dkk1, we observe extensive overlap of Sfrp3 and Dkk1 with endocrine cells in wild-type pancreata (Fig. 2.12B). Overall, *Hox6* mutant pancreata exhibited drastically reduced levels of Sfrp3 and Dkk1 (Fig. 2.12C, D). Close examination reveals co-localization of Sfrp3 and Dkk1 in a subset of Ngn3+ endocrine progenitor cells in the control and none in the *Hox6* mutant pancreas (Fig. 2.12E, F). This reduction of Wnt inhibitors continues through later stages of pancreas development (Fig. 2.13).

Wnt5a is the first mesodermally expressed Wnt ligand with disrupted expression in the *Hox6* mutant pancreas. If the lack of endocrine cell differentiation in the *Hox6* mutant pancreas stems from the lack of a sufficient amount of Wnt5a signaling from the mesoderm, we reasoned that it might be possible to rescue this phenotype with the addition of exogenous Wnt5a to the *Hox6* mutant pancreata. To test this, we dissected control and *Hox6* mutant pancreata at E12.5 and cultured them for six days in the presence and absence of exogenous Wnt5a (Fig 2.14A). Without exogenous Wnt5a in

the culture, we observed significant reduction of mature endocrine cells in the *Hox6* mutant pancreata compared to controls, similar to the *in vivo* phenotype (Fig. 2.14B and C). When recombinant Wnt5a protein was added to the culture media of *Hox6* mutant pancreata, the quantity of mature endocrine hormone staining was rescued to the same level as control pancreata (Fig. 2.14B and C).

Discussion

While the importance of the mesenchyme in pancreas development was demonstrated decades ago, the molecular mechanisms involved in the crosstalk between the epithelium and the mesenchyme are poorly understood. Here we report a novel role for *Hox6* genes in pancreas development as diagrammed in the model in Figure 2.18. Loss of *Hox6* function in the pancreatic mesoderm leads to mild defects in branching and delayed exocrine cell differentiation however, the pancreas achieves its normal size by newborn stages with extensive acinar cell formation. Endocrine cells are specified normally, but immature endocrine cells do not mature, resulting in a dramatic reduction of all types of fully differentiated endocrine cells, including >90% decreases in insulin and glucagon expression.

Specific components of the Wnt signaling pathway are disrupted by E12.5 while no changes in other critical developmental signaling pathways, including Fgf, Bmp, and Notch, are observed. Mesenchymal ligand expression of *Wnt5a* is significantly down-regulated (or absent) while epithelial expression of this ligand appears unperturbed. *Wnt5a* is expressed from E11 to the end of gestation with peak levels of expression at E12, similar to our measured *Hox6* gene expression profile (Heller et al., 2002). A study

examining MO knock-down of *Wnt5a* and *Fz-2* in zebrafish and global loss-of-function of *Wnt5a* in mice reported defects in islet formation in both organisms (Kim et al., 2005), consistent with loss of *Wnt5a* expression in the mesoderm contributing to the endocrine phenotype in our *Hox6* mutant animals.

In addition to loss of *Wnt5a* signaling in the pancreatic mesoderm, which may result directly from loss of *Hox6* function, there is a subsequent loss of expression of Wnt inhibitors, *Sfrp3* and *Dkk1,* specifically in delaminated Ngn3+ endocrine precursor cells as they are entering the mesoderm. As previous studies have demonstrated the importance of repressing Wnt signaling in developing endocrine cells (Pedersen and Heller, 2005), it is likely that loss of Wnt inhibitor induction in Ngn3+ progenitors leads directly to loss of further endocrine cell differentiation in *Hox6* mutant pancreata. Pharmacological treatment of *Hox6* mutant pancreata with exogenous Wnt5a protein is sufficient to restore endocrine cell differentiation, and demonstrates that Wnt5a is a critical mediator downstream of *Hox6* genes in the pancreatic mesenchyme during development. Collectively, our results suggest that *Hox6* genes are critical for the establishment of the Wnt mesenchymal-epithelial crosstalk necessary for pancreas development and endocrine cell specification.

Hox genes are important for many aspects of development and organogenesis, but no disruption of *Hox* function has previously been implicated in pancreas development. This work contributes to the growing understanding of pancreatic mesoderm signaling and the important roles the mesoderm plays in the development of both the endocrine and exocrine components of the pancreas. Here we show a direct link between *Hox* function and Wnt signaling; a theme that is reminiscent to a recent

report of loss of *Wnt2/2b* expression in the mesenchyme of *Hox5* triple mutant lungs during development (Hrycaj et al., 2015). Another recent study examining *Hoxd13* function in digit development reports that *Hoxd13* promotes expression of *Wnt5a in vitro* (Kuss et al., 2014). All Hox proteins bind to the same –ATTA- binding sequence and therefore it is plausible that *Wnt5a* is a direct target of Hox proteins during development. Future work will be required to establish possible direct regulation of *Wnt5a* by *Hox6*.

This study adds to growing evidence that *Hox* function in the mesoderm of several organ systems plays region-specific roles associated with the establishment of proper Wnt signaling crosstalk during organogenesis (Kuss et al., 2014; Hrycaj et al., 2015). A more complete elucidation of mesodermal-endodermal crosstalk during pancreas development is critical to the enhancement of *ex vivo* protocols for generating functional beta cells as a cellular therapy for the treatment of diabetes.

Materials and Methods

Generation of mouse mutants

Mice mutant for all three *Hox6* paralogous genes were generated using standard genetic crosses (Kostic and Capecchi, 1994; Rancourt et al., 1995; Garcia-Gasca and Spyropoulos, 2000). Hoxb6CreER^{T} mice were contributed by Dr. Susan Mackem (Nguyen et al., 2009). R26^{Tom} mice were obtained from The Jackson laboratory (Madisen et al., 2010). All experiments were performed following protocols approved by the University of Michigan's Institutional Committee on the Use and Care of Animals.

Tamoxifen injections

Tamoxifen and progesterone were dissolved in 100% ethanol and diluted in corn oil. Pregnant dams were given intraperitoneal injections with 1.5 mg of tamoxifen and 0.75 mg of progesterone on the days noted in Results.

In situ hybridization

For section *in situ* hybridization (ISH), embryos were collected in PBS and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Embryos were then rinsed in PBS and immersed in 30% sucrose at 4°C overnight before embedding into optimal cutting temperature (OCT) media. Frozen sections 20 µm in size were cut, and slides were stored at −80°C. Section ISH was performed as previously described (Mendelsohn et al., 1999; Di Giacomo et al., 2006). Detection of *Hox6* mRNA was done using probes generated against the 3' UTR of *Hoxa6, Hoxb6,* and *Hoxc6* or against the *Neo*^{r} cassette as previously described and with indistinguishable results from probes against *Hox6* gene mRNA (McIntyre et al., 2007). *Wnt5a* cDNA was ligated into PCR4-TOPO vector and reverse transcribed with T3 RNA polymerase. Sequenced plasmid aligns to mouse *Wnt5a:* GeneBank gi 46909566/NM 009524.2 from bp 406 to 1440. *Wnt2b, Wnt4, Wnt7b* and *Wnt11* ISH was performed with previously published riboprobes (Miller et al., 2012; Soofi et al., 2012; Ranghini and Dressler, 2015).

Immunofluorescent Staining

Mouse embryos were collected as described above. Frozen sections 12 µm in size were cut, and slides were stored at −80°C. Slides were blocked for one hour at room temperature in 0.1% or 0.5% Triton X-100 in PBS (PBS-T) with 1% donkey serum

and treated with primary antibody overnight at 4°C. On day two slides were washed in PBS-T, incubated with secondary antibody for two hours at room temperature, followed by a 10-minute wash in PBS-T with DAPI (Sigma Aldrich). Coverslips were added to the slides using Prolong Gold Antifade Reagent (Invitrogen). Primary and secondary antibodies used are listed in Table 2.1. Slides were imaged using either an Olympus BX-51 or Leica SP5X 2-Photon Confocal.

Whole mount Muc-1 staining

Tissue was fixed in 4% PFA for three hours at 4°C, dehydrated in MeOH and stored at

-20°C until stained. Fixed tissue was treated with Dent's bleach (MeOH: DMSO: H_2O_2, 4:1:1) for 2 hours at room temp, blocked with TNB (Perkin Elmer) and incubated overnight with anti-Muc-1 antibody in TNB at 4°C. Pancreata were then washed with PBS and incubated with secondary antibody in TNB overnight at 4°C. Pancreata were imaged in BABB (benzyl alcohol: benzyl benzoate, 1:2) on a Leica SP5X Inverted 2-Photon FLIM Confocal. Confocal Z-stacks were reconstructed using ImageJ. Antibodies are listed in Table 2.1.

RNA Isolation and Quantitative RT-PCR

RNA was isolated from mouse pancreata with the Qiagen RNeasy Micro Kit. Quantitative RT-PCR (qRT-PCR) was carried out using Roche FastStart SYBR Green Master Mix and the Applied Biosystems StepOnePlus Real-time PCR system (Life Technologies). Relative expression values were calculated as $2^{-\Delta\Delta Ct}$ and values of controls were normalized to 1. Rn18s served as an internal control for normalization in

all qRT-PCR experiments. All data are shown as the mean of at least three independent biological replicates; error bars represent s.e.m. Calculations and *P*-values (two-tailed, unpaired *t*-test) were generated in Microsoft Excel. Results were considered statistically significant at $P < 0.05$. Graphs were generated using Prism 6. Primer sequences are listed in Table 2.2.

Pancreas explant cultures and rescue

Pancreata were dissected at E12.5 and cultured at the air-media interface on a Nucleopore Track-Etched Membrane (Whatman) in DMEM / F12 with L-Glutamine and 15mM HEPES (Gibco) supplemented with Pen/Strep (Gibco). For rescue, the media was supplemented with 500ng/mL Recombinant Human/Mouse Wnt5a (R&D Systems) and refreshed with new media and Wnt5a protein on day two of culture. After six days in culture, pancreata were fixed at room temperature in 4% PFA for three hours, immersed in 30% sucrose at 4°C overnight and frozen in OCT the next day. Pancreas explants were cryosectioned at 12 μm and stained as described above for Ins/Gcg. ImageJ software was used to calculate the area of signal for DAPI and Ins/Gcg. Quantification is displayed as total Ins and Gcg signal / total DAPI signal for each explant.

Acknowledgments

We thank Dr. Susan Mackem for contributing Hoxb6CreER^T mice. We would also like to thank Dr. Ondine Cleaver for sharing the Muc-1 whole mount staining protocol and Dr. D. Gumucio, Dr. Å. Kolterud, and Dr. Kate Walton for the *Wnt5a* ISH probe. Dr. Gregory Dressler provided riboprobes for *Wnt4* and *Wnt11*. Dr. J. Spence provided several qRT-PCR primers. We thank Holly Fischer for model artwork. Bright Kim

provided technical assistance on parts of this work. The PECAM antibody developed by Steven A. Bogen was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. The NIH Cellular and Molecular Biology Training Grant T32- GM007315 and the Training Program in Organogenesis T32HD007505 supported BML. This work was supported by The American Diabetes Association ADA-7-13-BS-184, The Michigan Diabetes Research and Training Center Pilot and Feasibility Award, and by the National Heart, Lung, and Blood Institute (NHLBI) R01-HL119215. A Ruth L. Kirschstein National Research Service Award (NSRA) training Grant 5 T32 HL 7749-20 and a National Center for Research Resources grant number UL1RR024986 supported SMH.

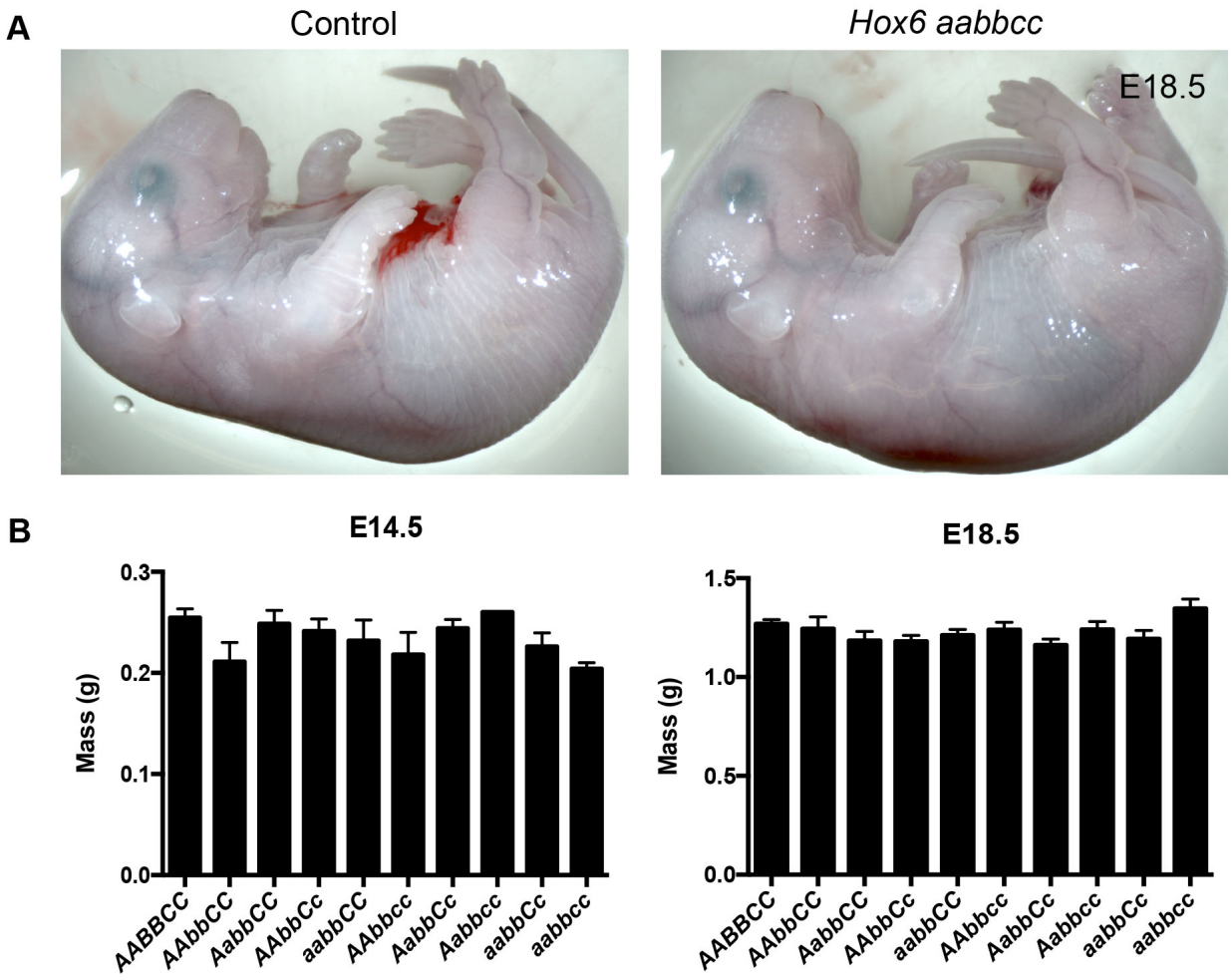

Figure 2.1. *Hox6* mutant embryos are externally indistinguishable from controls. (A) Dissected control and *Hox6* mutant embryos at E18.5. (B) Embryos were weighed at E14.5 and E18.5. There is no change in embryos mass at E14.5 or E18.5 regardless of *Hox6* mutation.

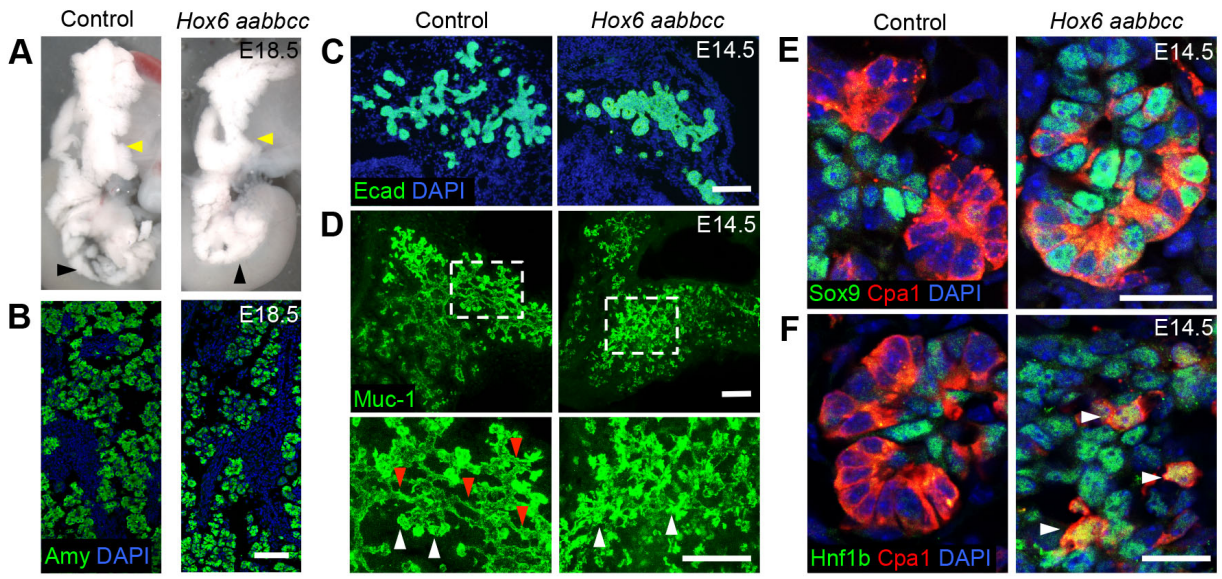

Figure 2.2. Loss of *Hox6* function results in abnormal pancreas morphology, but normal amylase expression.
(A) E18.5 dissected pancreata from control and *Hox6 aabbcc* embryos. *Hox6* null pancreata show abnormal pancreas morphology. Black arrowheads show compact ventral region of the pancreas in the mutant. Yellow arrowheads show narrower, more compact dorsal region with less lateral branching compared to controls. (B) Immunofluorescent staining for Amy (green) in sectioned E18.5 control and *Hox6 aabbcc* pancreata shows normal amylase patterns.
(C) Immunofluorescent staining for Ecad (green) shows that the epithelium of the *Hox6* null pancreas is more compact with less branching than the control pancreas at E14.5.
(D) Whole mount immunofluorescence for Muc-1 (green) at E14.5 shows that the control has a well-defined multi-lumen plexus whereas the *Hox6* null pancreas is less developed. Close up images show both ductal (red arrowheads) and tip (white arrowheads) regions are readily visible in the control pancreas however there are fewer ductal lumens apparent in *Hox6* mutant. (E) Immunofluorescent staining for Sox9 (green) and Cpa1 (red) shows no co-localization in control tip cells at E14.5 but many Cpa1/Sox9 double positive cells in the *Hox6* null pancreas.
(F) Immunofluorescent staining for Hnf1β (green) and Cpa1 (red) shows no co-localization in control tip cells at E14.5 but Cpa1/Hnf1β double positive cells in the *Hox6* null pancreas. Arrowheads indicate double positive cells. Scale bars, 100 μM (B and C); 50 μM (D); 20 μM (E); 15 μM (F).

