BMP SIGNALING THROUGH BMPR1A IS REQUIRED FOR ESTABLISHMENT OF PANCREATIC LATERALITY

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THIS DISSERTATION IS DEDICATED TO MY FAMILY,

PAST, PRESENT AND FUTURE

BMP SIGNALING THROUGH BMPR1A IS REQUIRED FOR ESTABLISHMENT OF PANCREATIC LATERALITY

by

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Pancreatic development begins in the mouse, at embryonic day 8.5, as two patches within the gut tube that are located between the stomach and the duodenum. These patches begin to bud, proliferate, and differentiate to become the dorsal and ventral pancreas. During pancreatic bud development several tissues that are adjacent to the gut tube provide important signals for pancreatic development. Various bone morphogenetic proteins (Bmps) and Bmp receptors (Bmprs) are expressed in the pancreas and in the tissues that flank the pancreatic bud during pancreatic development. *Bmp7*, *BmpR1a*, and *BmpRII* are expressed in the pancreatic endoderm during pancreatic bud development. *Bmp2, Bmp4, BmpR1a* and *BmpRII* are expressed in the dorsal aorta which transiently flanks the pancreatic bud. *Bmp4, Bmp7, BmpR1a, BmpR1b, and BmpRII* are expressed in the pre-pancreatic mesenchyme. Interestingly, *Bmp4* is expressed asymmetrically along the gut in the mesogastrium, preceeding gut turning. Transgenic knock down of BMP signaling via the secreted BMP antagonist Noggin in the pre-pancreatic milieu results in

reduced pancreas, spleen, stomach and in failure of pyloric sphincter formation. Interestingly, BMP knockdown also results in defects in lateral growth of the pancreas. Specifically, BMP knockdown prevents formation of the splanchnic mesodermal plate (SMP), the asymmetrically forming mesothelial structure that accompanies leftward growth of the pancreas. Conversely, over expression of *Bmp2* in the pre-pancreatic milieu does not result in defects in SMP formation or lateral growth of the pancreas. However, overexpression of *Bmp2* in the pancreas epithelium resulted in failure to differentiate into endocrine and exocrine cell lineages. Global knockout of *BmpR1a*, but not *BmprII*, prior to pancreatic bud development results in developmental defects in SMP formation and pancreatic laterality. *Bmpr1a* knockout results in reduced SMP expression of *Bapx1*, a gene required for SMP formation and lateral growth of the pancreas. Therefore, defects in lateral growth of the pancreas in *Bmpr1a* mutants are likely mediated by *Bapx1* and, accordingly, *Bmpr1a* mutant pancreata exhibit misregulation of genes that require *Bapx1*, including *Fgf9* and *Fgf10*. Additionally, deletion of *Bmpr1a* also leads to misregulation of *Barx1*, another gene with SMP restricted expression. Deletion of *BmpR1a* specifically from the pre-pancreatic endoderm or endothelial (aorta) cells did not show defects with respect to pancreatic growth, development, or differentiation. Finally, work herein shows that BMP-BMPR1A signaling to the endothelium specifically is required for vascularization of the pancreatic bud and SMP formation. This study presents the first evidence of a cell-cell signaling molecule playing a role in left-right patterning during organogenesis.

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LIST OF ABBREVIATIONS

Alk – Activin receptor-like kinase

bHLH – basic Helix-Loop-Helix

BMP – Bone Morphogenetic Protein

BRE – BMP Response Element

CCK – Cholecystokinin

Dpc – Days post coitum

DM – Diabetes Mellitus

E – Embryonic day

Fgf – Fibroblast growth factor

FSH – Follicle-Stimulating Hormone

GFP – Green Fluorescent Protein

GS – Glycine / Serine

GSIS – Glucose Stimulated Insulin Secretion

Hh – Hedgehog

Id – Inhibitor of differentiation

IDDM – Insulin-Dependent Diabetes Mellitus

IDF – International Diabetes Federation

Ihh – Indian hedgehog

ISMAD – Inhibitory SMAD

LPM – Lateral Plate Mesoderm

LR – Left-Right (asymmetry)

MH – Mad Homology

Ngn3 – Neurogenin 3

NIDDM – Non Insulin-Dependent Diabetes Mellitus

NVP – Nodal Vesicular Parcel

Pdx1 – Pancreatic and duodenal homeobox 1

PGC – Primordial Germ Cell

RSMAD – Regulatory SMAD

Shh – Sonic hedgehog

SPM – Spleno – Pancreatic Mesenchyme

SMP – Splanchnic Mesodermal Plate

TF – Transcription Factor

TGFβ – Transforming Growth Factor beta

WHO – World Health Organization

β-gal – β-galactosidase

CHAPTER ONE

Introduction

OVERVIEW

The focus of this dissertation is to determine the role of bone morphogenetic proteins (BMPs) during pancreatic development. BMPs are a class of secreted signaling proteins that have a variety of important roles in several developmental processes. BMPs bind to their receptors, activating downstream effectors known as SMADs, which in turn activate downstream inhibitor of differentiation (Id) proteins that function as transcriptional repressors to regulate cell fate, proliferation, differentiation and maintenance of several tissues during development. BMPs can function in a paracrine or autocrine manner by being secreted by select tissues and signaling to adjacent tissues during development. My work will show that several BMPs and their cognate receptors are expressed in dynamic patterns during the development of the mouse embryo and during organogenesis, as well as the tissues that make up and flank the developing pancreas.