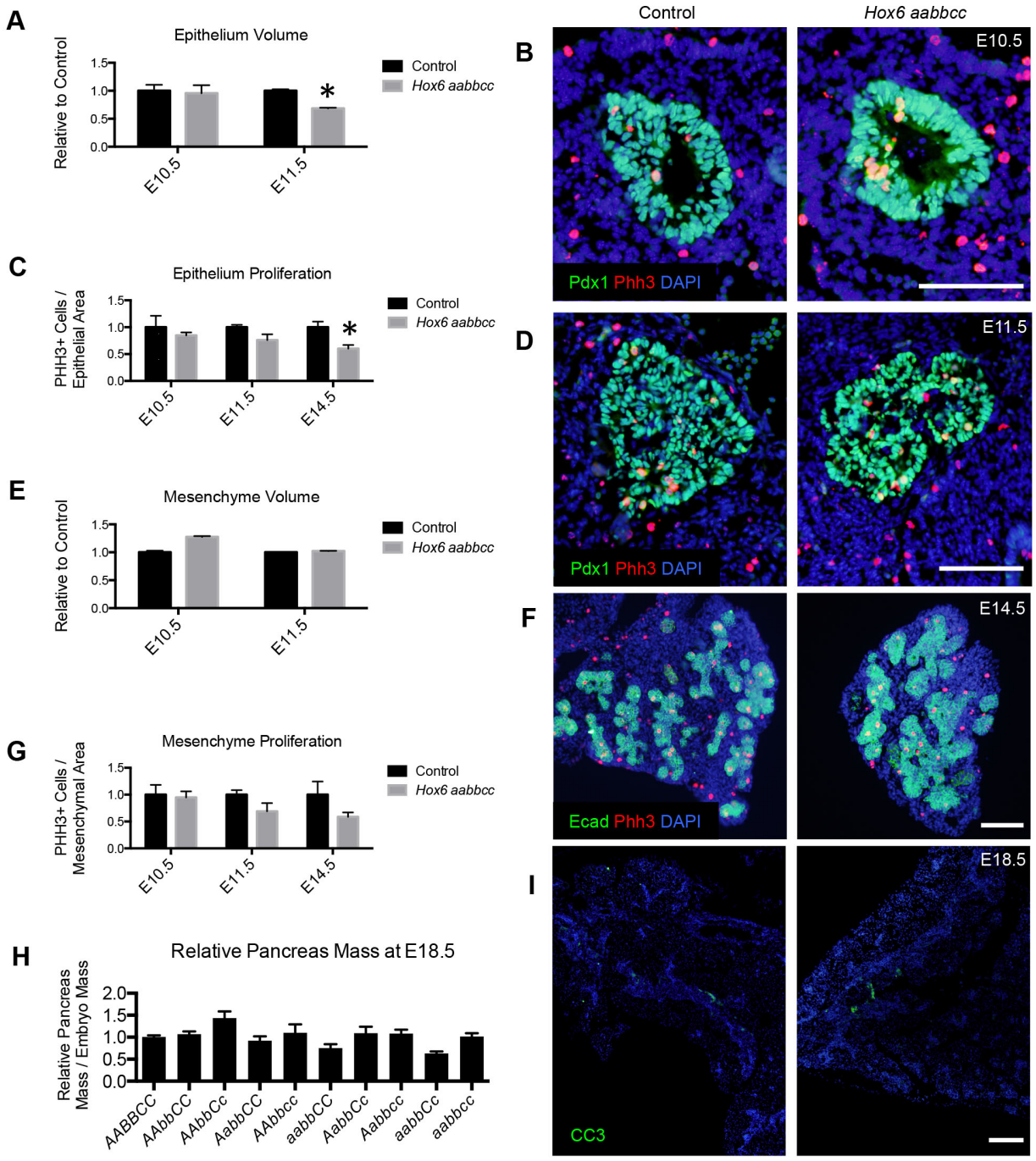

Figure 2.3. *Hox6* mutant pancreata have slightly reduced proliferation, but no overall changes in volume.
(A-G) Epithelial volume by Pdx1 staining at E10.5 (B) and E11.5 (D) quantified relative to control (A). Every section was imaged and the area of Pdx1+ staining was calculated with ImageJ to measure total volume. Epithelium proliferation was measured by

counting the number of Phh3+Pdx1+ cells and dividing by the Pdx1 volume calculated in panel A. At E14.5 the number of Phh3 cells in the epithelium was divided by Ecad+ volume calculated similarly to Pdx1 volume (C and F). There are no significant changes in mesenchyme volume (E) or proliferation (G). (H) There are no changes in pancreas mass regardless of *Hox6* mutation. Pancreata were weighed and divided by embryo mass. (I) Apoptosis was detected by antibody staining for CC3 at E18.5. There are no differences between control and mutant pancreata. Results are mean±s.e.m. **P*<0.05 calculated by Student's *t*-test (n≥3). Scale bars, 100 μM in (B, D, F, and I).

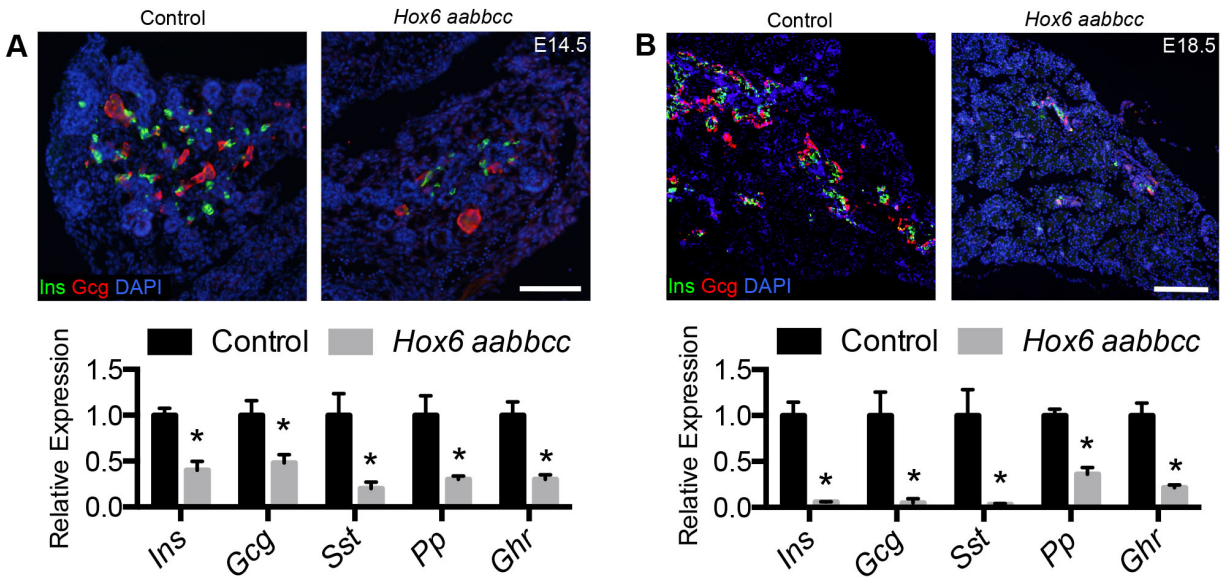

Figure 2.4. *Hox6* mutant pancreata demonstrate a drastic reduction of endocrine hormone expression.
(A) Immunofluorescent staining for Ins (green), Gcg (Red) and nuclei (blue) was performed at E14.5. qRT-PCR shows reduced expression of all five endocrine cell hormones at E14.5 in the mutant pancreas. (B) Immunofluorescence shows decreased staining for Ins (green) and Gcg (red) in the mutant pancreas at E18.5. There is a dramatic reduction of all five endocrine cell hormones at E18.5 in the mutant. Results are mean±s.e.m. **P*<0.05 calculated by Student's *t*-test (n≥3). Scale bars, 100 μM (A and B).

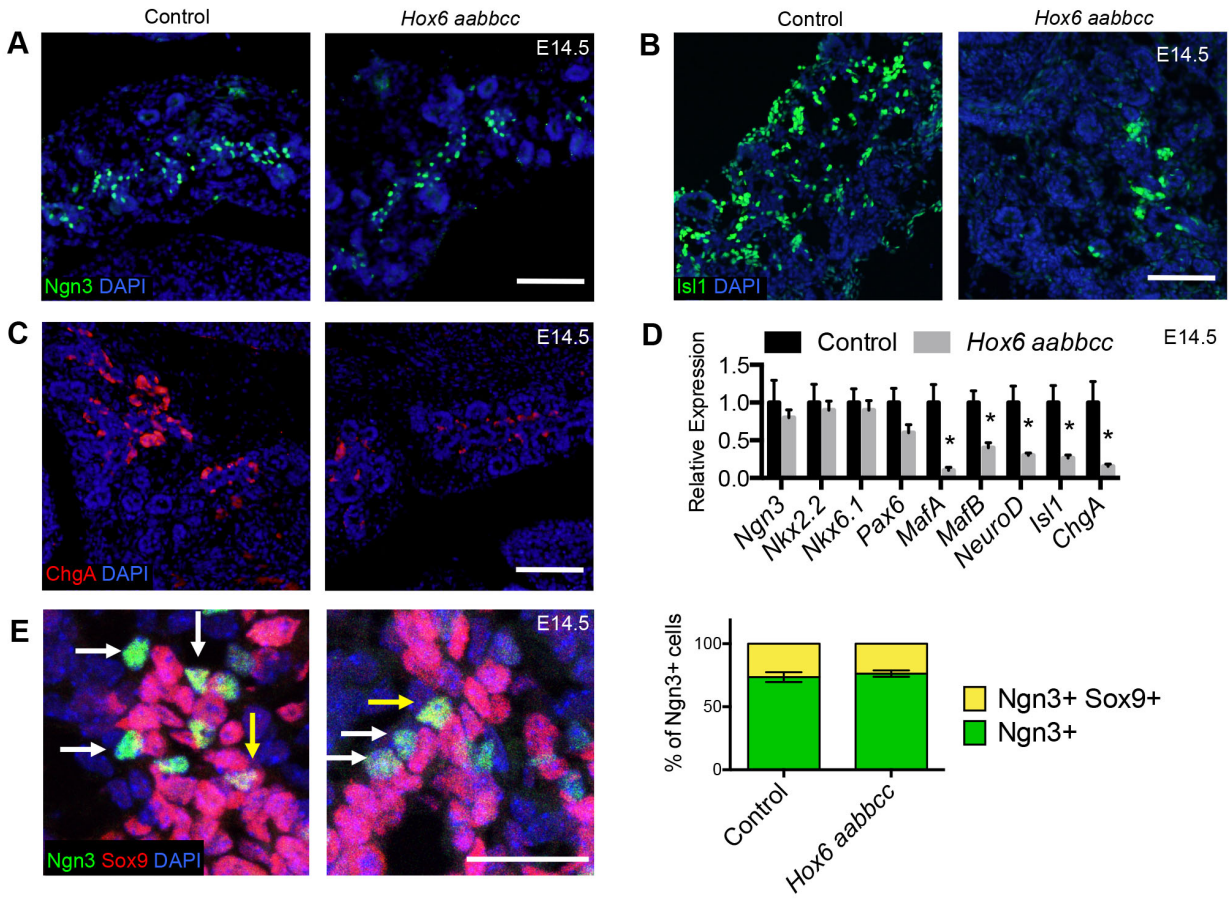

Figure 2.5. Immature endocrine cell genes are decreased in the mutant but there is no defect in endocrine progenitor delamination.
(A) Immunofluorescent staining for Ngn3 (green) and nuclei (blue) shows no change between control and *Hox6* null pancreata at E14.5. (B-C) Immunofluorescent staining shows a decrease in signal of both Isl1 (B) and ChgA (C). (D) qRT-PCR analyses show no change in the expression of *Ngn3, Nkx2.2, Nkx6.1* or *Pax6*, but there are significantly reduced levels of expression of *MafA, MafB, NeuroD, Isl1,* and *ChgA* in the *Hox6* mutant pancreas at E14.5. (E) Immunofluorescent staining for Sox9 (red) and Ngn3 (green) at E14.5 in control and *Hox6 aabbcc* pancreata. Quantification of Sox9+ and Ngn3+ cells shows that 25% of Ngn3+ (white arrows) cells in both the control and *Hox6* null pancreas are also Sox9+ (yellow arrows). Results are mean±s.e.m. **P*<0.05 calculated by Student's *t*-test (n≥3). Scale bars, 100 μM (A-E).

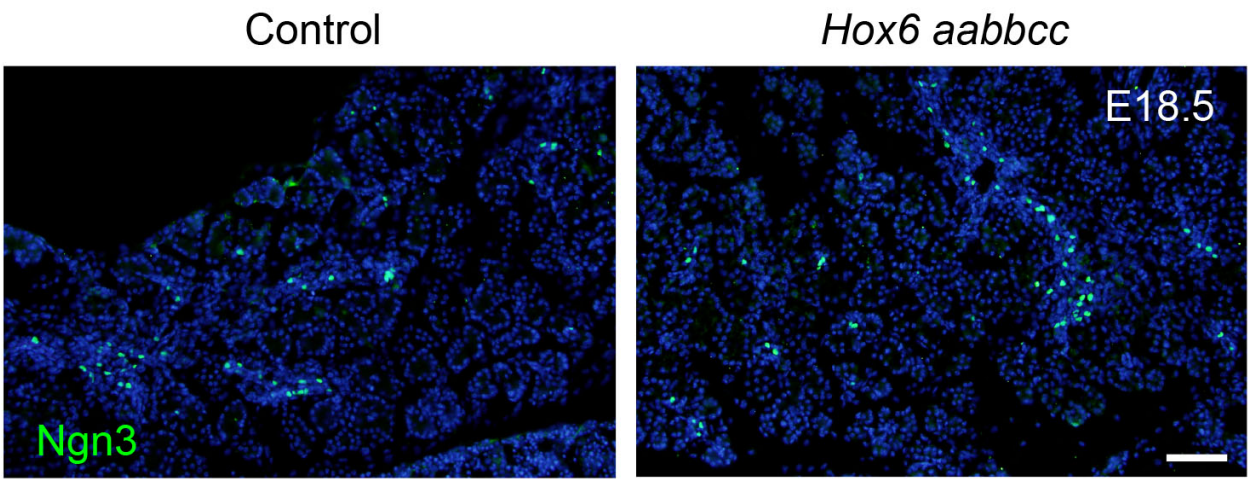

Figure 2.6. Ngn3 is unchanged at E18.5.
Immunofluorescence for Ngn3 shows no change between control and *Hox6* mutant pancreata at E18.5. Scale bar, 100 μM.

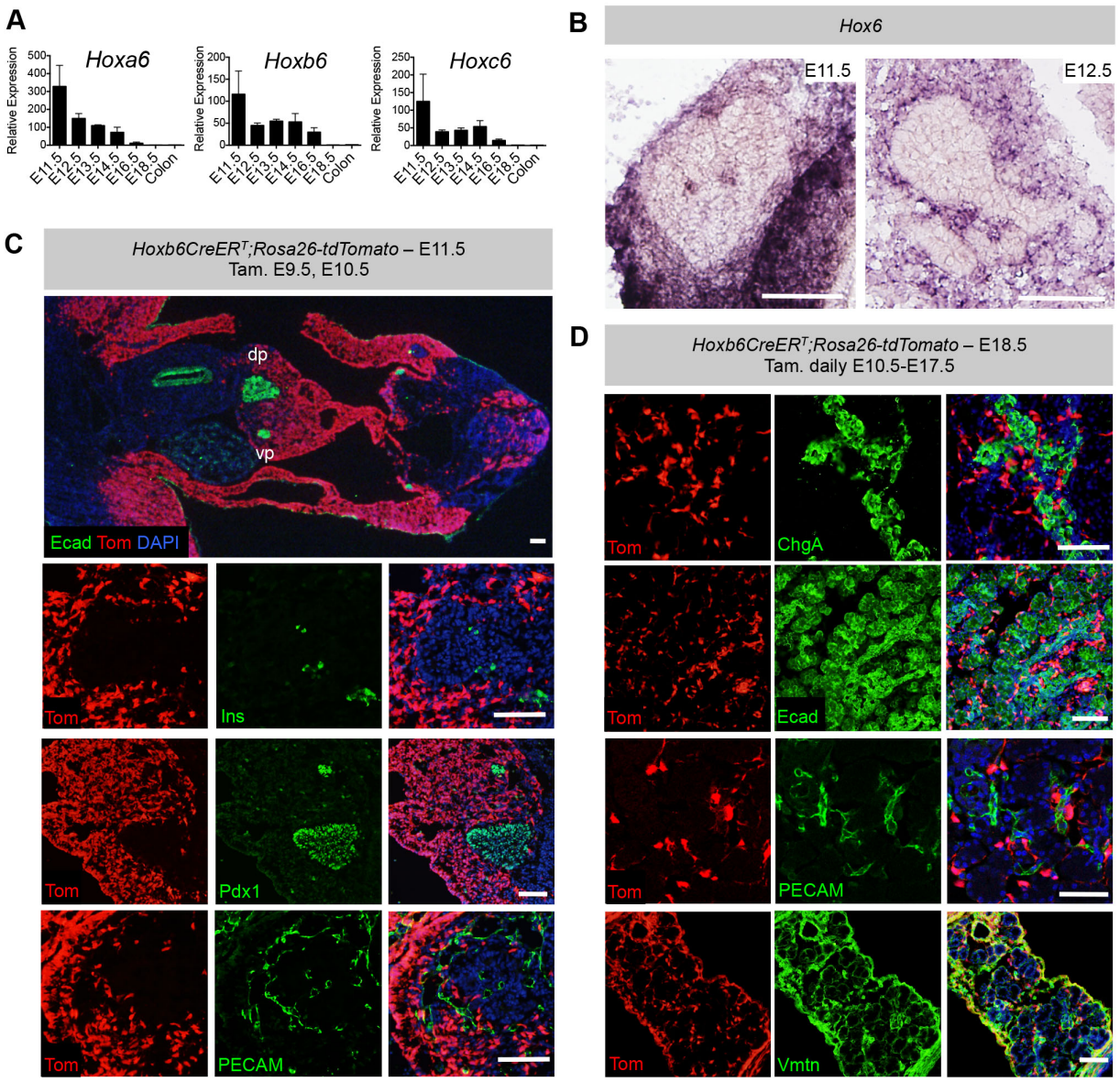

Figure 2.7. Hox6 expression is limited to the pancreas mesenchyme.
(A) qRT-PCR analysis shows expression of *Hoxa6, b6,* and *c6* in the pancreas throughout gestation with expression of all three genes highest early in development. No expression above the limit of detection was measured for any of the three *Hox6* genes at E18.5. Values are relative to the colon where there is a low level of *Hox6* expression. (B) ISH shows combined *Hox6* gene expression in the mesenchyme at E11.5 and E12.5. (C) *Hoxb6CreER*^{T} and *Rosa26-tdTomato* mice were crossed and tamoxifen was administered to pregnant dams via IP injection at E9.5 and E10.5 to examine reporter expression in the pancreas. Cre activity (red) was detected at an anterior limit in the lateral plate mesenchyme at the level of forelimb; the anterior limit in the somatoplueric mesenchyme is at the level of the pancreatic buds. The dorsal pancreas (dp) and ventral pancreas (vp) exhibits strong td-tomato (red) signal

throughout the mesenchyme. Epithelium is stained with Ecad (green). Cre activity is also excluded from Ins+ cells, Pdx1+ cells, and PECAM+ endothelium in the pancreas. (D) Daily tamoxifen administration to pregnant dams via IP injection from E10.5 to E17.5 shows reporter expression excluded from ChgA+ endocrine cells, Ecad+ epithelial cells, and PECAM+ endothelial cells in *Hoxb6CreER^{T};Rosa26-tdTomato* embryos. Cre activity is detected throughout the pancreas mesenchyme as co-labeled with Vmtn. Scale bars, 100 μM (B-D). Results are mean±s.e.m (n≥3).