The pancreas is a multifunctional organ that comprises exocrine and endocrine tissue. The primary functions of the pancreas are two-fold: the exocrine pancreas produces and secretes digestive enzymes and the endocrine pancreas produces and secretes hormones that regulate blood glucose levels. The pancreas develops from two patches of cells that evaginate from a region of the gut endoderm located between the future stomach and duodenum. As these patches of gut endoderm proliferate and evaginate, they become the dorsal and ventral pancreatic buds.

The aorta and pancreatic mesenchyme are tissues located adjacent to the dorsal patch of endoderm and have been shown to provide important signals that are required

for initial evagination and differentiation of the dorsal pancreatic bud. However, the factors that are secreted by these tissues and important reciprocal signaling events that occur during pancreatogenesis are still unknown. In the next chapter, I will provide a concise review of background information relevant to this dissertation. The literature review will address the general structure and function of the pancreas, discuss diseases associated with misregulation of the pancreas, introduce cellular and morphogenetic events that occur during pancreatic development, as well as cover the molecular basis for BMP signaling.

This thesis will detail the work I have conducted towards understanding the role of BMPs during pancreatic development. The first set of experiments address the questions: Where are BMPs expressed during murine embryogenesis? What BMPs are expressed in the pancreas and the adjacent tissues during pancreatic development? The goal of these studies is to place potential molecular players, spatially and temporally to better understand their potential roles. Detailed gene expression analysis for several BMP ligands and BMP receptors were carried out and interestingly, I found that several Bmps and their receptors were shown to be expressed in the pancreas and adjacent tissues throughout pancreatogenesis. Of particular interest was the gut expression pattern of *Bmp4*, which is expressed asymmetrically across the mesenchyme of the developing gut tube, suggesting a potential role of BMP4 in left-right (LR) determination of the gut.

The second set of experiments aims to address the role of BMP signaling during lateral growth of the pancreas. Transgenic mice were developed that express *Noggin*, a secreted BMP antagonist, in the pancreatic milieu. Transgenic embryos with disrupted BMP signaling displayed reduced pancreas, spleen, and stomach, and severe defects in

the pyloric sphincter. Interestingly, these mice also exhibit defects in the formation of the splanchnic mesodermal plate (SMP), (the asymmetrically forming mesothelial structure that drives leftward growth of the pancreas), and lateral growth of the pancreas. These defects demonstrate a role for BMP signaling in lateral growth of the pancreas. Additionally, transgenic mice were constructed that overexpress *Bmp2* in the pancreas. *Bmp2* overexpression on the other hand, did not result in defects in the lateral growth of the pancreas. However, *Bmp2* overexpressing transgenic pancreata were unable to differentiate into endocrine or exocrine cell types. This implies that in addition to a role in lateral growth of the pancreas, BMP signaling may also play a role during cell type specific differentiation of the pancreas.

The final set of experiments will address the questions: Which receptors are required for lateral development of the pancreas in response to BMP signals? What is the mechanism by which BMP signaling drives lateral growth of the pancreas? To determine the mechanism of BMP signaling during pancreatogenesis, we have employed several conditional knockout mouse studies disrupting BMP signaling to either the pancreas or adjacent tissues by deleting different Bmp receptors in different tissues. Global deletion of Bmp receptor II (*BmpRII*) had no affect on pancreatogenesis or lateral growth of the pancreas; however, global deletion of Bmp receptor 1a (*BmpR1a*) resulted in defects in lateral growth of the pancreas as well as mis-expression of a number of left-right (LR) markers, indicating defective initiation of lateral growth of the pancreas. For instance, *Bmpr1a* knockout resulted in reduced SMP expression of *Bapx1*, a gene required for SMP formation and lateral growth of the pancreas. Therefore, defects in lateral growth of the pancreas in *Bmpr1a* mutants are mediated by *Bapx1* and, accordingly, *Bmpr1a* mutant

pancreata exhibit misregulation of genes that require *Bapx1* including *Fgf9* and *Fgf10*. Additionally, deletion of *Bmpr1a* exhibits misregulation of *Barx1*, another gene with SMP restricted expression.

Endothelial specific *BmpR1a* knockout embryos showed defective SMP formation and pancreatic vascularization by E10.5. Additionally, endodermal deletion of *BmpR1a* showed no effect on lateral growth of the pancreas. This work reveals two distinct and previously unnoticed roles for BMP signaling during pancreatic development. Data presented in this thesis suggests that BMP signaling is required for the lateral growth of the pancreas.

Future studies will address the role of BMP signaling to the pancreatic mesenchyme and determine the mechanism by which BMP signaling drives lateral growth of the pancreas. Interestingly, I have shown that BMPs are functioning in an asymmetric manner in blood vessels during pancreatogenesis. It will be of interest to see whether BMP signaling mediates lateral growth of the pancreas through vascularization of the pancreatic bud. This work marks the first example of a cell-cell signaling molecule driving LR patterning in the embryo during organogenesis.

CHAPTER TWO

Literature Review

Structure and Function of the Pancreas

The pancreas is a multifunctional, highly branched gland that consists of exocrine and endocrine tissue. The exocrine tissue functions to secrete digestive enzymes that aide in the breakdown of carbohydrates, fats and proteins. The endocrine tissue of the pancreas secretes hormones such as insulin and glucagon which regulate the level of glucose in the blood. This first section of chapter 2 will address the functional aspects of the adult exocrine and endocrine pancreas.