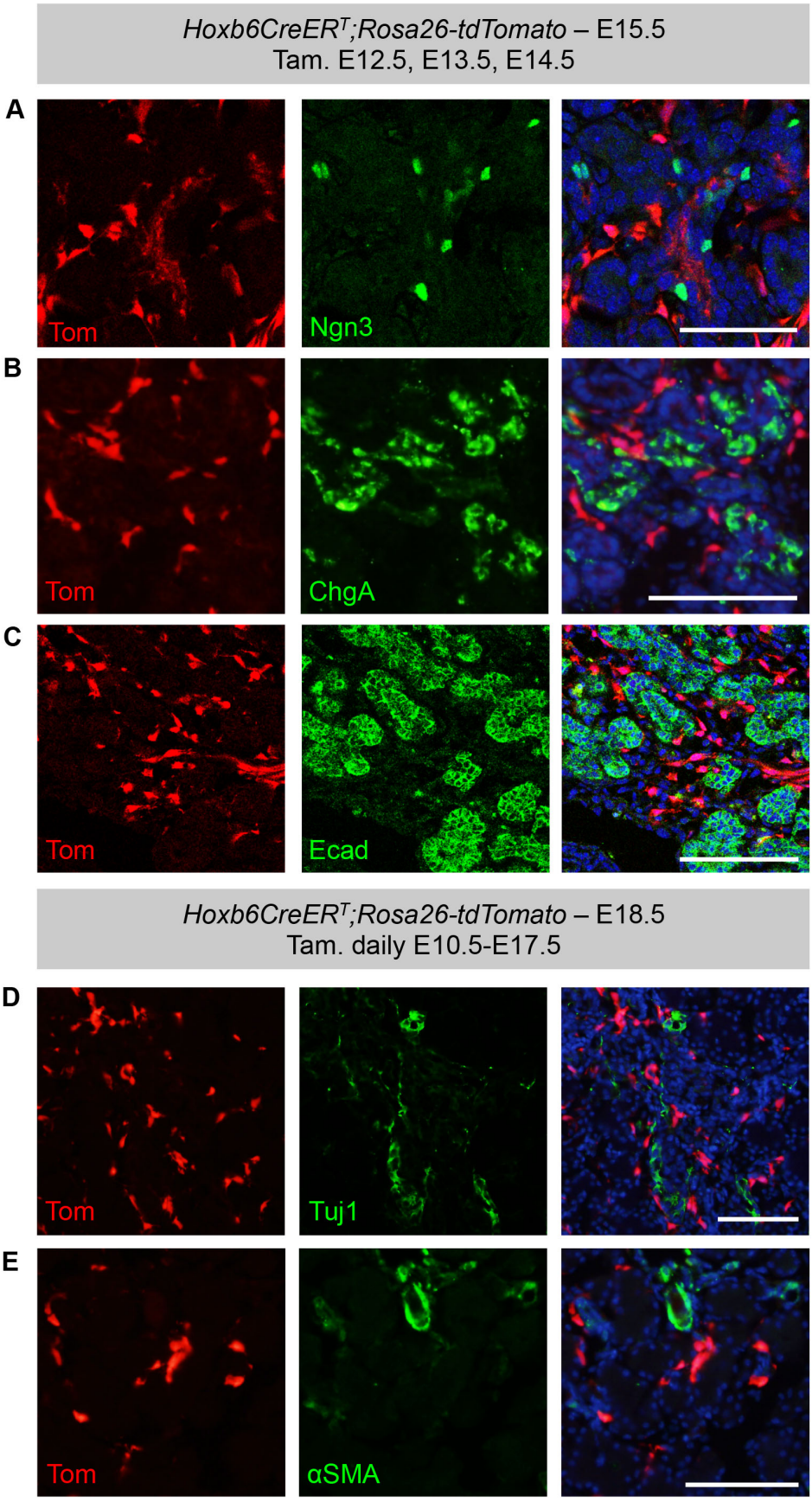
Hoxb6CreER^T;Rosa26-tdTomato – E15.5
Tam. E12.5, E13.5, E14.5
A
Tom
Ngn3
B
Tom
ChgA
C
Tom
Ecad
Hoxb6CreER^T;Rosa26-tdTomato – E18.5
Tam. daily E10.5-E17.5
D
Tom
Tuj1
E
Tom
αSMA

Figure 2.8. *Hox6* gene expression is restricted to the mesenchyme.
(A-C) Tamoxifen was injected at E12.5, E13.5, and E14.5. At E15.5 td-tomato (red) is excluded from Ngn3+ endocrine progenitor cells (A), ChgA+ endocrine cells (B) and the epithelium (C). To assess if *Hox6* expression could be detected in neurons or smooth muscle, tamoxifen was injected daily from E10.5 to E17.5 and embryos were analyzed at E18.5. There is no Cre recombination in Tuj1+ neurons (D) or αSMA+ smooth muscle (E). Scale bars, 100 μM (A-E).

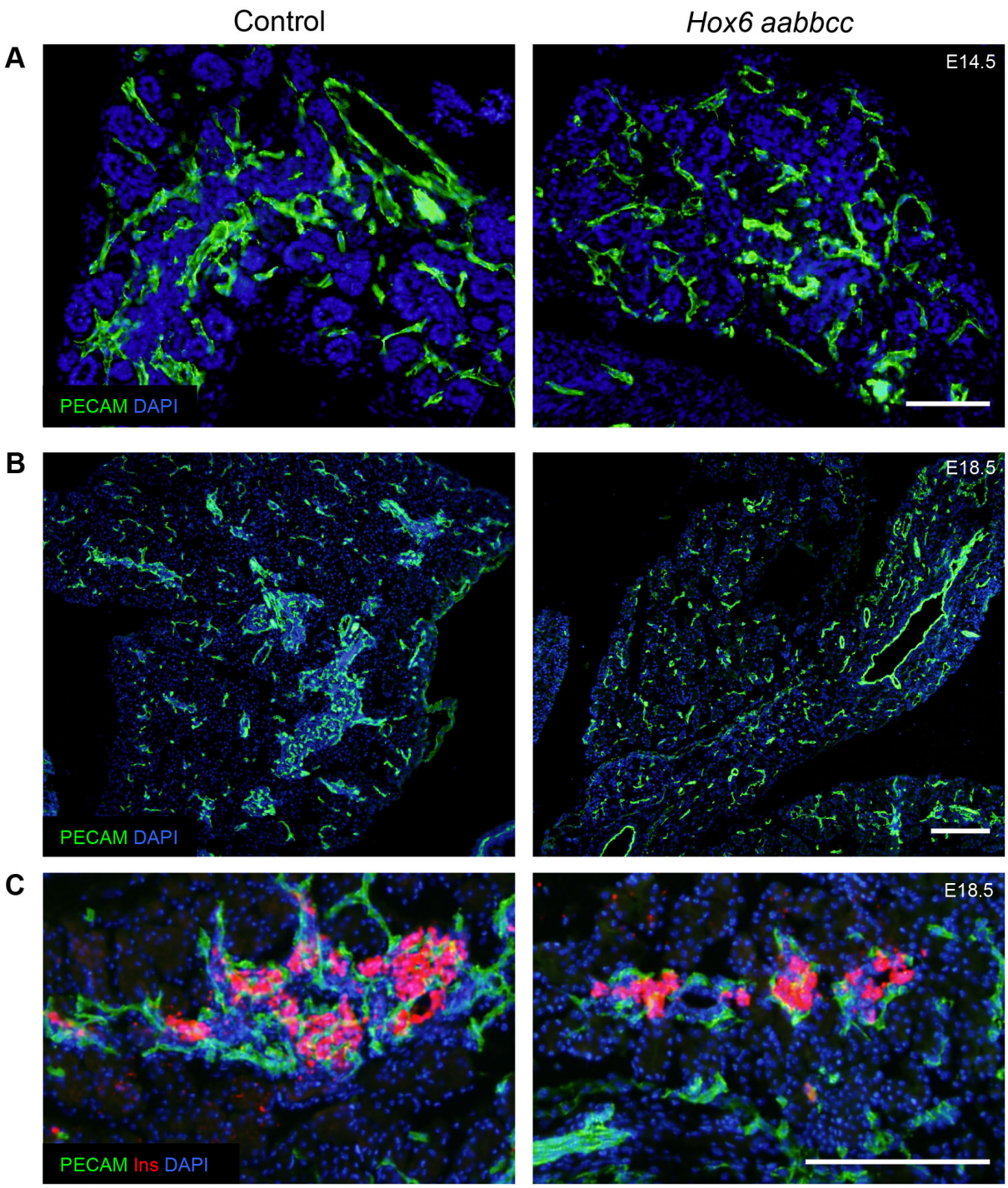

Figure 2.9. There is no change in vascularization in the *Hox6* null pancreas. (A-B) Immunofluorescent staining for PECAM at E14.5 (A) and E18.5 (B) shows no change between the control and *Hox6* null pancreas. (C) Immunofluorescent analysis shows Ins+ cells are closely associated with PECAM+ endothelial cells in both the control and *Hox6* null pancreas. Scale bars, 100 μM (A); 200 μM (B and C).

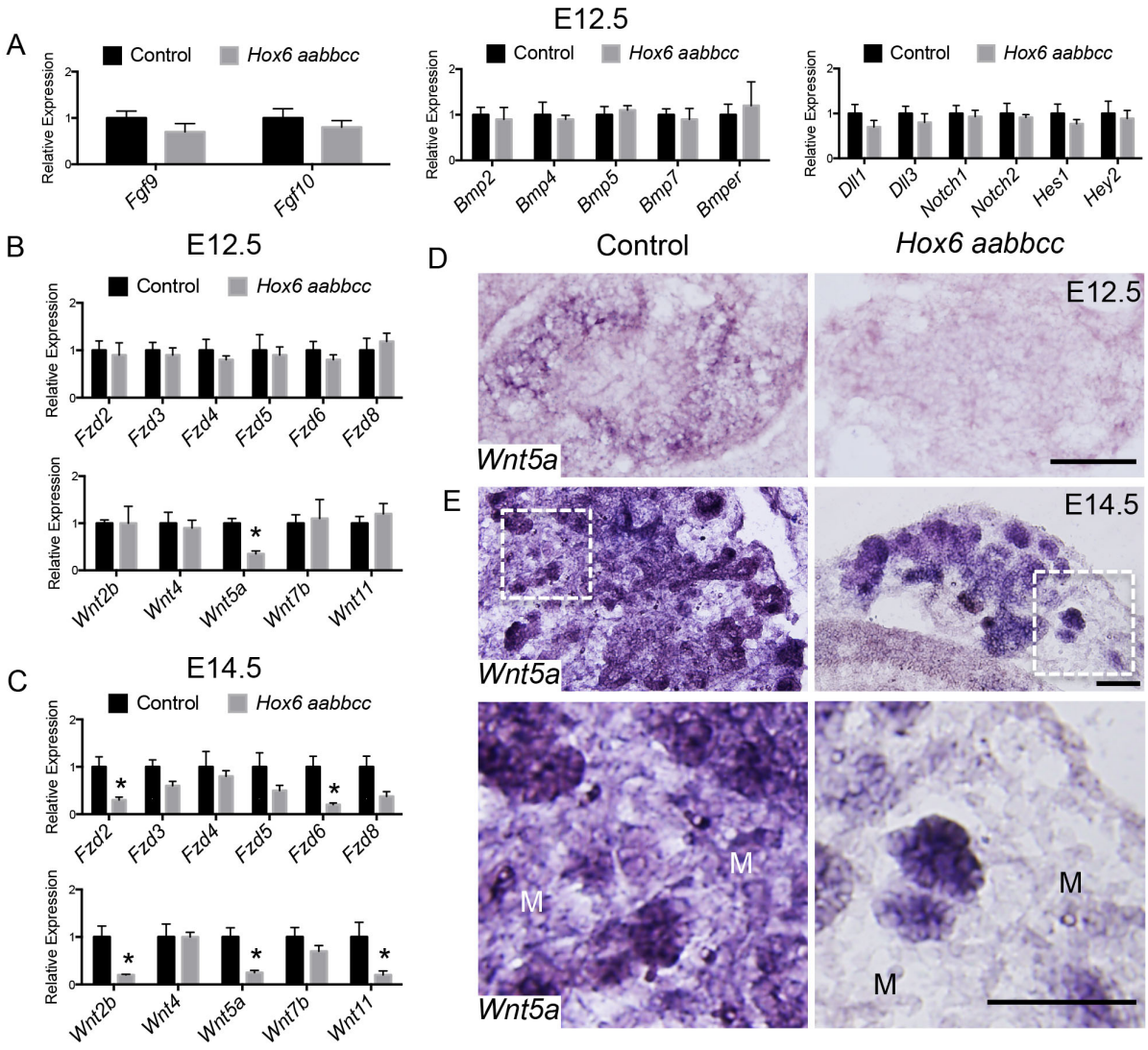

Figure 2.10. Expression of *Wnt5a* and Wnt inhibitors *Sfrp3* and *Dkk1* is lost in the *Hox6* mutant pancreas.
(A) qRT-PCR analysis of genes from the Fgf, Bmp, and Notch pathways at E12.5. (B-C) qRT-PCR analysis of Wnt receptor and ligand expression at E12.5 (B) and E14.5 (C). (D) ISH at E12.5 using probe for *Wnt5a* shows staining throughout the pancreas and a significant reduction of signal in the *Hox6* mutant pancreas. (E) At E14.5, *Wnt5a* is expressed in both the mesenchyme and epithelium of control pancreata. *Wnt5a* expression in the *Hox6* null pancreatic mesenchyme (M) is lost, but epithelial expression appears normal. Scale bars, 100 μM (D-E). Results are mean±s.e.m. **P*<0.05 calculated by Student's *t*-test (n≥3).