Exocrine Pancreas

Acinar and duct cells constitute the main types of cells that compose the exocrine tissue of the pancreas, comprising more than 90% of the pancreas (Githens 1988). The acinar cells of the pancreas secrete several digestive enzymes including: trypsin and chymotrypsin, enzymes that break down proteins; steapsin, which converts triglycerides into fatty acids; pancreatic amylase, which breaks down starches; and nucleases (DNAse and RNAse), which break down nucleic acids, among others. The exocrine pancreas, in concert with the liver and gallbladder, works to digest food as it enters the small intestine. The word acinar comes from the Latin word acinus, which means ‘grape’. This is fitting because the acinar cells are arranged in lobules around a highly branched array of pancreatic ducts resembling a bunch of grapes. The ducts of the pancreas are also part of

the exocrine pancreas and serve to shuttle pancreatic juice into the duodenum. Centroacinar cells are another type of exocrine cell type line the pancreatic ducts at the neck of the acinus and function to secrete mucin and a bicarbonate and salt solution into the small intestine to serve as a buffer to keep the environment in the small intestine at pH 7 and 8 thereby neutralizing stomach acid and attenuating pepsin activity. Pancreatic juice is clear and contains water, salts, sodium bicarbonate, and digestive enzymes.

Pancreatic juice flow is triggered when food enters the duodenum from the stomach. There are two avenues by which pancreatic juice release is triggered, via neuronal and hormonal induction. As food enters the gastric stomach, vagus nerves send impulses to the pancreas. These impulses instruct the pancreatic acini to increase enzyme secretion. Partially digested food then enters the duodenum from the stomach. Fatty acids and amino acids contained in the acidic chime from the stomach trigger cholecystokinin (CCK) to be released by enteroendocrine cells into the bloodstream. Additionally, the acidic chime promotes secretion of secretin into the blood by enteroendocrine cells. CCK functions to increase enzymes in the pancreatic juice, while secretin serves to increase bicarbonate ions in pancreatic juice.

Endocrine Pancreas

Endocrine cells comprise about 1 million clusters of cells which are scattered throughout the pancreas in structures called islets of Langerhans in humans. The endocrine pancreas constitutes only about 1.5-2% of the total mass of the pancreas and weighs only 1-2 grams in humans (Githens 1988). Islets consist of alpha, beta, delta,

epsilon and pancreatic polypeptide cells types (Tortora and Grabowski 1996; Collombat, Hecksher-Sorensen et al. 2006; Cleaver and MacDonald 2009). Alpha cells comprise 20% of the cells in the islet and secrete glucagon, which functions to raise blood glucose levels. Beta cells make up 70% of the islet and secrete insulin, which functions to lower blood glucose levels. These hormones drive the storage of glucose in times when energy is not needed and release glucose when it is. Delta cells comprise 5% of the islet and secrete somatostatin, a hormone that inhibits release of insulin and glucagon. Epsilon cells of the pancreas secrete ghrelin (Date, Nakazato et al. 2002; Gnanapavan, Kola et al. 2002; Volante, Allia et al. 2002; Wierup, Svensson et al. 2002), which functions to regulate insulin release by the islets by inhibiting glucose-induced insulin release (Dezaki, Hosoda et al. 2004) and glucose-stimulated insulin secretion (Colombo, Gregersen et al. 2003; Reimer, Pacini et al. 2003). Pancreatic polypeptide cells (F cells) make up 5% of the islet and function to regulate endocrine and exocrine secretion of the pancreas. Pancreatic polypeptide also promotes contraction of the gallbladder (Tortora and Grabowski 1996).

The process by which blood glucose homeostasis is regulated involves the constant and finely tuned dynamic and opposing action of insulin and glucagon. Low glucose levels in the blood (hypoglycemia) triggers release of glucagon from alpha cells. Glucagon serves as a hormone that signals to the liver (hepatocytes) to promote conversion of glycogen, lactic acid, and amino acids into glucose. This glucose is then released by the hepatocytes into the blood stream, resulting in increased blood glucose levels (hyperglycemia), and results in glucagon inhibition. As glucose levels rise, insulin is released from pancreatic beta cells. Insulin then functions mainly to promote uptake of

glucose by cells (especially skeletal muscle), and promotes conversion of glucose to glycogen for energy storage (Tortora and Grabowski 1996).

Pancreatic Dysfunction

The pancreas is critical for the production of digestive enzymes and hormones such as insulin and glucagon, which control blood sugar. Many severe human diseases are caused by the misregulation of pancreatic functions. Dysfunction of pancreatic cell types can lead to common diseases such as pancreatic cancer and diabetes. The goal of research in the pancreatic field of studies is the discovery of cures for these conditions. A better understanding of the cellular and molecular events that drive pancreatic development will aid in the design of treatments for these debilitating diseases.

Diabetes

Conditions that result in increased glucose in the blood are collectively called diabetes mellitus (DM) (Gavin, Alberti et al. 2003). Type 1 and type II diabetes mellitus are prevalent diseases. Type I diabetes is caused by insufficient levels of insulin in the bloodstream due to destruction of beta cells and is accordingly called insulin-dependent diabetes mellitus (IDDM). This type of diabetes is managed by regular insulin injections. IDDM is sometimes referred to as juvenile-onset diabetes due to its propensity for onset before 20 years of age. IDDM has both genetic and environmental components that are poorly understood and is usually caused by an autoimmune reaction by which the

immune system inappropriately destroys its own pancreatic beta cells (Gavin, Alberti et al. 2003). There is no cure for type I diabetes, but much progress is being made towards finding treatments, including cell replacement therapy to replace destroyed pancreatic beta cells (D'Amour, Bang et al. 2006; Zhou, Brown et al. 2008).

By far the most common form of diabetes is type II diabetes mellitus, comprising 90% of all known cases of diabetes. As opposed to type I diabetes, type II diabetes is mostly diagnosed after age 40, usually in overweight individuals and therefore is sometimes referred to as maturity-onset diabetes (Gavin, Alberti et al. 2003). Type II diabetes can usually be controlled by change of diet, exercise and weight loss; however, in extreme cases of type II diabetes, insulin is unable to function in the body due to a number of reasons including defects in insulin receptor expression or function. This is known as non insulin-dependent diabetes mellitus (NIDDM). This type of diabetes is the most difficult to manage and poses a great challenge for therapeutic intervention.

According to the International Diabetes Federation (www.IDF.org), diabetes was the cause of 3.8 million deaths worldwide in 2007, accounting for about 6% of total global mortality. The same number of people succumb to HIV/AIDS every year. Currently, there are no cures for diabetes; however, further understanding of pancreatic development will aid in the effort to find treatments for diabetes.

Pancreatic Cancer

According to the National Cancer Institute (www.cancer.gov), more than 29,000 people are diagnosed with pancreatic cancer per year in the United States. Patients

diagnosed with pancreatic cancer have only a 5% chance to live more than 5-years according to the World Health Organization (www.WHO.int/en/) and (Jemal, Siegel et al. 2008). Although pancreatic cancer constitutes only 2 percent of all cancers diagnosed, pancreatic cancer is the fourth leading cause of cancer death due to its low survival rate (American Cancer Society). Pancreatic cancer is usually caused by misregulation of exocrine cell proliferation, and many of these cancers are malignant. 95% of exocrine pancreatic cancers are adenocarcinomas, a cancer type that usually originates in the duct cells of the pancreas. Less frequently, exocrine cancers arise from acinar cells of the pancreas (ACS 2008). Far less common are endocrine pancreatic cancers, known as neuroendocrine tumors or islet cell tumors. These tumors can be further distinguished based on their ability to function. Islet cell tumors that can still function to secrete hormones such as insulin are termed "functioning tumors" and are usually benign. Non functioning islet cell tumors on the other hand are unable to secrete hormones and have a propensity to be malignant (ACS 2008). Interestingly, pancreatic dysfunction has a propensity to affect multiple processes of the pancreas. For example, 80% of patients with pancreatic cancer also display signs of diabetes, and accordingly, DM is considered a risk factor for pancreatic cancer (Permert, Ihse et al. 1993). Understanding pancreatic regulation during development will aid in strategies for the treatment of pancreatic cancer.

Development of the Pancreas

The pancreas is a compound gland that contains exocrine tissue, including acini and ducts, and endocrine tissue interspersed in clusters of cells called islets of Langerhans. The pancreas forms from a patch of embryonic endoderm in response to numerous extrinsic signaling events in the form of cell-cell signaling molecules that are exchanged between the endoderm, the notochord, the pancreatic mesenchyme, and the dorsal aorta (Fig.2.1 and (Kim and MacDonald 2002)). In addition, several intrinsic factors such as transcription factors have been identified that play important roles during the growth and differentiation of the pancreas (Kim and MacDonald 2002; Oliver-Krasinski and Stoffers 2008). The goal of research in pancreatic development is to elucidate the relationships between extrinsic and intrinsic events exchanged by the developing pancreas and adjacent tissues and understand the role that each play to drive the transformation of early endoderm into a mature, functioning pancreas (Cleaver and MacDonald 2009). Understanding the symphony of signaling and regulatory events required during pancreatic differentiation will lead researchers to the ultimate goal of facilitating therapeutic countermeasures for debilitating diseases such as diabetes mellitus and pancreatic cancer.

Pancreatogenesis overview

Shortly after gastrulation, the definitive endoderm consists of a single layer of epithelial cells which transforms from a sheet into a tube. At one point along this hollow tube, the pancreatic anlagen forms at around embryonic day 8.75 (E8.75) and begins to express pancreatic duodenal homeobox 1 (Pdx1). The pancreas arises from two patches

of endoderm located between the developing stomach and duodenum. One patch is located dorsally and another ventrally along the gut tube, these patches give rise to the dorsal and ventral pancreas, respectively. The work discussed in this thesis focuses on the development of murine dorsal pancreas, which will eventually give rise to the gastric and splenic lobes of the pancreas. Any reference to pancreatic development made from hereon should be assumed to be with respect to dorsal pancreatic development. At day 9 of pancreatic development, the pancreatic endoderm begins to proliferate, resulting in an outgrowth of endodermal epithelia surrounded by pancreatic mesenchyme known as the 'pancreatic bud'. Very little is known about the early inductive signals that are required for pancreatic development. However, it has been shown that tissues that lay adjacent to the bud are required for the specification and development of the pancreas (Slack 1995; Kim and MacDonald 2002).

Early pancreatic development requires signals from the adjacent notochord (Kim, Hebrok et al. 1997; Hebrok, Kim et al. 1998). Sonic Hedgehog (*Shh*) is absent from the pancreatic endoderm during initiation of the pancreatic bud and ectopic expression of *Shh* prevents pancreatic development. The notochord is responsible for secreting activin-βB and FGF2, factors which function to down regulate *Shh* in the endoderm and allow pancreatic development to proceed (Hebrok, Kim et al. 1998).