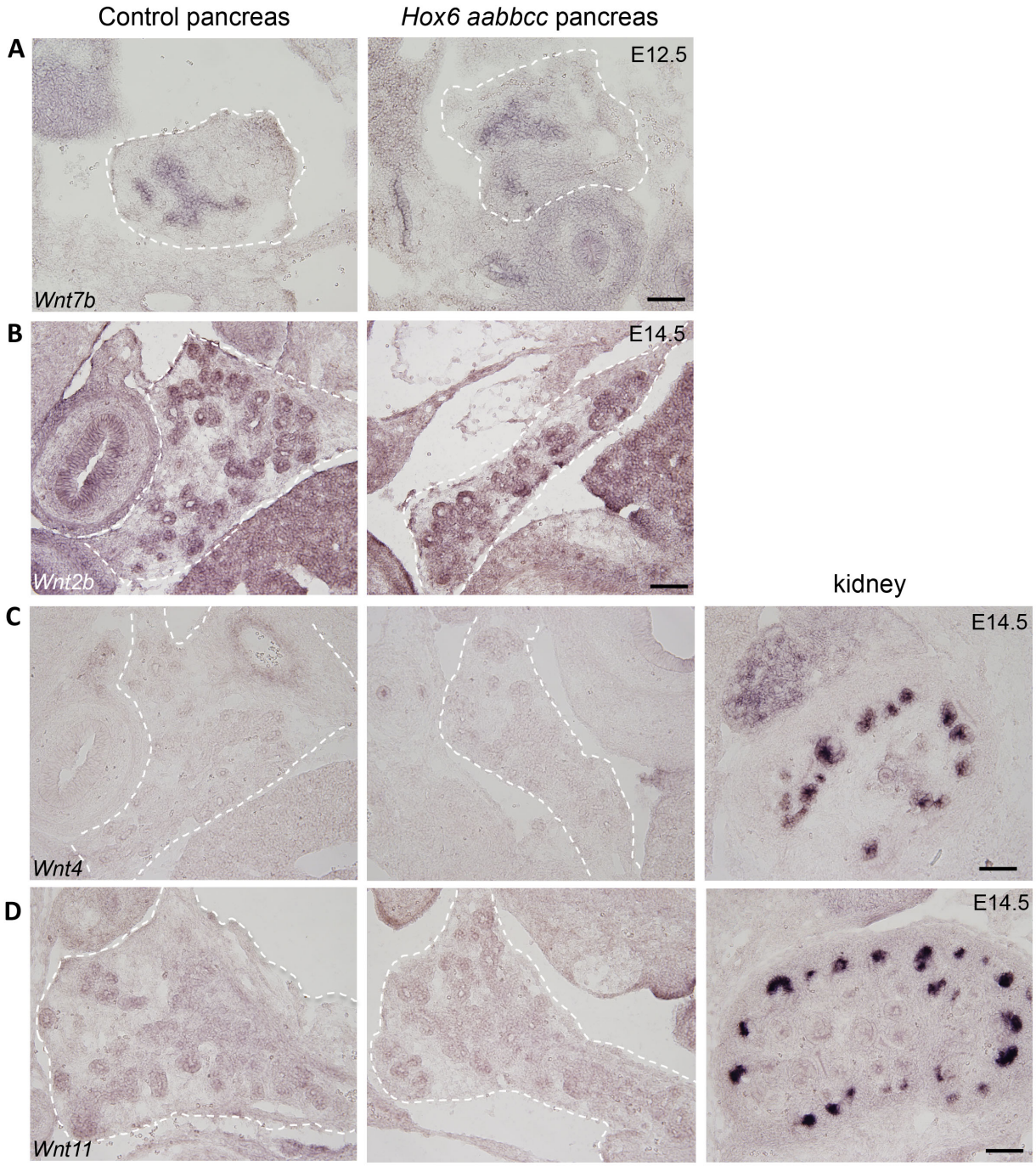

Figure 2.11. Expression of *Wnt2b, Wnt4, Wnt7b,* and *Wnt11* appears similar between control and *Hox6* mutant pancreata.
(A-D) At E12.5 the expression of *Wnt7b* is epithelial in the developing pancreas and there is no difference between control and mutant pancreas (A) as shown by ISH. (B) ISH at E14.5 for *Wnt2b* does not show differences in expression pattern between control and *Hox6* mutants. There was very little signal for expression of *Wnt4* (C) and

Wnt11 (D) in the developing pancreas by ISH despite very strong signal in the kidney on the same or adjacent sections. Scale bars, 100 μM (A-D).

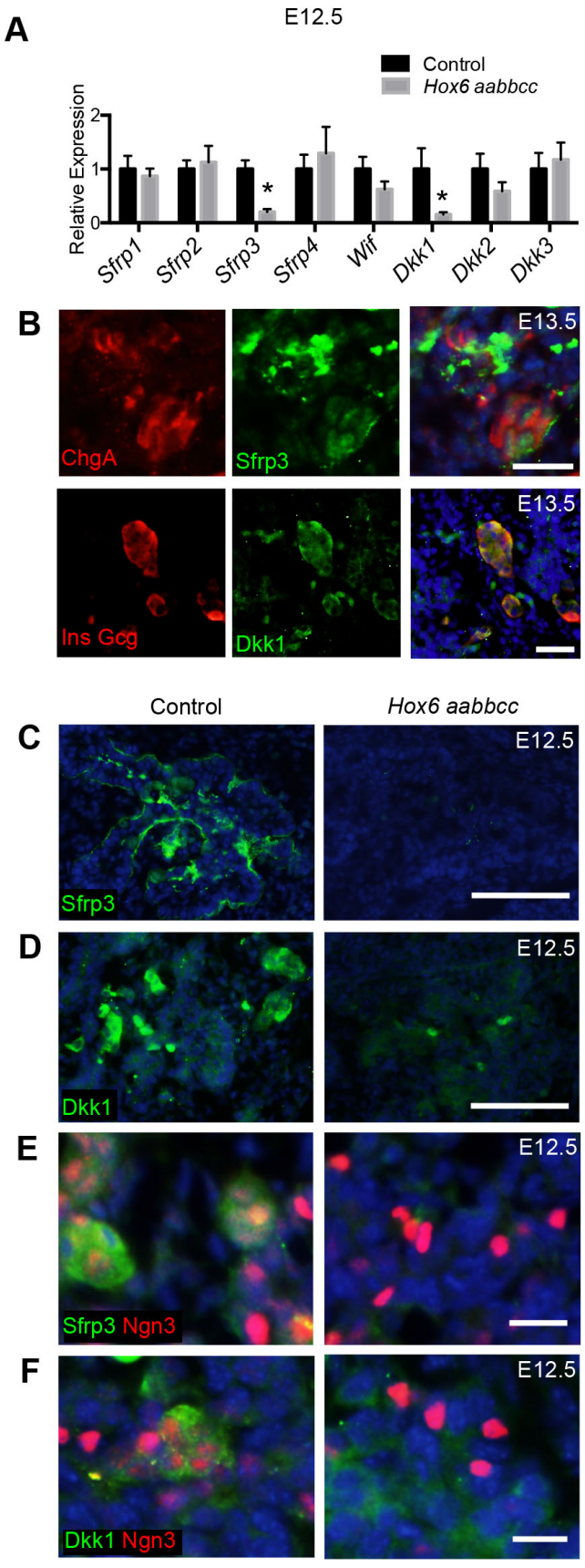
A
E12.5
Control
Hox6 aabbcc
Relative Expression
2
1
0
*
*
Sfrp1
Sfrp2
Sfrp3
Sfrp4
Wif
Dkk1
Dkk2
Dkk3
B
ChgA
Sfrp3
E13.5
Ins Gcg
Dkk1
E13.5
Control
Hox6 aabbcc
C
Sfrp3
E12.5
D
Dkk1
E12.5
E
Sfrp3 Ngn3
E12.5
F
Dkk1 Ngn3
E12.5

Figure 2.12. Expression Wnt inhibitors *Sfrp3* and *Dkk1* is lost in endocrine progenitors in the *Hox6* mutant pancreas.
(A) qRT-PCR analysis of Wnt inhibitors in control and *Hox6* mutant pancreata at E12.5. (B) Immunofluorescent staining for Wnt inhibitors Sfrp3 (green) and Dkk1 (green) with endocrine markers ChgA (red) and a combination of antibodies for Ins and Gcg (red) shows consistent overlap of staining in E13.5 mouse pancreas. (C-D) Immunofluorescent staining for Sfrp3 (C) and Dkk1 (D) in control and *Hox6 aabbcc* pancreata. There is a drastic reduction of signal for both Wnt inhibitors in the mutant pancreas at E12.5. (E-F) Sfrp3 (E, green), Dkk1 (F, green) and Ngn3 (E and F, red) in control and *Hox6* mutant pancreata at E12.5. Signal for Sfrp3 and Dkk1 is absent in Ngn3+ endocrine progenitor cells in *Hox6* mutant pancreata. Scale bars, 100 μM (C and D) and 25 μM (B, E and F). Results are mean±s.e.m. **P*<0.05 calculated by Student's *t*-test (n≥3).

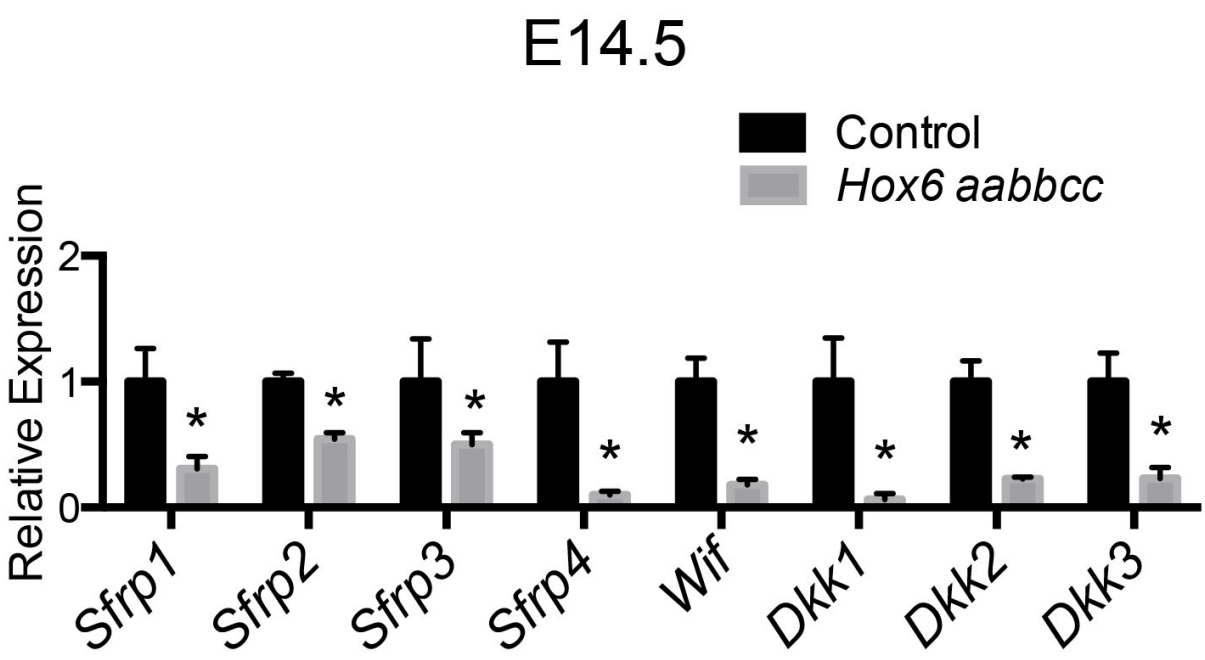

Figure 2.13. Wnt inhibitor expression is reduced in the *Hox6* mutant pancreas. qRT-PCR analysis at E14.5 between control and *Hox6* mutant pancreata shows a significant decrease in expression of all Wnt inhibitor genes examined. Results are mean±s.e.m. **P*<0.05 calculated by Student's *t*-test (n≥3).

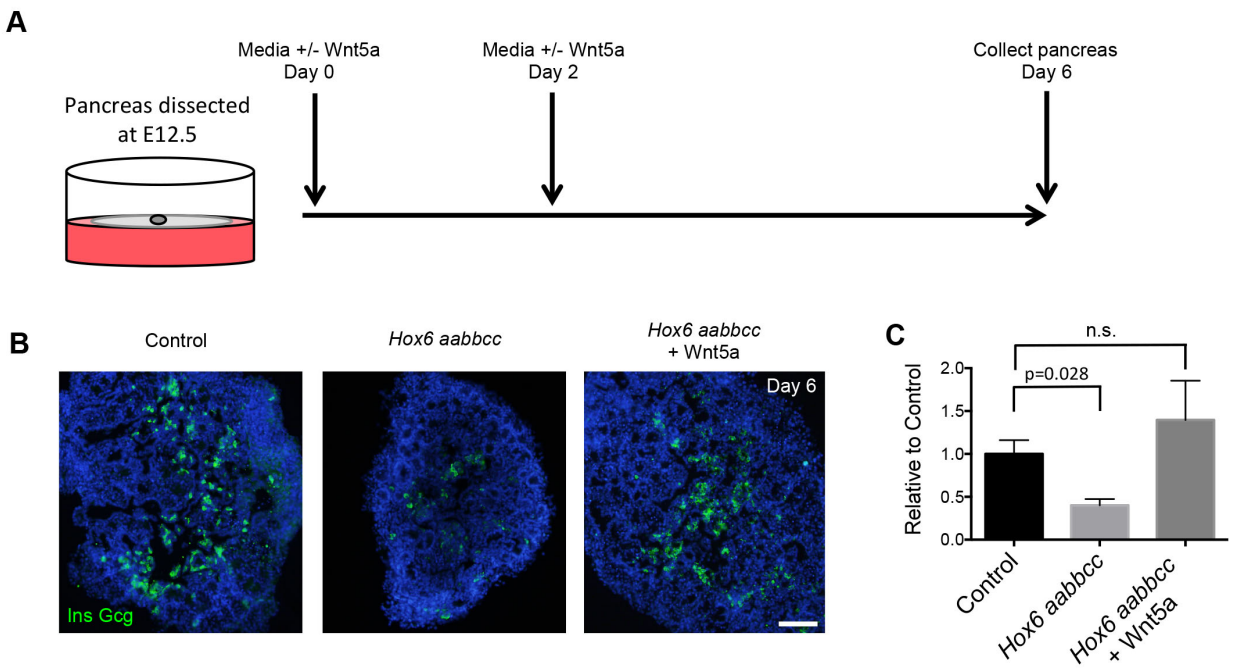

Figure 2.14. Exogenous Wnt5a treatment rescues endocrine cell differentiation in *Hox6* mutant pancreas.
(A) Control and *Hox6* mutant pancreata were dissected at E12.5 and cultured for six days in media with and without recombinant Wnt5a protein. On day two of culture, fresh media with or without Wnt5a was added to the explants. (B) Immunofluorescent staining for combination of Ins and Gcg primary antibodies on pancreas explants collected after six days in culture. (C) Quantification of immunofluorescent staining using ImageJ software shows that untreated *Hox6* mutant pancreata have significantly less endocrine staining per total DAPI area than control pancreata. *Hox6* mutant pancreata treated with Wnt5a protein have the same amount of endocrine staining per DAPI area as controls. n=3 for each condition/genotype. Scale bar is 100 μM (B). Results are mean±s.e.m. *P* values were calculated by Student's *t*-test.

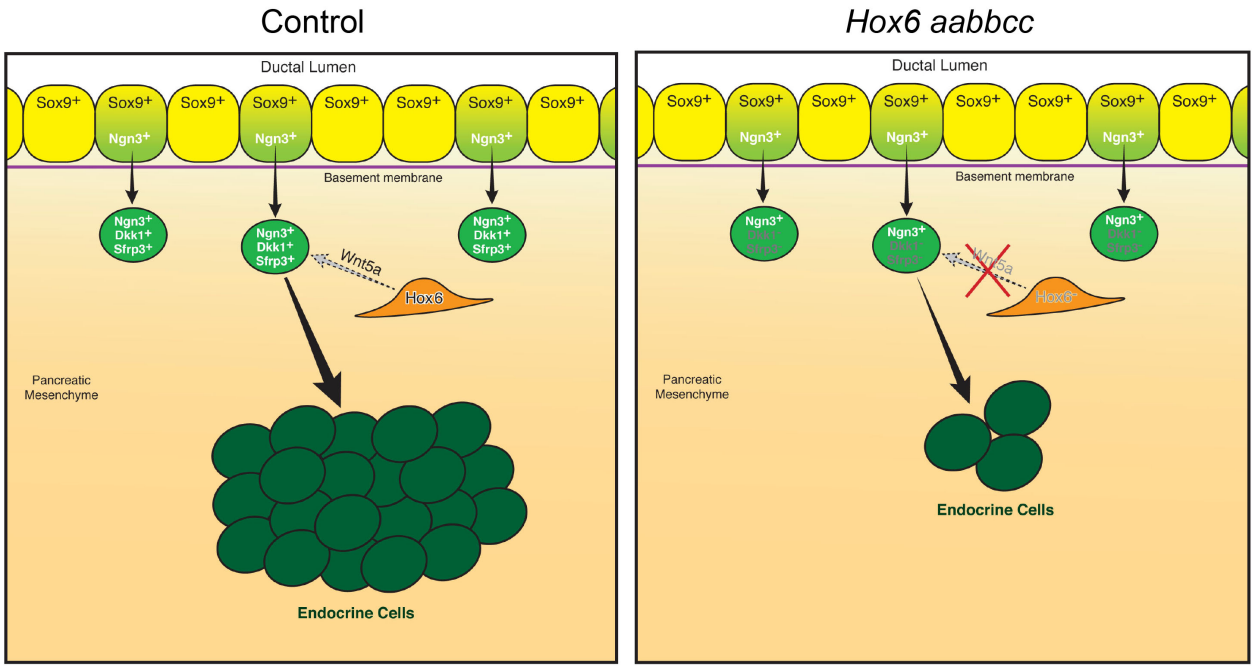

Figure 2.15. *Hox6* regulates mesenchymal *Wnt5a* expression to promote endocrine cell differentiation.
Mesenchymal *Hox6* promotes the expression of *Wnt5a* in the mesenchyme, which signals to endocrine precursor cells to initiate expression of *Sfrp3* and *Dkk1* to allow differentiation to more mature, hormone producing endocrine cells. When *Hox6* function is disrupted, expression of *Wnt5a, Sfrp3,* and *Dkk1* is lost and differentiation of mature endocrine cells is inhibited in the *Hox6* mutant pancreas.