As the pancreatic bud grows larger, the notochord is displaced from the dorsal pancreas by the fusing aorta, which comes to lay dorsal to the dorsal pancreatic bud epithelia (Pictet and Rutter 1972). At this point, is has been shown that the dorsal aorta also provides important signals which allow pancreatic development to proceed. Removal of aortic precursor cells from *Xenopus laevis* embryos resulted in failure of the pancreas

to form insulin producing β-cells, and co-culture of blood vessel endothelium and pancreatic endoderm explants induces insulin expression. Additionally, ectopic vascularization of the pancreatic bud results in ectopic insulin expression and islet hyperplasia (Lammert, Cleaver et al. 2001). The dorsal aorta is also required for pancreatic development in mice by maintaining Pdx-1 expression in the dorsal pancreatic bud (Yoshitomi and Zaret 2004). The dorsal aorta also promotes survival of Isl-1 positive dorsal mesenchyme during pancreatic bud outgrowth (Jacquemin, Yoshitomi et al. 2006). Interestingly, no endothelial signaling molecule has yet been identified to be required for pancreatic development.

The pancreatic mesoderm is also adjacent to the pancreatic endoderm and provides vital signals that are required for pancreatic development (Kumar, Jordan et al. 2003; Duvillie, Attali et al. 2006). The lateral plate mesoderm (LPM), which over lays the pancreatic endoderm provides signals that are required induction of pancreatic genes *Pdx1*, *p48*, *Nkx6*.1, *glucagon*, and *insulin* (Kumar, Jordan et al. 2003). Interestingly, activin, BMP, or retinoic acid signaling is sufficient to promote growth of pancreatic endoderm in explant cultures and induce Pdx1 expression (Kumar, Jordan et al. 2003). The pancreatic mesoderm secretes fibroblast growth factor 7, FGF7 and FGF10, which are required for proliferation of pancreatic epithelial cells (Ye, Duvillie et al. 2005). *Fgf10* and *Fgf24* expression in the LPM are also required for development of the ventral pancreas (Manfroid, Delporte et al. 2007).

The pancreas, like many other organs such as the heart, the brain, and the lungs, exhibits left-right (LR), or situs specific patterning during development. This means that the organ does not develop symmetrically in the body. The pancreatic bud begins to grow

leftward at E10.0 (Fig.2.1). Eventually, the pancreas will come to rest on the left side of the stomach spanning between the gastric stomach and the spleen (Fig.2.2). The signals that drive situs specific patterning during organogenesis are unknown. However, lateral growth of the pancreas depends on the asymmetric thickening and growth of the mesothelial layer of cells that encase the pancreatic mesoderm forming a mesothelial cap structure (Fig.2.1). Thickening of the mesothelial layer in the pancreatic mesoderm is called the 'splanchnic mesodermal plate', or SMP, and occurs only on the left side of the embryo. Asymmetric thickening of the SMP is required for lateral growth of the pancreas (Fig.2.1). Asymmetric organogenesis and specifically pancreatogenesis will be discussed in more detail later in this chapter.

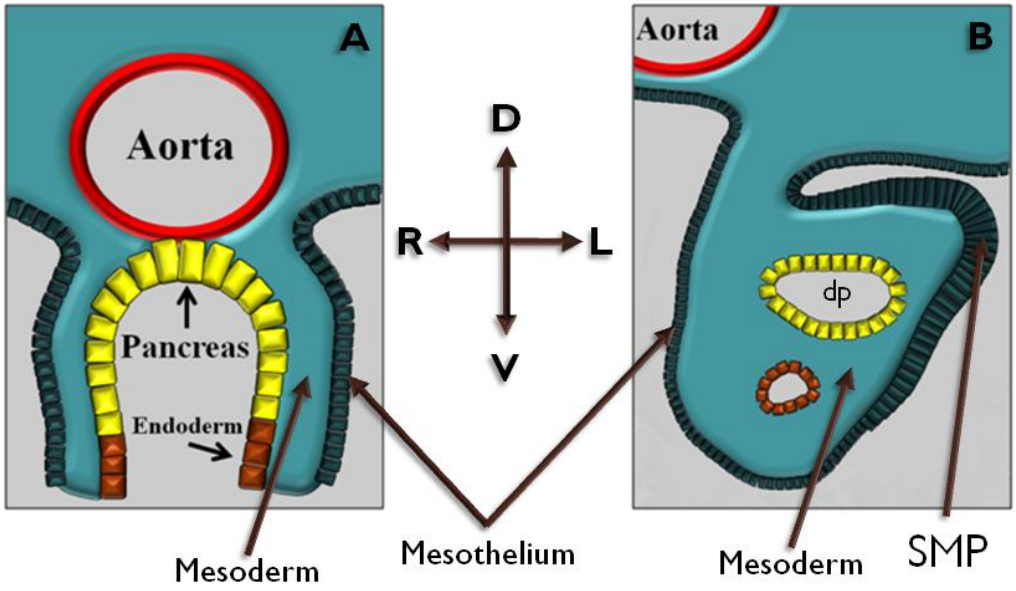

Figure 2.1 Asymmetric pancreatic growth. Cartoon depicting a transverse section through murine pancreatic bud during bud evagination and onset of lateral growth. Dorsal is up and left side of embryo is shown on the right. A) The dorsal pancreatic bud epithelium evaginates symmetrically towards the dorsal aorta at E9.5. At this time, the pancreatic endoderm is in direct contact with the dorsal aorta and adjacent mesoderm. An epithelial layer (the mesothelium) coats the pancreatic mesoderm, lining the embryonic coelom. As the pancreatic bud grows, the mesothelium on the left side of the bud thickens and the mesothelial cells become columnar, while mesothelial cells of the right side of the bud remain cuboidal. B) At E10.5 asymmetric growth of the mesothelium results in leftward rotation of the pancreatic bud in a clockwise direction with respect to the gut tube. The mesothelium on the left side of the pancreatic bud has thickened to form a mesothelial cap called the splanchnic mesodermal plate (SMP), which drives leftward growth of the pancreas.

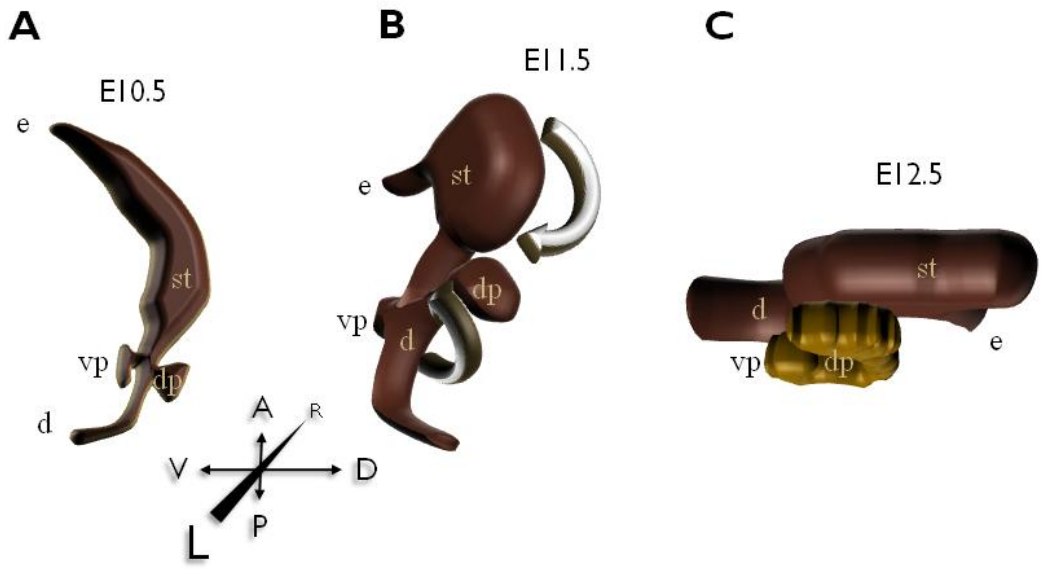

Figure 2.2 Midgut development and turning. Cartoon depicting murine gut tube during pancreatic budding and subsequent lateral growth. Only the gut epithelium is shown. (A-C) Compass denotes fixed orientation of the embryo during midgut looping. A) At E10.5, the dorsal pancreatic bud has expanded and is ready to rotate leftward. The ventral pancreatic bud has also undergone evagination and begins rotating to the right towards the dorsal pancreas. B) Midgut development at E11.5 is marked by extensive gut rotation and rearrangement. The dorsal and ventral pancreas travel towards each other around the radius of the duodenum. Meanwhile, the stomach also begins to rotate on a course to intersect with the dorsal pancreas. C) By E12.5 the proximal ducts of the dorsal and ventral pancreatic buds have come together and fused. The dorsal pancreas now lies on the left gastric side of the stomach, which is now perpendicular to the A-P plane of the embryo. d, duodenum; dp, dorsal pancreas, e, esophagus; st, stomach; vp, ventral pancreas. Model is based on observations by M.C. Jorgensen. (Jorgensen, Ahnfelt-Ronne et al. 2007)

Shortly after the pancreatic bud evaginates, lateral growth of the pancreas begins, and the pancreatic epithelium proliferates by E10.5 (Pictet and Rutter 1972). There is evidence to suggest that epithelial stratification and microlumen formation is the mechanism by which the pancreas is growing at this point (Jensen 2004). In rodents, this period of pancreatic development is called the 'primary transition' due to the transient appearance of early glucagon (endocrine) cells (Pictet and Rutter 1972). Interestingly, this wave of early endocrine differentiation is absent in humans. By E11.5, the ventral pancreas has rotated around the gut tube and the ventral and dorsal pancreatic ducts have fused proximally near the duodenum (Figure 2.2). Additionally, the dorsal pancreas has continued to grow and branch.

Later, at E12.5 the dorsal and ventral pancreata have fused and the pancreas begins to undergo 'secondary transition' (Pictet and Rutter 1972). During the secondary transition, multipotent precursor cells located at the tips of the pancreatic epithelium begin to grow outward to produce the branches of the pancreas. As these specialized multipotent cells grow outwards, the tip cells start to undergo differentiation into acini. Therefore, acinar differentiation takes place in a growing wave from the center towards the periphery of the growing pancreas. As a result of branching, the pancreas begins to adopt its characteristic triangular shape at this point, with the more distal parts of the pancreas consisting of mostly acini, and the islets and ducts forming near the center. The islets of the pancreas arise from epithelial cells located near blood vessels. These pre-endocrine epithelial cells are released from the pancreatic epithelium through delamination. Delamination occurs through one of two mechanisms, either an epithelial-to-mesenchymal transition, or EMT (Rukstalis and Habener 2007), or by a change in the

axis of cell division (orthogonal cell division) (Pictet and Rutter 1972), both are possible mechanisms that result in early endocrine cells escaping from the pancreatic epithelium. These delaminated cells coalesce to form small clusters of cells that make up the islets. Once together, these clumps of cells once again form epithelial contacts. Therefore, the pancreas consists of acini and ducts that make up the epithelial framework of the pancreas and the islets, which are delaminated and interspersed clusters of cells within the pancreas. By E15.5, the pancreas is a highly branched organ containing a large number of pre-islet clusters. This time point is marked by extensive acinar cell differentiation. The islets also continue to mature until after birth.

The process by which early endoderm is instructed to grow, branch, and differentiate to form the mature pancreas is complex. However, research in developmental biology aims to decipher these complex processes to allow us to better understand diseases of the pancreas and help us towards cures for those diseases.