Table 2.1. List of primary and secondary antibodies

Antibody	Dilution	Vendor	Catalog Number
Rabbit anti-Amy	1:100	Sigma Aldrich	090M4801
Guinea pig anti-Ins	1:500	Dako	A0564
Guinea pig anti-Pdx1	1:500	Abcam	AB47308
Armenian hamster anti-PECAM	1:50	DSHB	AB_2161039
Goat anti-Ngn3	1:100	Santa Cruz	sc-13793
Goat anti-ChgA	1:100	Santa Cruz	sc-1488
Mouse anti-Vmtn	1:200	Sigma Aldrich	091M4791
Rabbit anti-Cpa1	1:100	Rockland Immunochemicals	100-4152
Goat anti-Hnf1β	1:100	Santa Cruz	sc-7411
Rabbit anti-Gcg	1:100	Millipore	AB932
Goat anti-Isl1	1:100	R&D Systems	AF1837
Rabbit anti-Sox9	1:500	Millipore	AB5535
Goat anti-Ecad	1:500	R&D Systems	AF748
Rabbit anti-Sfrp3/Frzb	1:100	Genway	GWB-MT441I
Hamster anti-Muc1	1:200	NeoMarkers	HM-1630-P
Rabbit anti-Cleaved Caspase-3	1:100	Cell Signaling	9661
Mouse anti-Tuj1	1:500	Promega	G7121
Rabbit anti-αSMA	1:100	Abcam	AB5694
Alexa Fluor 488 anti-goat	1:500	Jackson Immunoresearch	705-545-003
Cy3 anti-goat	1:500	Jackson Immunoresearch	705-165-003
Cy3 anti-rabbit	1:500	Jackson Immunoresearch	711-165-152
DyLight 488 anti-guinea pig	1:500	Jackson Immunoresearch	706-545-148
Alexa Fluor 488 anti-Armenian hamster	1:500	Jackson Immunoresearch	127-545-160
AlexaFluor 488 anti-rabbit	1:500	Invitrogen	A21206
AlexaFluor 488 anti-mouse	1:500	Invitrogen	A31570

Table 2.2. List of qRT-PCR primers for embryonic tissue

Target	Forward Primer	Reverse Primer
Bmp2	AGACCACCGGCTGGAGAG	TTTCCCACTCATCTCTGGAA
Bmp4	TGGACTGTTATTATGCCTTGTTT	ATCAAACTAGCATGGCTCGC
Bmp5	CATGGTCATGAGCTTTGTCA	CTCCATGTGGAATCTGGGTC
Bmp7	TGGTCATGAGCTTCGTCAAC	TGGAAAGATCAAACCGGAAC
Bmper	TCCAGACCTGCCACTCTACA	CGCCAGAAATGACTCACAGTA
ChgA	ATGACAAAAGGGGACACCAA	GTCTCCAGACACTCAGGGCT
Dkk1	ATGAGGCACGCTATGTGCT	ATGCTTTCCTCAATTTCCCC
Dkk2	CTCAGTCAGCCAACCGATCT	ATCACTGCTGCAAGGGTAGG
Dkk3	CCAGAGTGGACAGGTGGTCT	GGCCCACAGTCTTCATCAAT
Dll1	GGAGAAGATGTGCGACCCT	CTCCCCTGGTTTGTCACAGT
Dll3	TCCCTGTCTCCACCAGTAGC	GTTCCCATCACAAGGTCCAG
Fgf9	ACGAGAAGGGGGAGCTGTAT	AGAGGTTGGAAGAGTAGGTGTTG
Fgf10	GCAACAACTCCGATTTCCAC	GATTGAGAAGAACGGCAAGG
Fzd2	ACATCGCCTACAACCAGACC	CACCAGCGGGTAGAACTGAT
Fzd3	CATCTGGGAGACAACATGGA	GAATCAGGTCTGGACGACTCA
Fzd4	TGCAGCTGACAACTTTCACG	TCTTCTCTGTGCACATTGGC
Fzd5	TTGTCGTTAAACTTTCCCAGC	CTCCGACTCCAAGGACAGAA
Fzd6	ATTATGACCAGGGGATCGCT	AAAAGCTTGGCAAAGGAACA
Fzd8	TCCGTTCAGTCATCAAGCAG	CGTGTAGAGCACGGTGAAGA
Gcg	ACTCCCGCCGTGCCCAAGAT	AGGAGCCATCAGCGTGCCTG
Ghr	ACCCAGAGGACAGAGGACAA	CTGAGCTCCTGACAGCTTGA
Hes1	GGCAGACATTCTGGAAATGA	GGTATTTCCCCAACACGCT
Hey2	TGAAGATGCTCCAGGCTACA	TCTGTCAAGCACTCTCGGAA
Hoxa6	GTCTGGTAGCGCGTGTAGGT	CCCTGTTTACCCCTGGATG
Hoxb6	GAGACCGAGGAGCAGAAGTG	CAGGGTCTGGTAGCGTGTG
Hoxc6	CAGGGTCTGGTACCGAGAGTA	TCCAGATTTACCCCTGGATG
Ins	CAAACAGCAAAGTCCAGGGGGC	TCCACTTCACGGCGGGACTTG
Isl1	GGTTAGGGATGGGAAAACCT	CACGAAGTCGTTCTTGCTGA
MafA	GAGGAGGTCATCCGACTGAA	TTCTCGCTCTCCAGAATGTG
MafB	TCCACCTCTTGCTACGTGTG	CGTTAGTTGCCAATGTGTGG
NeuroD	ACGCAGAAGGCAAGGTGTC	CGCTCTCGCTGTATGATTTG
Ngn3	ACCCTATCCACTGCTGCTTG	AGGTTGTTGTGTCTCTGGGG
Nkx2.2	CCGAGGGCCTCCAATACT	TTGTCATTGTCCGGTGACTC
Nkx6.1	GGATGACGGAGAGTCAGGTC	CGAGTCCTGCTTCTTCTTGG
Notch1	GAATGGAGGTAGGTGCGAAG	CTGAGGCAAGGATTGGAGTC
Notch2	TGCTGTGGCTCTGGCTGT	TGTGCCGTTGTGGTAGGTAA
Pax6	CGGGACTTCAGTACCAGGG	CTTCATCCGAGTCTTCTCCG
Pp	CTGGGCCCAACACTCACTA	CAGAGCCACCCAAGTGGATA
Sfrp1	CATCTCTGTGCAAGCGAGTT	GGGTTTCTTCTTCTTGGGGA
Sfrp2	CGACATCATGGAAACCCTTT	TTGCTCTTTGTCTCCAGGATG
Sfrp3	AGTACTGGACACTGCAGAGGG	AGCCCGGATGACATAGTTGT
Sfrp4	TCGAACACAAGTCCCTCTCA	ATCATCCTTGAACGCCACTC

Sst	CAGACTCCGTCAGTTTCTGC	TTCTCTGTCTGGTTGGGCTC
Wif	CCATCAGGCTAGAGTGCTCA	GCATTCTTTGTTGGGCTTTC
Wnt1	AAATGGCAATTCCGAAACC	GAAGATGAACGCTGTTTCTCG
Wnt2b	CTGCTGCTGCTACTCCTGACT	GGGGATGTTGTCACAGATCA
Wnt4	CCTGCGACTCCTCGTCTTC	GTTTCTCGCACGTCTCCTCT
Wnt5a	ACGCTTCGCTTGAATTCCT	CCGGGCTTAATATTCCAATG
Wnt7b	ACGTGTTTCTCTGCTTTGGC	CCAGGCCAGGAATCTTGTT
Wnt8b	CCCGTGTGCGTTCTTCTAGT	AGACCAGGTAAGCCTTTGGA
Wnt11	CTGCGAGGCTCTGCTCTTT	TCTGATTCAGTGCCAAGGCT

CHAPTER 3

HOX6 FUNCTION IN THE *IN VITRO* DIFFERENTIATION OF MESCS TO INSULIN-PRODUCING CELLS

Summary

The differentiation of glucose-responsive, insulin-producing cells from ESCs *in vitro* is promising as a cellular therapy for the treatment of diabetes, a devastating and common disease. Pancreatic β-cells are derived from the endoderm *in vivo* and therefore most current protocols attempt to generate a pure population of first endoderm, then pancreas epithelium, and finally insulin-producing cells. Despite this, differentiation protocols result in mixed populations of cells that are often poorly defined, but also contain mesoderm. Here, we describe a mouse ESC differentiation method that results in endoderm derived insulin-producing cells, as well as mesoderm with an appropriate region-specific *Hox* gene expression profile. Loss of function of all three *Hox6* genes impairs the ability of mESCs to differentiate to insulin-producing cells *in vitro* as we have observed previously *in vivo*. Together, we describe a method to generate pancreatic endoderm and mesoderm in mouse ESCs, and we show that mesenchyme-specific perturbations in pancreas development *in vivo* are recapitulated *in vitro* using this model. This system will be useful for elucidating mechanisms involved in mesodermal support of pancreatic endoderm and insulin-producing cells.

Introduction

Diabetes affects more than 25 million Americans and over 300 million people worldwide (Pagliuca and Melton, 2013). The cause of Type 1 diabetes is the autoimmune destruction of insulin-producing pancreatic β-cells (Abdelalim and Emara, 2015). Two major treatments for Type 1 diabetes include the maintenance of blood sugar levels by injection of exogenous insulin throughout each day and/or the transplantation of cadaveric insulin-producing β-cells (Shapiro et al., 2000; Pagliuca et al., 2014). There are inherent problems with these treatments including the invasiveness of constant injections, problems with improper management of blood glucose levels, the current lack of cadaveric β-cells, potential graft versus host disease and long-term immunosuppression (Russ et al., 2015). A promising treatment for diabetes is the generation of glucose-responsive, insulin-producing cells from human embryonic stem cells (hESCs) and/or induced pluripotent stem cells (iPSCs). This could alleviate the need for exogenous insulin injections and cadaveric β-cells (Pagliuca and Melton, 2013).

The pancreas is composed of exocrine and endocrine components. Exocrine cells secrete digestive enzymes into the small intestine to assist in nutrient metabolism. There are five types of endocrine cells that each secretes a separate endocrine hormone. Insulin-producing β-cells are one of the five distinct endocrine cells in the pancreas. All exocrine and endocrine cells of the pancreas arise from the foregut endoderm of the embryo (Gittes, 2009; Puri and Hebrok, 2010). While all of the major functional components of the pancreas are derived from the endoderm, the surrounding mesodermally-derived mesenchyme is critical for the growth and development of these

cell types (Golosow and Grobstein, 1962; Gittes et al., 1996; Miralles et al., 1998; Bhushan et al., 2001; Attali et al., 2007; Gittes, 2009; Puri and Hebrok, 2010). Explant studies were first used to demonstrate the importance of the mesenchyme. When pancreas epithelium is cultured in the absence of its surrounding mesenchyme, both endocrine and exocrine development is arrested with defects in growth and differentiation. These defects can be rescued by recombination with pancreatic and other types of mesenchyme (Golosow and Grobstein, 1962; Wessells and Cohen, 1967). When the pancreas mesenchyme is genetically ablated *in vivo,* a similar failure in development of the exocrine and endocrine components results (Landsman et al., 2011). Critical signals from the surrounding mesoderm that inform pancreas specification and development include fibroblast growth factor (FGF), bone morphogenetic protein (Bmp), and retinoic acid (RA) (Bhushan et al., 2001; Stafford and Prince, 2002; Martin et al., 2005; Molotkov et al., 2005; D'Amour et al., 2006; Nostro et al., 2011). Moreover, co-culture with pancreatic mesenchyme significantly expands pancreatic progenitor cell populations *in vitro,* indicating that mesenchyme may be critical for generating the large number of cells needed for cell replacement therapy (Sneddon et al., 2012). While the role of mesenchymal to epithelial crosstalk has long been appreciated and some of the signaling mechanisms have been described, the mesenchymal signals required for differentiation of pancreatic endocrine cells are still not fully understood.

Recently, we described a critical role for *Hox6* gene function in the pancreatic mesenchyme for the proper differentiation of all five endocrine cell types (Larsen et al.,

2015). The *Hox6* paralogous group is composed of three genes; *Hoxa6, Hoxb6* and *Hoxc6.* In the pancreas, *Hox6* genes are expressed only in the mesenchyme.

Based on our recent *in vivo* data, we hypothesized that *Hox6* genes also play a role in endocrine cell differentiation *in vitro.* We further hypothesized that enhancing mesodermal differentiation during *in vitro* pancreas differentiation would lead to an increase in efficiency of endocrine cell differentiation. Here, we modified existing protocols (D'Amour et al., 2006; Kroon et al., 2008) and developed a method to direct differentiation of mESCs into pancreatic endoderm and mesoderm. We also show that *Hox* genes normally expressed in the pancreatic region *in vivo* are also expressed *in vitro*, indicating that both pancreatic endoderm and mesoderm are differentiating and obtain the proper regional identity. In contrast, the most anterior and posteriorly expressed *Hox* genes are not present *in vitro*. In addition, we derived several *Hox6* triple-null mESC lines, along with controls, and show that *Hox6* mutant mESCs have a decreased capacity for differentiation into insulin-producing cells *in vitro* compared to wild-type mESCs. Taken together, we show a critical role for mesodermally expressed *Hox6* genes in the differentiation of endodermally derived insulin-producing cells *in vitro*.

Results

Differentiation of mESCs using three-dimensional culture

In order to stimulate cell-cell interactions and multi-lineage differentiation, our differentiation method utilized the hanging drop technique to first form embryoid bodies (EBs), followed by the implementation of a directed differentiation protocol that has been used in monolayer cultures for directed differentiation of human ESCs (D'Amour et

al., 2006; Kroon et al., 2008)(Figure 3.1). Briefly, after two days in hanging drop suspensions, EBs were embedded in a droplet of Matrigel in order to provide the cells with a supportive matrix in which to grow and maintain their three-dimensional structure. The EBs were then differentiated to definitive endoderm by treatment with 100ng/mL Activin A for four days. Following Activin A, EBs were analyzed for endodermal and mesodermal markers. Compared to mESCs, Activin A treated cells show significantly increased mRNA expression and protein levels of the endoderm markers Foxa2 (Fig. 3.2A) and Sox17 (Fig. 3.2B)(Ang and Rossant, 1994; Weinstein et al., 1994; Kanai-Azuma et al., 2002). When treated with Activin A, EBs do not show an increased induction of mesenchyme when compared to mESCs based on mesoderm marker Vimentin (Vim) mRNA expression, however scattered Vim-positive cells were observed by protein staining (Fig. 3.2C).

Foregut and pancreas differentiation was further induced by treatment with retinoic acid (RA,10μM), the Hedgehog inhibitor SANT-2 (10μM), and bFGF (50ng/mL) (RSF media). After two days in RSF media, media was replaced and EBs were cultured in “basal media” (see Methods) without growth factors for up to three weeks (Fig. 3.1). *Pdx1* is the first marker of pancreas differentiation and is expressed in the epithelium by E9.5 during mouse development. We detected Pdx1 protein and found a significant increase in expression of *Pdx1* at three weeks post-treatment with RSF media when compared to EBs (Fig. 3.3A). Similarly, the endocrine hormone Insulin (*Ins*) is expressed after three weeks post-RSF treatment (Fig. 3.3B). We also detected C-peptide immunostaining in these conditions, indicating that pro-insulin protein is being produced and cleaved (Fig. 3.3B). Pancreatic exocrine cells are also produced after

RSF treatment. We observe significant increases in mRNA expression and antibody staining of *Amy2* and *Cpa1* three weeks post-RSF treatment (Fig. 3.3C, D). In addition to pancreatic lineage markers, we interrogated our cultures to determine the presence of other endodermal lineages. We examined the thyroid marker *Pax8,* lung marker, *Nkx2.1* and intestinal marker *Cdx2* (Plachov et al., 1990; Kimura et al., 1996; Silberg et al., 2000). Of these lineages, we only detected an enrichment of *Pax8* relative to EBs (Fig. 3.4).

Hox6 genes are critical for the differentiation of insulin-producing cells in vitro

We have recently shown that *Hox6* genes are critical for the differentiation of β-cells in the developing mouse pancreas *in vivo* (Larsen et al. 2015). We therefore sought to determine if mesenchymal factors such as *Hox6* genes influence the differentiation of endocrine cells *in vitro*. To confirm that *Hox* genes were present in differentiated EBs, we performed qRT-PCR analyses for a series of *Hox* genes that are expressed at different levels along the anterior-posterior axis of the embryo *in vivo* (Fig. 3.5). Examining several stages throughout the differentiation protocol (EB, Endoderm, 2 weeks post RSF media, 3 weeks post RSF media), we observed that *Hox* gene expression *in vitro* mimics *Hox* gene expression at the level of the pancreas in the embryo (Fig. 3.5)(Larsen et al., 2015). In the pancreatic foregut region of the embryo, *Hox5* and *Hox6* genes are robustly expressed whereas *Hox1* and *Hox2* genes are expressed more anteriorly and *Hox11* and *Hox13* genes are expressed more posteriorly (Fig. 3.5)(Dressler and Gruss, 1989; Duboule and Dolle, 1989; Graham et al., 1989). We detected a significant enrichment of all *Hox5* and *Hox6* genes post-RSF media (at 2

and 3 weeks in culture) compared to EB and endoderm stages whereas *Hox1, Hox2, Hox11,* or *Hox13* expression was not enriched at any stage (Fig. 3.5). Collectively, our data suggests that the induction protocol we have implemented successfully generated pancreatic endoderm (Fig. 3.3) and mesoderm with the appropriate region-specific *Hox* gene expression (Fig. 3.5)

To investigate the role of *Hox6* genes in differentiation of pancreatic insulin-producing cells, we derived three lines of *Hox6* mutant mESCs from previously described *Hox6* mutant crosses (McIntyre et al., 2007). In *Hox6* mutant mESC lines, we observed expression levels of endodermal genes *Foxa2* and *Sox17* similar to control mESC lines following Activin A treatment, indicating that endoderm induction is not impaired in these cells (Fig. 3.6A).

Following endoderm induction, control and *Hox6* mutant cells were cultured in RSF media followed by basal media (as in Figure 1). Cells were collected after three weeks and examined for expression of endocrine and exocrine pancreas genes. At three weeks post-RSF treatment we observed similar increases in expression of *Pdx1* in control cells and in *Hox6* mutant cells relative to the EB stage (Fig. 3.6B). However, *Ins* mRNA and protein expression is markedly decreased in the *Hox6* mutant cells compared to controls (Fig. 3.6C). In contrast, exocrine genes *Amy2* (Fig. 3.6D) and *Cpa1* (Fig. 3.6E) were expressed at comparable levels in both control and *Hox6* mutant cultures (Fig. 3.65E). Amy2- and Cpa1-positive staining appears to occur only on epithelial-like cells of these cultures as would be expected (Fig. 3.6D and E). These data show that *Hox6* mutant mESCs are capable of differentiating to exocrine cells

competently but are defective in their ability to differentiate into insulin-producing cells. This is the same phenotype observed in *Hox6* null embryos *in vivo* (Larsen et al., 2015).

Increased mesoderm differentiation does not improve differentiation efficiency

Our results (Fig. 3.6) suggest that genes expressed solely in the mesenchyme (*Hox6* genes) have an influence on the ability of mESCs to differentiate to pancreatic endocrine cells *in vitro*. To test if increasing the population of mesoderm early in differentiation would be able to provide better support for endodermal progenitor cell differentiation to insulin-producing cells, we added Bmp4 to the media at the same time as Activin A. Bmp4 has been previously shown to enrich mesoderm differentiation (Purpura et al., 2008; Sakurai et al., 2009). The addition of Bmp4 does not significantly alter the differentiation of endodermal cells as shown by equivalent levels of expression of *Foxa2* in cells treated with Activin A alone or with Activin A + Bmp4 (Fig. 3.7A). Cells treated with Bmp4 have significantly higher expression of *Vim* shown by qRT-PCR and antibody staining, indicating an increase in the amount of mesoderm generated (Fig. 3.7B). The induction of *Ins* expression did not appear to be affected by increasing the mesodermal population early in development (Fig. 3.7C). The addition of Bmp4 significantly increased the population of mesoderm early in differentiation, however; at two and three weeks after treatment with RSF media, the amount of mesenchyme is indistinguishable in cultures treated with Activin A alone, or with Activin A and Bmp4 (Fig. 3.7D). Taken together our data supports a role for mesenchymal factors, specifically *Hox6,* in endocrine, but not exocrine pancreas development *in vitro,* similar to what is observed *in vivo.*

Discussion

Here, we use a three-dimensional method of mESC differentiation to pancreas cells, including insulin-producing cells. By mimicking *in vivo* processes of differentiation, we are able to produce cells of both the exocrine and endocrine pancreatic lineages. We also show that *Hox* genes, critically important developmental transcription factors, initiate the expression patterns expected for the AP position of the pancreas during this differentiation procedure. In vivo, we have shown that *Hox6* genes are important for the differentiation of insulin-producing cells, but not exocrine cells (Larsen et al., 2015). This shows that previously unappreciated factors produced in the pancreatic mesenchyme, such as *Hox6* genes, may be critical to improve the generation of functional pancreatic tissue *in vitro*. Further study of the effects of multiple tissue types, including the mesenchyme, on mESC differentiation may lead to increased functional pancreatic cells and more fully differentiated tissue *in vitro*.