Extrinsic and intrinsic factors during pancreatogenesis

The process by which the embryo undergoes organogenesis involves extrinsic (cell-cell communication) and intrinsic (transcription factors) factors within each cell to work together to drive development (Oliver-Krasinski and Stoffers 2008; Cleaver and MacDonald 2009). It is the interplay between these extrinsic and intrinsic factors that drives organogenesis by changing gene transcription and thereby modifying cellular outcomes. This dynamic interplay between cells and tissues during embryonic development drives organogenesis. The intrinsic state of a cell at any particular time

during development is determined by its molecular history, or lineage (Cleaver and MacDonald 2009). This lineage gives every cell a specific intrinsic state, allowing it to respond differently to various external stimuli, thereby determining the competence of that cell towards various stimuli (Cleaver and MacDonald 2009).

The aim of developmental research of the pancreas is to determine how two small patches of endodermal epithelium influence their surroundings and conversely, how cells within these patches are influenced by other cells over time to drive the formation of the complex multifunctional mature pancreas. One such family of signaling molecules is known as the bone morphogenetic protein (BMP) family, part of the Transforming Growth Factor B (TGF-B) superfamily of signaling molecules. Recent work has suggested that BMPs may play a role during pancreatic development (Dichmann, Miller et al. 2003; Chung, Shin et al. 2008), and the focus of work in this thesis is the elucidation of that role.

Background: Bone Morphogenetic Proteins

Signaling

Bone morphogenetic proteins (BMPs) were originally identified for their ability to induce bone differentiation when introduced into rodents (Urist 1965). Bone morphogenetic proteins comprise a subgroup of the transforming growth factor-β (TGF-β) gene superfamily. This subgroup consists of over 20 members, including the decapentaplegic sub family, BMP2 and 4, the 60A sub family, which includes BMP5,6,7,8A, and 8B, screw (Drosophila), growth and differentiation factors (GDFs),

Vg1 (frog), Dorsalin-1 (chick), Univin (Sea urchin), Daf-7 (Caenorhabditis elegans), nodal and lefty genes (Kingsley 1994; Zhao 2003). BMPs are translated and then folded into large homo- or heterodimeric pro-proteins which are then cleaved by proteases and secreted by the cell. After being secreted, BMPs form morphogen gradients that can reach nearby cells. Upon reaching a target cell, the BMP dimer binds to membrane heterodimeric receptor complexes that must contain both type I and type II serine/threonine kinase subunits for downstream signaling (Wrana, Attisano et al. 1992; Nellen, Affolter et al. 1994; Wrana, Attisano et al. 1994; Ruberte, Marty et al. 1995; Hogan 1996; ten Dijke, Miyazono et al. 1996; Weis-Garcia and Massague 1996).

There are several receptors that transduce BMP signals: type II BMP receptors are constitutively active kinases that include BMPRII, ActRIIA, and ActRIIB, and type I receptors that contain Gly-Ser (GS) domains that are phosphorylated by type II receptors, these include ALK1 (activin receptor-like kinase 1), ALK2, BMPR1A, and BMPR1B (Suzuki, Thies et al. 1994; Dewulf, Verschueren et al. 1995; Kawabata, Chytil et al. 1995; Liu, Ventura et al. 1995). BMP2 and BMP4 signal through BMPR1A and BMPRII; however, BMP2 can also signal through BMPR1B. BMP5, BMP6, and BMP7 on the other hand, prefer to bind to ALK2 and BMPR1B (Miyazono, Maeda et al. 2005). Typically, type I receptors exhibit more restricted expression patterns than type II receptors and are consequently responsible for modulating BMP signal transduction.

Upon BMP ligand binding, type II receptors phosphorylate type I receptors which in turn phosphorylate downstream effectors known as SMADs. In the case of BMPs, SMAD1, SMAD5, and SMAD8 serve as effectors, whereas other SMADs transduce signals from other TGFB family members (Derynck and Zhang 1996; Graff,

Bansal et al. 1996; Massague 1996; Macias-Silva, Hoodless et al. 1998). Once phosphorylated on their C-terminus by type I receptors, SMAD1, SMAD5, or SMAD8 effectors heterodimerize with the common mediator SMAD, SMAD4 (also known as co-SMAD) and this complex then shuttles into the nucleus to activate or inhibit transcription of various downstream target genes (Liu, Ventura et al. 1995; Graff, Bansal et al. 1996; Hoodless, Haerry et al. 1996; Heldin, Miyazono et al. 1997). These SMAD complexes can either directly bind to DNA or indirectly through their mad homology 1 (MH1) domain by interacting with transcription factors (TFs) such as Menin, Runx2, Yin Yang 1, or Hoxc8 (Miyazono, Maeda et al. 2005). BMPs have also been shown to signal through the p38, RAS, and Erk pathways; however, signaling through these pathways has been far less characterized (Yamaguchi, Shirakabe et al. 1995; Moriguchi, Gotoh et al. 1996; Shirakabe, Yamaguchi et al. 1997; Shibuya, Iwata et al. 1998; Kimura, Matsuo et al. 2000; Lou, Tu et al. 2000; Mulder 2000; Lai and Cheng 2002).

The main targets of the BMP transduction pathway are the *Id* (inhibitor of differentiation or inhibitor of DNA binding) genes (Ogata, Wozney et al. 1993). Typically, *Id* genes (*Id1-4*) function to prevent cellular differentiation and promote cellular proliferation (Yokota and Mori 2002). Id proteins contain helix-loop-helix (HLH) dimerization domains and function as repressors through their interaction with HLH transcription factors in the cell, thereby blocking transcription of their target genes. Genes that are under the control of HLH transcription factors contain E box regions in their promoters. Transcription of these genes requires two types of HLH transcription factors to bind concurrently to the E box, a ubiquitously expressed HLH protein and a tissue specific HLH protein. Id proteins usually bind to ubiquitously expressed HLH proteins,

which causes the tissue specific HLH protein to be degraded, thereby preventing gene transcription (Miyazono, Maeda et al. 2005).