Treating mESCs with Bmp4 to increase the amount of early mesenchyme induced during differentiation did not increase the efficiency of differentiation of insulin-producing cells. While there was a significant increase in the amount of mesenchyme generated immediately following Bmp4 treatment, this early induction of mesenchymal differentiation was not maintained and was insufficient in improving mESC differentiation to pancreatic tissue or, specifically, insulin-producing cells. Replacing the mesenchyme with recombinant proteins produced by the pancreatic mesenchyme *in vivo* improves pancreas cell differentiation *in vitro*; however, currently unidentified factors produced in pancreatic mesenchyme are likely to be important for improved differentiation of completely functional insulin-producing cells *in vitro.*

In addition to generating glucose-responsive, insulin-producing cells to treat diabetes, *in vitro* engineering a pancreatic organ has many uses for drug development and disease modeling. In addition to diabetes, treatments for other devastating pancreatic diseases such as pancreatitis and pancreatic cancer can be studied through the use of functional pancreatic tissue *in vitro.* The presence of ductal and exocrine cells will be critical for the accuracy of diagnostics using engineered pancreatic tissue. The inclusion of mesenchyme and also endothelial cells will be needed to further improve a useful *in vitro* pancreatic model (Lammert et al., 2001; Sneddon et al., 2012). While there have recently been significant strides towards the generation of functional pancreatic cells *in vitro* for the treatment of human disease, there is still more knowledge to be gained from studying the effects of all pancreatic tissue types in *in vitro* differentiation strategies.

Materials and Methods

Derivation of mESCs

Mice mutant for all three *Hox6* paralogous genes were generated using standard genetic crosses (Kostic and Capecchi, 1994; Rancourt et al., 1995; Garcia-Gasca and Spyropoulos, 2000). All experiments were performed following protocols approved by the University of Michigan's Institutional Committee on the Use and Care of Animals.

Differentiation Protocol

mESCs were cultured in DMEM (Gibco) supplemented with LIF (Millipore), 15% FBS (Gibco), Pen/Strep (Gibco), Glutamax (Gibco), NEAA (Gibco), Sodium Pyruvate

(Gibco), and β-mercaptoethanol (Sigma) on gelatin coated tissue culture plates with mitotically inactivated MEFs in a 37ºC incubator with 5% CO_2. EBs were formed by pipetting 2,000 mESCs in 20µl drops of culture media without LIF onto a tissue culture plate and suspended upside-down for two days. EBs were collected by rinsing the plate with media. EBs were then suspended in 20µl drops of Matrigel (Fisher) in 24-well tissue culture plates. EBs were cultured in DMEM with 3% KOSR (Gibco), Pen/Strep, Glutamax, NEAA, Sodium Pyruvate, and β-mercaptoethanol with 100ng/ml Activin A and some with 100ng/ml Activin A (R&D Systems) and 26ng/ml Bmp4 (R&D Systems) for four days. Media was changed after two days. EBs were then treated with All-Trans RA (Stemgent), SANTII, and bFGF (R&D Systems) for two days after which cells were grown in EB culture media with no supplements (Basal media) for 19 more days. Media was changed every other day.

RNA Isolation and Quantitative RT-PCR

EBs were flash frozen immediately upon collection and stored at -80°C. RNA was isolated from cells with the Qiagen RNeasy Micro Kit or MagMax RNA Isolation Kit. Quantitative RT-PCR (qRT-PCR) was carried out using Roche FastStart SYBR Green Master Mix and the Applied Biosystems StepOnePlus Real-time PCR system (Life Technologies). Relative expression values were calculated as $2^{-\Delta\Delta Ct}$ and values of controls were normalized to 1. Rn18s served as an internal control for normalization in all qRT-PCR experiments. All data are shown as the mean of three independent biological replicates; error bars represent s.e.m. Calculations and *P*-values (two-tailed, unpaired *t*-test) were generated in Microsoft Excel. Results were considered statistically

significant at $P < 0.05$. Graphs were generated using Prism 6. Primer sequences are listed in Table 3.1.

Immunofluorescent Staining

For section *in situ* hybridization (ISH), differentiated EBs were fixed for 3 hours in 4% paraformaldehyde in PBS at room temperature. EBs were then rinsed in PBS and immersed in 30% sucrose at 4°C overnight before embedding into optimal cutting temperature (OCT) media. Frozen sections 12 μm in size were cut, and slides were stored at −80°C. Slides were blocked for one hour at room temperature in 0.1% or 0.5% Triton X-100 in PBS (PBS-T) with 1% donkey serum and treated with primary antibody overnight at 4°C. On day two slides were washed in PBS-T, incubated with secondary antibody for two hours at room temperature, followed by a 10-minute wash in PBS-T with DAPI (Sigma Aldrich). Coverslips were added to the slides using Prolong Gold Antifade Reagent (Invitrogen). Primary and secondary antibodies used are listed in Supplementary Table 1. Slides were imaged using an Olympus BX-51 microscope.

Acknowledgments

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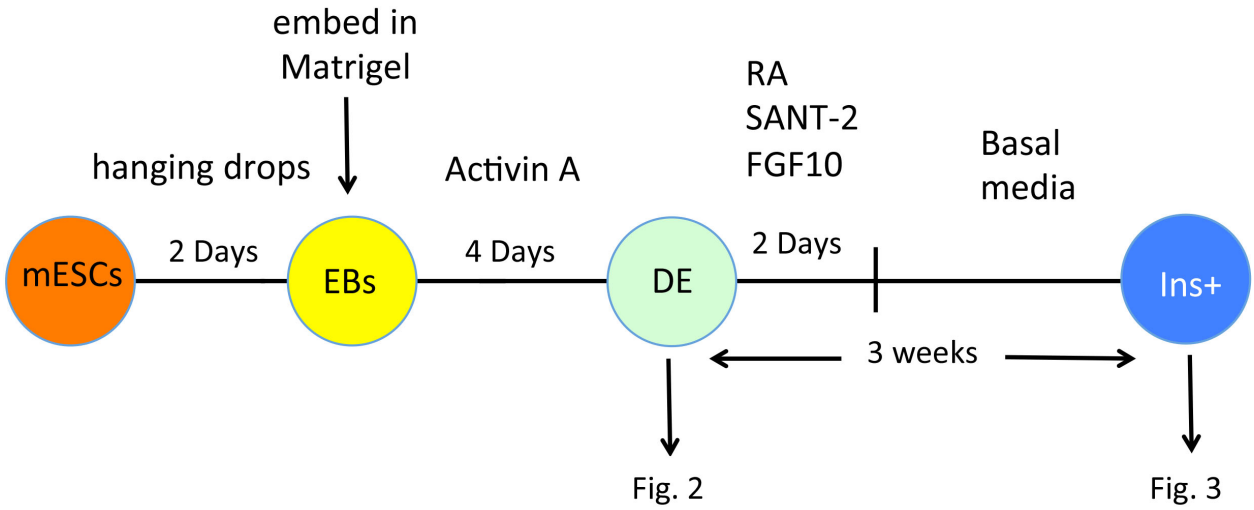

Figure 3.1. Differentiation protocol.
EBs were formed from mESCs using hanging drops for two days. EBs were then embedded in Matrigel and treated with Activin A for four days to induce endodermal differentiation. RA, SANT-2, and bFGF were added for two days to induce foregut, and then pancreas differentiation. After two days of treatment, EBs were cultured in media without growth factors and cells were collected for final analysis three weeks after RA, SANT-2, and bFGF treatment was started.

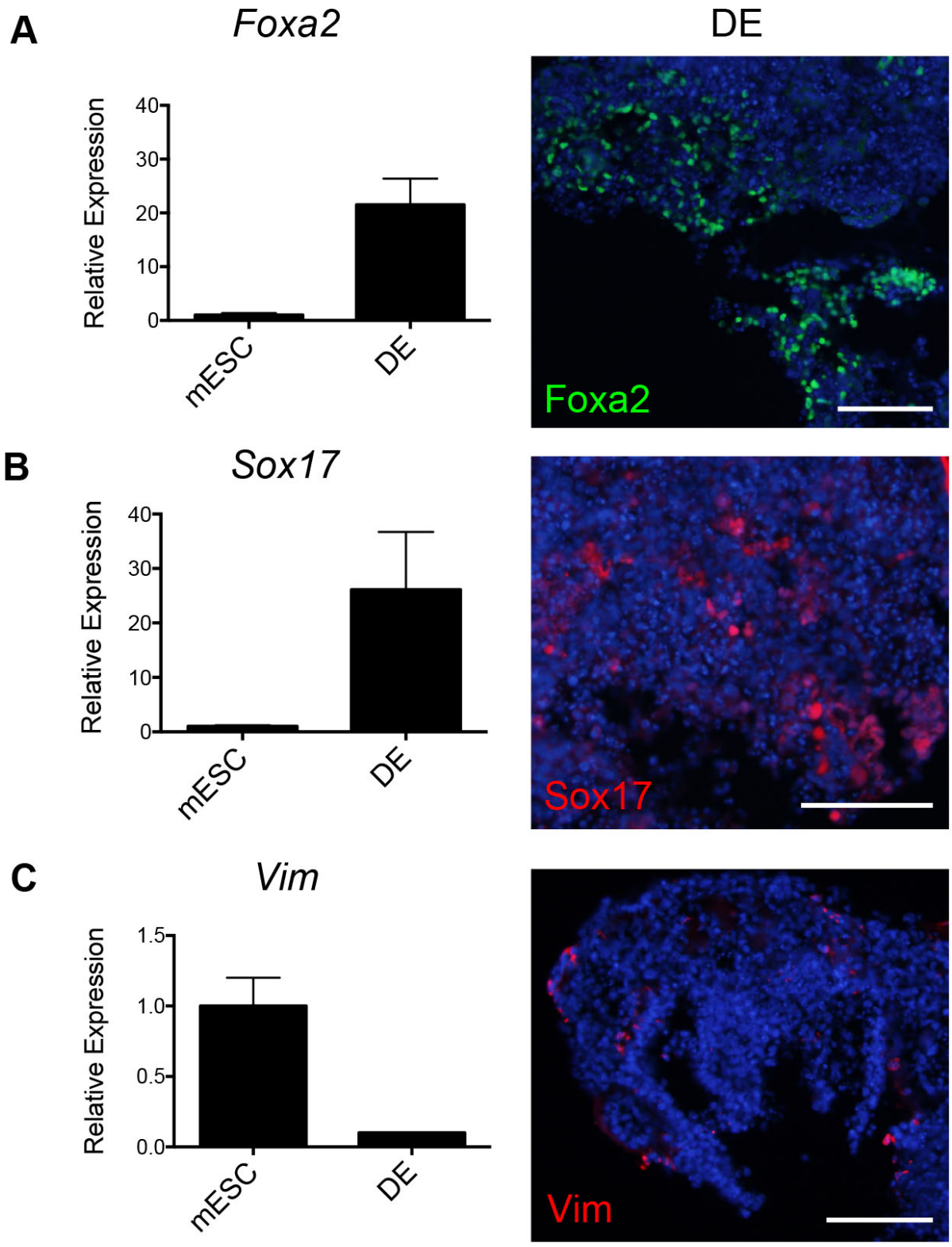
A
Foxa2
DE
Relative Expression
40
30
20
10
0
mESC
DE
Foxa2
B
Sox17
Relative Expression
40
30
20
10
0
mESC
DE
Sox17
C
Vim
Relative Expression
1.5
1.0
0.5
0.0
mESC
DE
Vim

Figure 3.2. mESCs differentiate to endoderm.
qRT-PCR and immunofluorescence analyses shows the expression of endodermal markers *Foxa2* (A) and *Sox17* (B). There is a decrease in expression of *Vim* compared to mESCs however there are mesenchymal cells present as shown by antibody staining for Vim (C). Scale bars 100 μM. Results are mean±s.e.m. (n≥4).

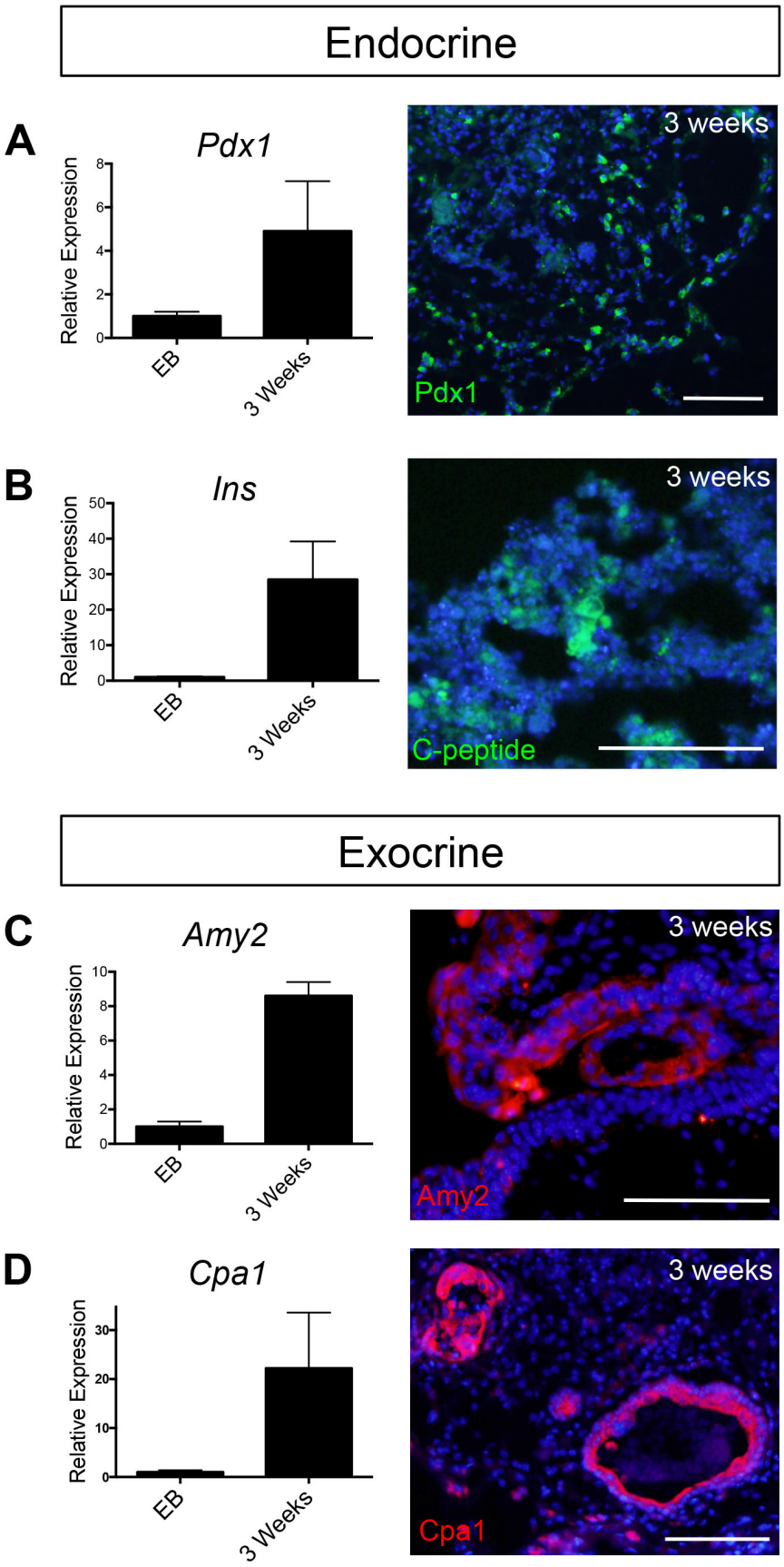
Endocrine
A
Pdx1
Relative Expression
8
6
4
2
0
EB
3 Weeks
3 weeks
Pdx1
B
Ins
Relative Expression
50
40
30
20
10
0
EB
3 Weeks
3 weeks
C-peptide
Exocrine
C
Amy2
Relative Expression
10
8
6
4
2
0
EB
3 Weeks
3 weeks
Amy2
D
Cpa1
Relative Expression
30
20
10
0
EB
3 Weeks
3 weeks
Cpa1

Figure 3.3. Both exocrine and endocrine pancreas cells are represented in *in vitro* differentiation.
qRT-PCR shows that both *Pdx1* (A) and *Ins* (B) are expressed and immunofluorescence analyses confirms the presence of Pdx1- (A) and C-peptide-positive (B) cells three weeks after treatment with RSF media. Exocrine cells are also represented as shown by the expression and protein staining of both *Amy2* (C) and *Cpa1* (D). Scale bars 100 μM. Results are mean±s.e.m. (n≥3).

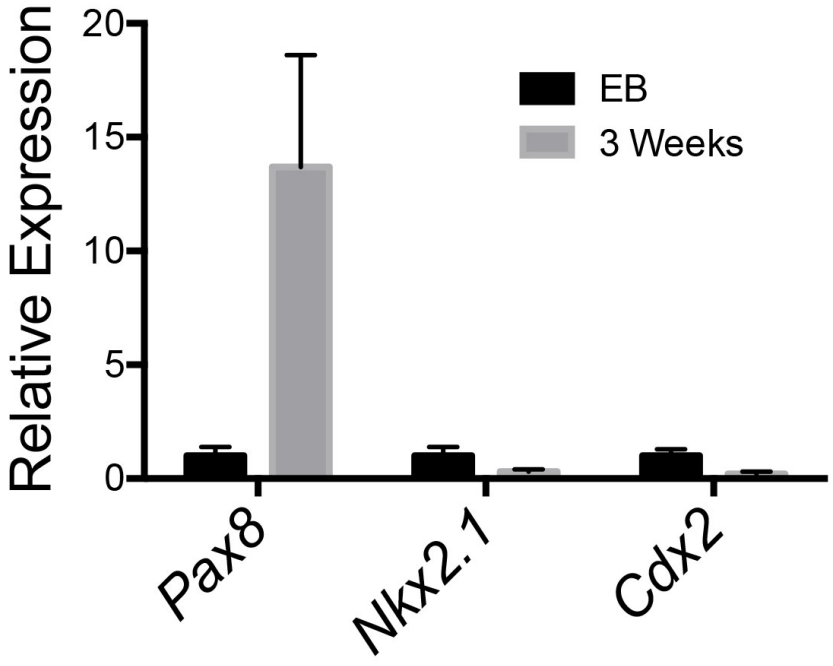

Figure 3.4. qRT-PCR analyses for genes primarily expressed in other foregut derived organs.
Pax8 (thymus) is expressed after 3 weeks of differentiation but *Nkx2.1* (lung) and *Cdx2* (gut) are not expressed. Results are mean±s.e.m. (n≥5).

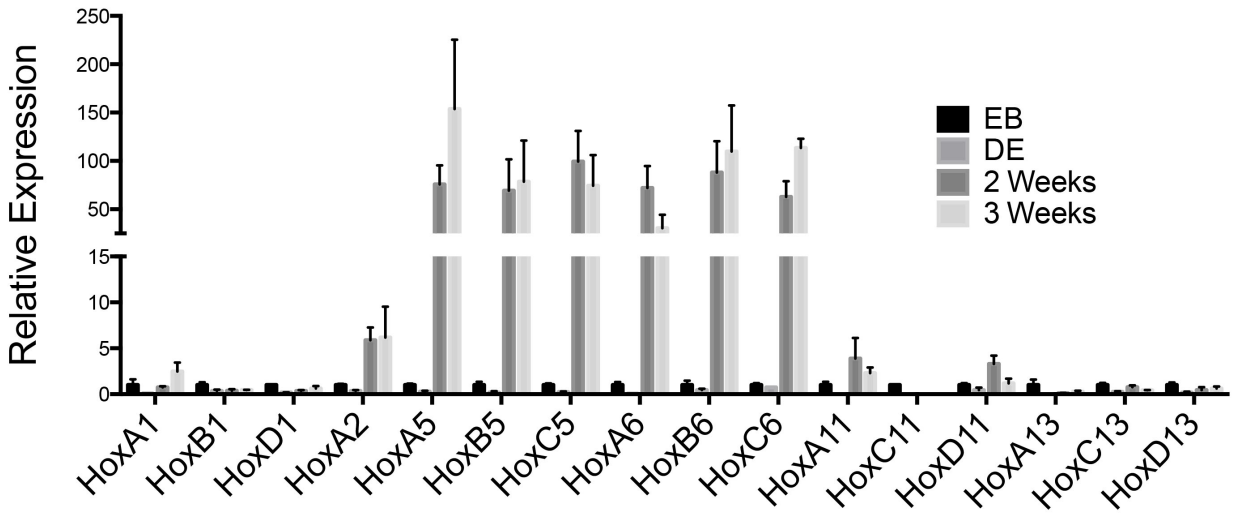

Figure 3.5. *Hox* genes are expressed and the *Hox* code is maintained *in vitro*. qRT-PCR analyses of the *Hox 1, 2, 5, 6, 11,* and *13* paralogous groups show that genes expressed in the pancreatic region of the embryo are expressed in culture at two and three weeks of differentiation. The *Hox 1, 2, 11,* and *13* groups, which function in the anterior and posterior regions of the embryo, are expressed at much lower levels compared to EB and endodermal stages. Results are mean±s.e.m. (n≥3).

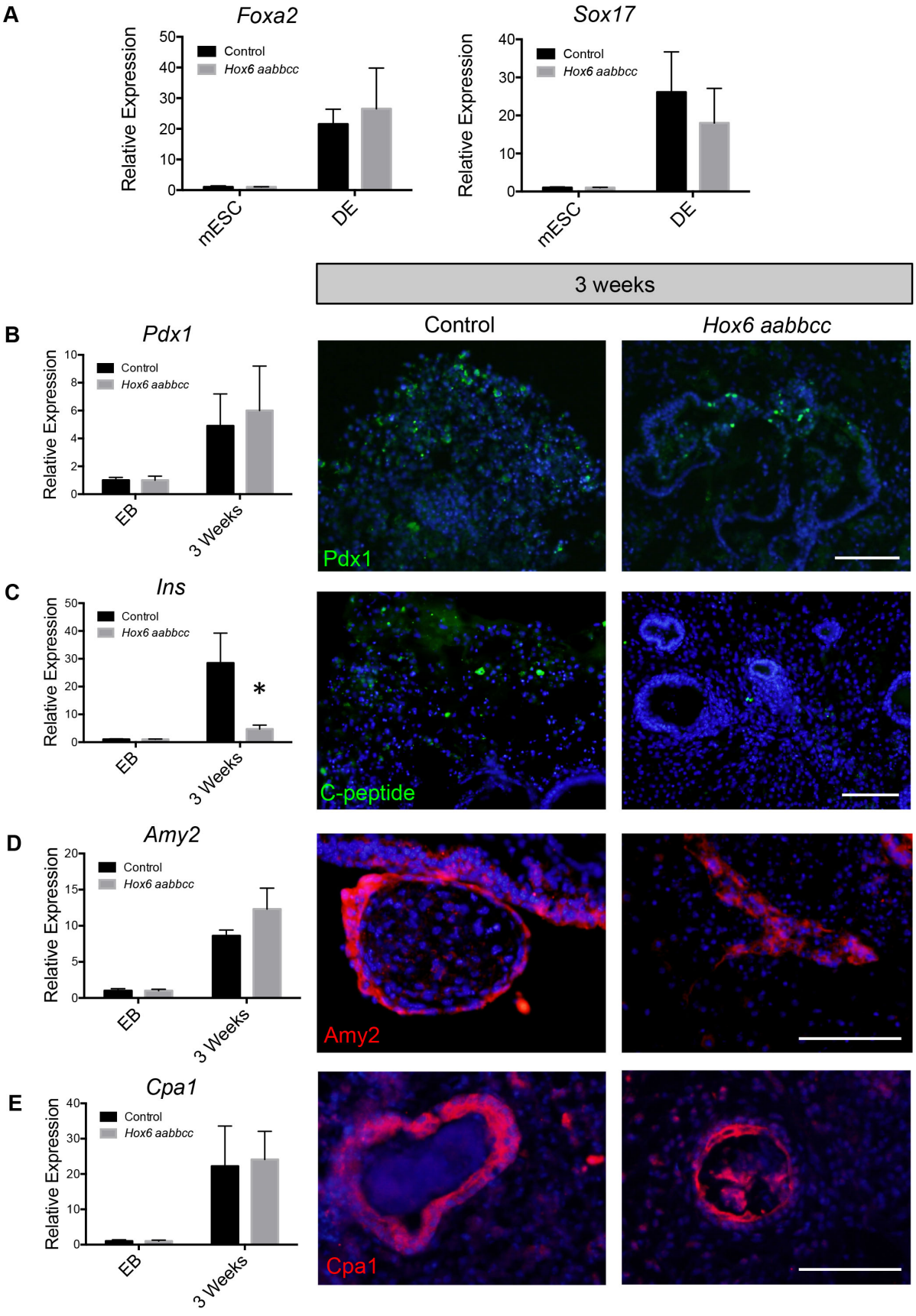
A
Foxa2
Relative Expression
Control
Hox6 aabbcc
mESC
DE
Sox17
Relative Expression
Control
Hox6 aabbcc
mESC
DE
3 weeks
Control
Hox6 aabbcc
B
Pdx1
Relative Expression
Control
Hox6 aabbcc
EB
3 Weeks
Pdx1
C
Ins
Relative Expression
Control
Hox6 aabbcc
*
EB
3 Weeks
C-peptide
D
Amy2
Relative Expression
Control
Hox6 aabbcc
EB
3 Weeks
Amy2
E
Cpa1
Relative Expression
Control
Hox6 aabbcc
EB
3 Weeks
Cpa1

Figure 3.6. *Hox6* mutant ESCs differentiate to endoderm and exocrine cells normally but exhibit lower Ins+ cell populations.
Endodermal genes *Foxa2* and *Sox17* are expressed comparably in both WT control and *Hox6* mutant mESCs after treatment with Activin A (A). *Hox6* mutant cells express similar amounts of *Pdx1* (B) but less *Ins* (C) than WT control cells. qRT-PCR and immunofluorescence analyses show that there is no difference in the competency of *Hox6* mutant and control mESCs differentiate to Amy2-positive (D) and Cpa1–positive (E) exocrine cells three weeks post RSF treatment. Scale bars 100 μM. Results are mean±s.e.m. **P*<0.05 calculated by Student's *t*-test (n≥3).

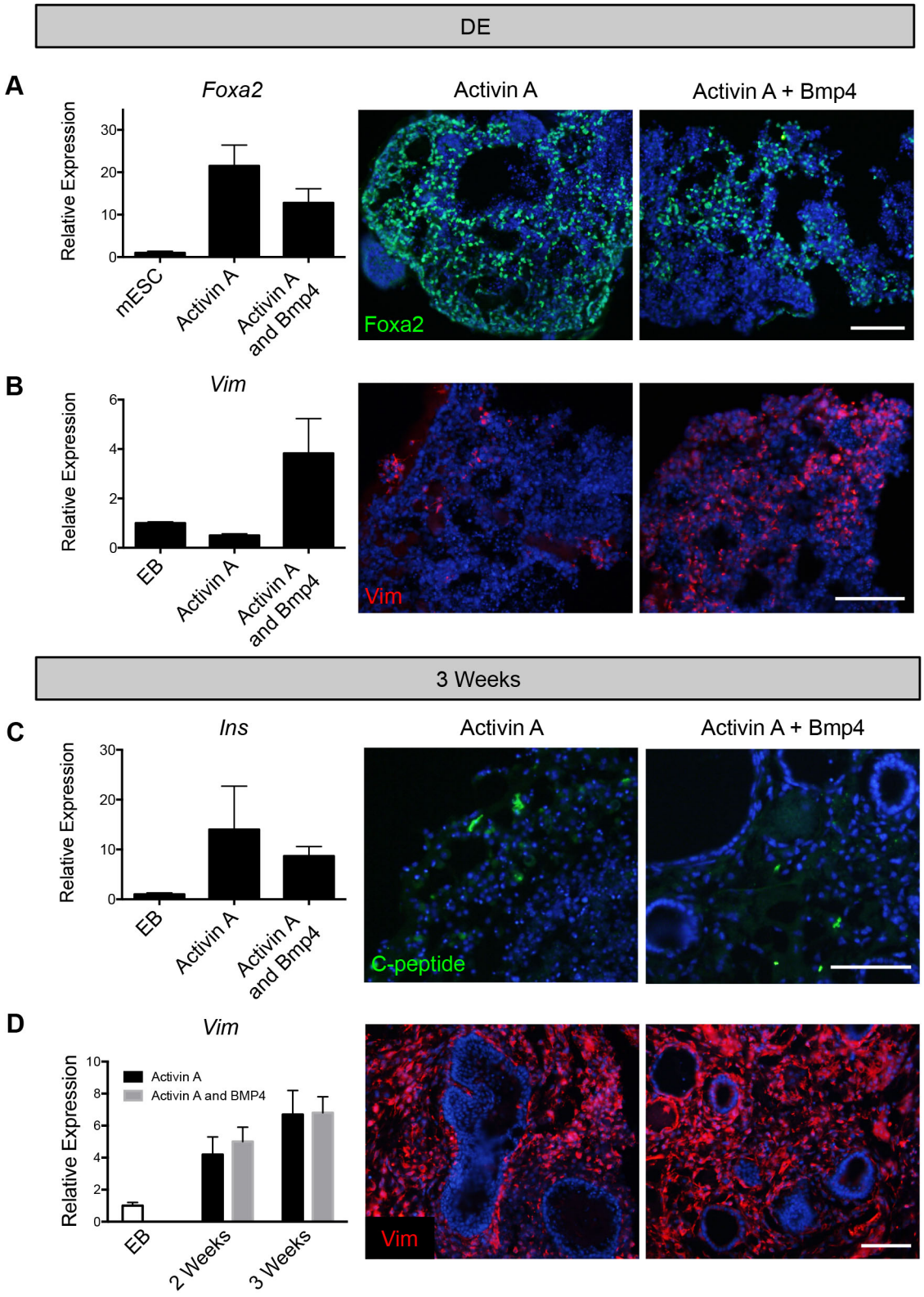
DE
A
Foxa2
Activin A
Activin A + Bmp4
Relative Expression
30
20
10
0
mESC
Activin A
Activin A and Bmp4
Foxa2
B
Vim
6
4
2
0
EB
Activin A
Activin A and Bmp4
Vim
3 Weeks
C
Ins
Activin A
Activin A + Bmp4
EB
Activin A
Activin A and Bmp4
C-peptide
D
Vim
Activin A
Activin A and BMP4
10
8
6
4
2
0
EB
2 Weeks
3 Weeks
Vim

Figure 3.7. BMP4 increases the amount of mesoderm early in differentiation but does not increase the amount of *Ins* expression overall.
Treatment of cells with both Activin A alone and with Activin A and Bmp4 produces populations with proportional amounts of endodermal *Foxa2* gene expression (A). Treatment of cells with Bmp4 in addition to Activin A significantly increases the *Vim*-positive population of mesenchymal cells (B). The amount of *Ins* expression and C-peptide staining remains unchanged when cells are treated with Activin A alone or with both Activin A and Bmp4 (C). Two weeks after RSF treatment, the amount of *Vim* expression and Vim-positive mesenchyme (shown at three weeks post RSF treatment) is comparable between cells treated with and without Bmp4 (D). Scale bars 100 μM. Results are mean±s.e.m. (n≥4).

Table 3.1. List of qRT-PCR primers for cultured cells

Gene	Forward Primer	Reverse Primer
Sox17	GGCACAGCAGAACCCAGAT	TTGTAGTTGGGGTGGTCCTG
Vim	AGAGAGAGGAAGCCGAAAGC	TCCACTTTCCGTTCAAGGTC
Foxa2	TAAAGTATGCTGGGAGCCGT	TCATGTTGCTCACGGAAGAG
Cdx2	AAACCTGTGCGAGTGGATG	TCTGTGTACACCACCCGGTA
Pax8	CGGCGATGCCTCACAACTCG	CCGGATGCTGCCAGTCTCGT
Nkx2.1	TACTGCAACGGCAACCTG	GCCATGTTCTTGCTCACGTC
Pdx1	CCACCCCAGTTTACAAGCTC	TGTAGGCAGTACGGGTCCTC
Ins	GTGACCAGCTATAATCAGAGACCA	CTCCCAAAGGGCAAGCAG
Amy2	AGCCTTTTTCAAACTGGTGG	TAAAGTGGCTGACAAAGCCC
Cpa1	ACACGGGACCAAGTTCAAGT	ACTTGATGCCCTGGCTGTAG
HoxA1	AAAAGAAACCCTCCCAAAACA	AGCTCTGTGAGCTGCTTGGT
HoxB1	TTCGACTGGATGAAGGTCAA	GGTGAAGTTTGTGCGGAGAC
HoxD1	TGAAAGTGAAGAGGAACGCC	TCTGTCAGTTGCTTGGTGCT
HoxA2	GATGAAGGAGAAGAAGGCGG	TGCCATCAGCTATTTCCAGG
HoxA5	CAGGGTCTGGTAGCGAGTGT	CTCAGCCCCAGATCTACCC
HoxB5	CTGGTAGCGAGTATAGGCGG	AGGGGCAGACTCCACAGATA
HoxC5	TTCTCGAGTTCCAGGGTCTG	ATTTACCCGTGGATGACCAA
HoxA6	GTCTGGTAGCGCGTGTAGGT	CCCTGTTTACCCCTGGATG
HoxB6	GAGACCGAGGAGCAGAAGTG	CAGGGTCTGGTAGCGTGTG
HoxC6	CAGGGTCTGGTACCGAGAGTA	TCCAGATTTACCCCTGGATG
HoxA11	AGGCTCCAGCCTACTGGAAT	CCTTTTCCAAGTCGCAATGT
HoxC11	GCGGCCGACGAGCTTAT	TTTTTCATGAGGATCTCAGTGACTGT
HoxD11	ACACCAAGTACCAGATCCG	AGTGAGGTTGAGCATCCGAG
HoxA13	AGCGGCTACTACCCGTGC	CGGTGTCCATGTACTTGTCG
HoxC13	TCAGGTGTACTGCTCCAAGG	CTCACTTCGGGCTGTAGAGG
HoxD13	CCCATTTTTGGAAATCATCC	TGGTGTAAGGCACCCTTTTC

Table 3.2. Primary antibodies

Antibody	Dilution	Vendor	Catalog Number
Rabbit anti-Amy	1:100	Sigma Aldrich	090M4801
Guinea pig anti-Pdx1	1:500	Abcam	AB47308
Mouse anti-Vim	1:200	Sigma Aldrich	091M4791
Rabbit anti-Cpa1	1:100	Rockland Immunochemicals	100-4152
Sox17	1:500	R&D Systems	AF1924
C-peptide	1:100	Cell Signaling	4593S
Foxa2	1:1,000	Seven Hills Bioreagents	WRAB-FOXA2

CHAPTER 4

CONCLUSION

Summary of findings

Hox6 genes are expressed in the mesenchyme of the pancreas and play a critical role in the differentiation of endocrine cells. At no point did we observe co-expression of *Hox6* with markers for endocrine cells, epithelium, blood vessels, neurons, or smooth muscle in the pancreas at embryonic stages. Although the exocrine compartment of the *Hox6* mutant pancreas is largely normal, we observed some minor early developmental defects. The *Hox6* mutant pancreas exhibited impaired branching of the epithelium at early stages with an apparent disruption of proper ductal plexus remodeling at early embryonic stages. This defect may be caused by the failure of MPCs of the pancreas to differentiate normally, as suggested by prolonged co-localization of Sox9 and Hnf1β. These proteins are associated with multipotency in the tip cells in the pancreas. At later stages, the expression of *Sox9* and *Hnf1β* normalizes, and these proteins are excluded from the tip cells, suggesting a delay in the transition of tip MPCs to unipotent acinar cells. This delay in MPC maturation does not result in extensive branching defects overall.

The endocrine compartment is significantly reduced throughout development and by E18.5, the *Hox6* mutant pancreas exhibits a greater than 90% decrease in

expression levels of all five endocrine hormones. This does not appear to be a defect in either the specification or delamination of Ngn3+ endocrine progenitor cells. We were unable to detect any defects in *Ngn3* expression, the abundance of Ngn3+ cells, or the ability of Ngn3+ cells to successfully turn off *Sox9* expression as they leave the ductal epithelium. There is a significant loss of several endocrine specific genes required for proper endocrine cell maturation such as *MafA, MafB, Nkx6.1, NeuroD, Isl1,* and *ChgA*. This suggests that the loss of mature endocrine cells occurs during the maturation process following the proper delamination of Ngn3+ endocrine progenitors from the ductal epithelium.

There are multiple developmental signaling pathways active in the mesenchyme that are critical for pancreas development including Fgf, Bmp, Notch, and Wnt (Puri and Hebrok, 2010). Microarray analysis performed at E12.5 comparing control and *Hox6* mutant pancreata identified defects in the Wnt signaling pathway, while other developmental pathways were relatively normal. Consistent with the microarray analysis, qRT-PCR showed significant decreases in expression of Wnt signaling pathway members *Wnt5a, Sfrp3,* and *Dkk1* at E12.5 in the *Hox6* mutant pancreas while members of the Fgf, Bmp and Notch pathways were unchanged.

Wnt5a is a ligand expressed in both the epithelium and mesenchyme of the pancreas, and *Sfrp3* and *Dkk1* are Wnt inhibitors expressed in endocrine cells and the epithelium of the pancreas (Heller et al., 2002). ISH analyses showed that expression of *Wnt5a* is dramatically decreased in the pancreas mesenchyme in the *Hox6* mutant pancreas while expression of *Wnt5a* in the epithelium was unchanged. These data

support a role for *Wnt5a* downstream of *Hox6* in the mesenchyme and may in fact be a direct target.

In addition to demonstrating the importance of *Hox6* genes in endocrine pancreas development *in vivo*, we show a role for *Hox6* in the *in vitro* differentiation of insulin-producing cells. We utilized a method of three-dimensional culture to encourage cell-cell interactions as a way to better mimic the *in vivo* environment of β-cell development and encouraged the formation of mesoderm, in addition to endoderm, in order to investigate the effect of genes expressed solely in the mesoderm. We first induced mESCs to form EBs, which are three-dimensional spheres capable of giving rise to all three germ layers. Next, we compared the ability of WT and *Hox6* null mESCs to differentiate to insulin-producing cells and found that there was significantly less *Ins* expression in the *Hox6* mutant cells. Equivalent amounts of endoderm markers, exocrine markers, and *Pdx1* was detected in control and mutant cultures indicating that the lack of *Ins* expression in the mutant cultures was not due to an inability of these mutant cells to differentiate in general. These data support an important role for *Hox6* genes, expressed just in the mesoderm, in the efficiency of mESCs to differentiate to insulin-producing cell *in vitro.*

Given the important role of the mesenchyme in β-cell differentiation, we also investigated the effect of manipulating the amount of mesenchyme throughout our differentiation protocol. Treating cells with Bmp4 has been shown to induce mesoderm differentiation in culture (Purpura et al., 2008; Sakurai et al., 2009). We treated the EBs with both Bmp4 and Activin A to induce a combination of mesoderm and endoderm respectively. Our data show that this significantly increases the population of mesoderm

without significantly inhibiting endoderm differentiation. Despite a significant increase in the abundance of mesoderm early in differentiation, there was no significant increase in the level of *Ins* expression compared to cultures without Bmp4 treatment. Additional analyses revealed that there was a comparable amount of mesenchyme produced regardless of the initial endoderm/mesoderm differentiation (Activin A or Activin A and Bmp4 treatment). This, unfortunately, may have masked any role of additional early mesoderm differentiation, as the signals for insulin-producing cells are critical at later stages in differentiation when the amount of mesenchyme was comparable between treatment conditions. Taken together, we have shown that mesenchymal cells present during directed differentiation cultures have a significant influence on the differentiation of the surrounding cells. Cell-cell signaling between different populations can have significant effects on the differentiation of desired cells, and including the proper complementary cells in culture may greatly impact the efficiency of successfully generating cells for use in the treatment of diabetes.

Contributions to the field

While the importance of the mesenchyme in pancreas development has been appreciated for more than 50 years, the specific molecular signals involved in mesenchymal to epithelial crosstalk remain largely unknown. Here we show that *Hox6* genes are expressed in the pancreas mesenchyme and are critical for endocrine pancreas development both *in vivo* and *in vitro*. Prior data on the functional roles for *Hox6* was limited to anteroposterior patterning, rib formation, and neuronal development (McIntyre et al., 2007; Mallo et al., 2010; Philippidou and Dasen, 2013). Our data now

identify a novel role for *Hox6* genes in the pancreas, an organ without a previously reported role for *Hox* genes. Specifically, we show that *Hox6* is required for proper *Wnt5a* expression in the mesenchyme, which directs endocrine cell differentiation in the mouse. These data show a regulatory relationship between *Hox* and Wnt signaling in organ development, a theme that is becoming more apparent (Kuss et al., 2014; Hrycaj et al., 2015).

In addition to their critical role in endocrine pancreas development *in vivo*, we also show that *Hox6* mutant mESCs are deficient in their ability to differentiate to insulin-producing cells *in vitro*. These data show a critical role for signals produced just in the mesoderm in directed differentiation experiments. The *in vitro* differentiation of functional β-cells is a very promising strategy to treat diabetes. Knowledge of the mesenchymal signals that promote β-cell differentiation *in vivo* have been critical to developing efficient protocols of differentiating insulin-producing cells from hESCs and iPSCs (Pagliuca and Melton, 2013). Uncovering the mesenchymal signals critical for β-cell differentiation, maturation, and function will be paramount to successfully developing functional β-cells to treat diabetes. Our work adds to this knowledge by uncovering another mesenchymal factor, *Hox6*, with a critical role in β-cell development.

Future Directions

Our work in pancreas development *in vivo* has shown a critical interaction between *Hox6* and Wnt signaling in the mesenchyme and endocrine cells of the pancreas. With the inactivation of *Hox6* in the mesenchyme and subsequent disruption in Wnt signaling, there is a dramatic reduction in the abundance of hormone producing endocrine cells. Despite this dramatic loss of endocrine cells, there was no change in

Ngn3-positive progenitor cells, proliferation of endocrine progenitors or mature endocrine cells, or increased apoptosis. One question that remains is the fate of those endocrine progenitor cells that do not mature to hormone producing cells. The fate of these cells could be investigated using lineage trace techniques. The *Ngn3*-Cre allele (Desgraz and Herrera, 2009) could be bred into the *Hox6* mutant mouse colony to drive expression of a reporter allele in *Hox6* triple mutants. We could then co-stain with a pan-endocrine marker such as ChgA to identify those reporter-positive cells that have matured to endocrine cells, while also identifying reporter-positive, ChgA-negative endocrine progenitor cells that did not mature. It will be interesting to determine if these cells remain semi-differentiated in the mesenchyme, re-insert back into the duct, or localize somewhere else.

Our data has shown that with the inactivation of *Hox6* function, there is a loss of *Wnt5a* expression in the mesenchyme and a dramatic loss of hormone-producing endocrine cells *in vivo.* We demonstrate the importance of Wnt5a in pancreas explant cultures showing that Wnt5a is sufficient to rescue endocrine cell differentiation. To further investigate the role of Wnt5a in the mesenchyme, we could use one of two techniques. First, we could take *Wnt5a* null mesenchyme from *Wnt5a* global knockout embryos and recombine it with WT, stage matched epithelium in culture. Second, we could genetically inactivate *Wnt5a* function specifically in the mesenchyme of the pancreas. Utilizing tools such as the *Nkx3.2*-Cre (Landsman et al., 2011) mouse and crossing it to a conditional *Wnt5a* knockout mouse (Miyoshi et al., 2012) would allow us to examine the specific role of *Wnt5a* function in the mesenchyme. Using one (or both) of these methods, we would first compare the phenotypes from mesenchymal *Wnt5a*

knockout mice to phenotypes observed in the *Hox6* mutants including, endocrine cell abundance, Wnt inhibitor expression, and branching. The results of this experiment would allow us to assess several points. First, does *Wnt5a* affect the expression of Wnt inhibitors *Sfrp3* and *Dkk1*? Second, is the loss of endocrine cells and branching disruptions both caused by the loss of *Wnt5a*? Third, is *Wnt5a* necessary for endocrine cell differentiation, or just sufficient to rescue the *Hox6* mediated loss of endocrine cell differentiation? If the phenotypes observed in mesenchyme specific loss of *Wnt5a* do not match that of the *Hox6* phenotypes, we would hypothesize that there are other factors affected in the pancreas mesenchyme that act in parallel to *Wnt5a.* These may include additional Wnt ligands such as *Wnt2b* or *Wnt11,* which have reduced levels of expression in the Hox6 mutant pancreas at later stages than the initial reduction of *Wnt5a* expression (Chapter 2).

It has been recently shown that Hox proteins are capable of inducing the expression of *Wnt5a* (Kuss et al., 2014), but further work is needed to determine if *Wnt5a* is a direct target of Hox6 proteins in the pancreas mesenchyme. Unfortunately, the lack of reliable antibodies for ChIP experiments makes this difficult. An alternative approach to determine the binding sites of Hox6 is to generate tagged Hox6 proteins *in vitro* as a method to utilize commercially available antibodies for ChIP and/or ChIP-Seq.

We have shown a loss of *Sfrp3* and *Dkk1* expression in Ngn3-positive endocrine progenitor cells at early stages in development. We do not know if expression of these inhibitors is activated in Wnt5a mediated rescue of endocrine cell differentiation due to the experimental design. Since pancreas explants were collected six days after dissection, at what equates to E18.5, we were unable to detect Wnt inhibitor

localization. We could test the capability of Wnt5a treatment to promote Wnt inhibitor expression by collecting the pancreas explants after two days in culture instead of six. The quantity of Wnt inhibitor detection could be quantified similarly to how endocrine cell abundance was analyzed to assess inhibitor abundance between control and *Hox6* mutant pancreata.

While we have shown that several members of the Wnt signaling pathway are disrupted in the *Hox6* mutant pancreas, we have been unable to demonstrate changes in overall Wnt signaling activity. qRT-PCR for readouts such as *Axin2* has not shown any significant changes in expression between control and *Hox6* mutant pancreata. This may be due to the small number of cells (i.e. the endocrine progenitor cells) with disrupted Wnt activity, while the rest of the pancreas is relatively unaffected. One method to assess changes in Wnt activity in specific cells would be to cross our *Hox6* mutant mice with Wnt reporter mice such as the BAT-gal or *Axin2*-LacZ animals (Jonckheere et al., 2008) to investigate the presence or absence of Wnt activity in specific cells such as Ngn3-positive endocrine progenitor cells. These reporter mice could support our hypothesis that Ngn3-positive endocrine progenitor cells in the *Hox6* mutant have excess Wnt signaling, whereas Ngn3-positive cells in the control lack Wnt activity.

The role of Wnt inhibitors in endocrine cell differentiation is also largely uncharacterized, and to provide insights into their function we could investigate endocrine cell defects in *Dkk1* and *Sfrp3* knockout mice. Mice have been generated with a knockout of each of these genes (Mukhopadhyay et al., 2001; Jang et al., 2013), but there has been no analysis of the endocrine pancreas in either. It could be that a

double knockout of both *Dkk1* and *Sfrp3* is necessary to see an effect due to functional redundancy of these inhibitors. If there is no detectable endocrine cell phenotype in the single mutant of either *Dkk1* or *Sfrp3* mutant mice, it would be interesting to cross these two knockout lines together to determine if the endocrine pancreas is significantly affected. This would show a critical role for Wnt inhibitors in the differentiation of endocrine cells of the pancreas.

Hox genes have been shown to be critical for AP patterning, and the LOF of *Hox* paralogous groups results in anterior homeotic transformations (Lewis, 1978). One possible cause of the observed pancreas phenotypes might be the anterior homeotic transformation of the pancreas mesenchyme to another type of mesenchyme normally produced in an anterior region. Further analysis of our microarray data could provide clues as to the nature of the mesenchyme present in *Hox6* mutant pancreata compared to controls. Microarray analyses could also be performed on physically separated mesenchyme from E11.5 pancreata and other organs such as E11.5 lungs. We could then compare the expression profiles of mesenchyme from *Hox6* mutant pancreas, WT control pancreas, and other WT organs. This would allow us to determine if the *Hox6* mutant pancreas mesenchyme is more similar to control mesenchyme or to that of other organs.

We have shown that *Hox6* genes are expressed through most of development, however their expression declines and is eventually undetected at later stages of development by qRT-PCR. It would be interesting to investigate possible *Hox6* expression in further detail at these later stages and at adult stages. It has been previously shown that the percentage of the pancreas that is made up of mesenchyme

dramatically decreases over time (Landsman et al., 2011). Does the level of *Hox6* expression actually decreases in the pancreas mesenchyme or is this an artifact of the increase in the proportion of *Hox6*-negative epithelium compared to *Hox6*-positive mesenchyme? The physical composition of the organ makes it extremely difficult, if not impossible, to dissect the mesenchyme from the epithelium at these late stages. To circumvent this difficulty, we could utilize *Hoxb6CreER^{T};Rosa26-tdTomato* embryos and induce recombination at late stages to determine if *Hox6* is expressed at later stages of pancreas development. For more detailed analyses of mesenchymal expression, cell sorting could be utilized to separate the mesenchyme from the epithelium by the detection of the red fluorescent protein. qRT-PCR analysis of RNA extracted just from pancreas mesenchyme would allow us to determine if *Hox6* expression does in fact decline through development, or if the signal is lost in the overwhelming majority of the *Hox6*-negative pancreas epithelium.

We could also take advantage of *Hoxb6CreER^{T};Rosa26-tdTomato* mice to examine *Hox6* expression in adult stages. If *Hox6* expression does not persist into adult stages it would be interesting to assess whether *Hox6* expression can be reactivated in pancreas injury. We could investigate whether the developmental pathways involving Hox6 function in the mesenchyme are utilized in adult stages to help to repair the pancreas. The generation of a mouse with a fluorescent protein knocked in to a *Hox6* locus would also allow us to visualize real time expression of *Hox6* at any time point desired, and to perform live imaging after pancreas injury.

In addition to the necessary role of mesenchymal factors in endocrine cell development *in vivo,* our work in the differentiation of mESCs to insulin-producing cells

has shown a significant role for factors produced by the mesenchyme *in vitro*. While our study showed that inducing mesenchyme differentiation early in the protocol had no effect on the efficiency of IPC differentiation, it is possible that manipulating the mesenchyme at different stages could play an important role. Sneddon et al. showed that providing progenitor cells at different stages of differentiation with the appropriate mesenchyme significantly enhanced the proliferative capacity of these cells (Sneddon et al., 2012). It is possible that inducing or providing appropriate mesenchyme to progenitor cells may greatly increase the number of functional β-cells generated, providing a greater pool of cells to use in cellular therapies of diabetes.

After demonstrating a critical role for Wnt5a in endocrine cell differentiation *in vivo,* it would be interesting to investigate whether Wnt5a can have an effect on insulin-producing cell differentiation *in vitro*. Wnt5a could be added to the media after treatment with RA, bFGF, and SANT-2 when endocrine cell differentiation is starting to occur. We could compare the efficiency of differentiation in WT control cells with or without Wnt5a in the media. This would allow us to assess any beneficial role of adding a non-canonical Wnt ligand to the media. We could also add Wnt5a to the differentiation media in *Hox6* mutant mESC cultures to investigate whether Wnt5a is capable of rescuing endocrine cell differentiation *in vitro* similar to *in vivo.*

Utilizing animal models, such as the mouse and zebrafish, has provided insights into pancreas and endocrine cell development, however there are critical differences between these animals and human endocrine pancreas development (Nair and Hebrok, 2015). While animal models are important and accessible tools for the study of β-cell development and function, more will need to be learned about human β-cell

development and function to provide greater insights into developing new treatments for diabetes. It will be interesting to analyze genetic data from diabetic patients to determine if any contain mutations of *Hox6* genes. While many *Hox6* genes will need to be disrupted to see any dramatic phenotype by themselves, it is possible that LOF of one or more *Hox6* genes in parallel to other critical endocrine cell differentiation factors may have a cumulative effect on β-cell production in Type I diabetic patients.

Current protocols to develop functional β-cells *in vitro* have made significant strides to generate cells for treatment, although optimization of these protocols is needed (Pagliuca et al., 2014). One major issue will be the generation of a pure population of insulin-producing cells. This will be necessary to prevent the presence of remaining multipotent progenitor cells from forming cysts or tumors in the patient (Pagliuca and Melton, 2013). A more complete understanding of the signals critical for directing endocrine cell differentiation via mesenchymal to epithelial crosstalk may be the key factor in establishing clinical protocols for the generation of functional β-cells to treat diabetes.

APPENDIX: PUBLICATIONS AND MANUSCRIPTS

Chapter 2 is based on a manuscript in revision titled “Mesenchymal *Hox6* Function is Required for Pancreatic Endocrine Cell Differentiation” with authors listed as Brian M. Larsen, Steven M. Hrycaj, Micaleah Newman, Ye Li and Deneen M. Wellik.

Chapter 3 is based on a manuscript in submission titled “*Hox6* function in the *in vitro* differentiation of mESCs to insulin-producing cells” with authors listed as Brian M. Larsen, Micaleah Newman, Derek T. Lukacs, Jason R. Spence, and Deneen M. Wellik.

Hrycaj SM, Dye BR, Baker NC, Larsen BM, Burke AC, Spence JR, Wellik DM. Hox5 Genes Regulate the Wnt2/2b-Bmp4-Signaling Axis during Lung Development. Cell Rep. 2015 Aug 11;12(6):903-12. doi: 10.1016/j.celrep.2015.07.020. Epub 2015 Jul 30. PubMed PMID: 26235626; PubMed Central PMCID: PMC4536095.

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