BMPs can regulate various downstream target genes depending on the presence of specific type II and type I receptors, various receptor regulated SMADs (R-SMADs) or inhibitor SMADs (I-SMADs) that are expressed in the particular target cell. However, BMP signaling is also modulated by extracellular mechanisms in addition to these intracellular mechanisms. Extracellularly, BMP signaling is regulated by secreted antagonists that bind to BMP ligands with high affinity, and prevent signal transduction. These antagonists include Cerberus, Dan, Gremlin, and Noggin (Balemans and Van Hul 2002; Gazzerro and Canalis 2006). Perhaps the best studied BMP antagonist, NOGGIN has been shown to directly bind to BMP ligands including BMP2,4,5,6,7, GDF5,6, and Vg-1, prevent receptor activation (Holley, Neul et al. 1996; Piccolo, Sasai et al. 1996; Zimmerman, De Jesus-Escobar et al. 1996). X-ray crystallography analysis of a complex of BMP7 and NOGGIN shows that NOGGIN inhibits BMP7 signaling by blocking binding epitoptes necessary for binding to both type II and type I BMP receptor, thus sequestering BMP7 in an inactive complex (Groppe, Greenwald et al. 2002). Interestingly, BMP signals are able to cross talk with other pathways as well.

BMP cross-talk

Several molecular pathways are affected by BMP signaling, and this crosstalk between pathways is required for development and regulation of the growth and function of many tissues. BMP signaling pathways can intersect with Wnt, Activin, Notch, and

STAT pathways. Wnt signals stabilize B-catenin, which then forms a complex with lymphoid enhancer binding factor 1 / T cell specific factor (Lef1/Tcf) and this complex promotes transcription of downstream genes including c-Myc and cyclin D1 (Kikuchi 1999). Various SMADs can directly interact with members of the Wnt pathway. SMAD3 binds to Lef1 resulting in increased transcription of Lef1 target genes (Labbe, Letamendia et al. 2000), additionally SMAD4 can directly bind to both Lef1 and B-Catenin (Nishita, Hashimoto et al. 2000).

Activins can also interact with components of the BMP pathway. Activins and certain BMPs share some of the same receptors (ALK2, ACTRIIA, and ACTRIIB) and SMADs (SMAD4) (Candia, Watabe et al. 1997).

Notch signals have been shown to either promote or repress BMP signal transduction. BMP4 activation of SMAD1 can promote transcription of *Hey1* and *Hes1*, which are downstream targets of the Notch pathway (Dahlqvist, Blokzijl et al. 2003). Conversely, the notch pathway can repress BMP signaling. BMP2 has also been shown to activate Hey1 which in turn represses *Runx2*, preventing further BMP signaling (Itoh, Itoh et al. 2004).

The BMP and STAT pathways also interact. STAT1 is induced by IFN-a/b / y, and then binds to and sequesters Runx2, thereby blocking downstream BMP signaling (Nakashima, Yanagisawa et al. 1999; Ulloa, Doody et al. 1999; Kim, Koga et al. 2003).

In addition to the ability of most BMPs to induce bone formation, they are also required for the development of several organs during embryogenesis (Hogan 1996; Whitman 1998; Mishina 2003).

Role of BMPs in Development

Since their discovery about 40 years ago for their ability to induce bone formation in rodents (Urist 1965), BMPs have been shown to be involved in countless other vital processes during development including cell proliferation, differentiation, and survival (Hogan 1996; Zhao 2003; Gazzerro and Canalis 2006). The nearly 7,000 published articles involving BMPs attest to the fact that there is a lot more to BMP signaling than just bone formation. Many BMP ligands and receptors are required for vital functions and deletion of these genes in mice results in early embryonic lethality. The following list details some of the phenotypic abnormalities seen in embryos in which various *Bmps* are mutated.

BMP Ligands

- Knockout of *Bmp2* results in embryonic lethality by E8.5. Embryos failed to close the proamniotic canal, resulting in amnion/chorion defects. Additionally, *Bmp2*-deficient mice exhibit heart defects, specifically with respect to the development of the exocoelomic cavity (Zhang and Bradley 1996). Additionally, these mutant embryos exhibit shortening of the allantois and in primordial germ cell (PGC) generation (Ying and Zhao 2001).

- BMP3 is the most abundant BMP found in bone and knockout of *Bmp3* results in two-fold increase in trabecular bone formation compared to wild-type mice, suggesting that BMP3 is a negative regulator of bone density (Daluiski, Engstrand et al. 2001).

- Knockout of *Bmp4* results in embryonic lethality between E6.5 to E9.5 for a variety of reasons including defects in gastrulation and mesoderm formation. Mutants are unable to express *Brachyury*, the mesodermal marker T, and have general absence of mesodermal differentiation. Additionally, there are defects in posterior specification and in the extraembryonic mesoderm (Winnier, Blessing et al. 1995). These mutants lack primordial germ cells (PGCs) and fail to form an allantois (Lawson, Dunn et al. 1999). *Bmp4* is also required for development of the vertebrate lens (Furuta and Hogan 1998). In *Bmp4* heterozygotes, deletion of *Bmp4*, mutants displayed cystic kidneys, craniofacial defects, microphtalmia and defects in limb development, suggesting that BMP4 is required for the development of several organs (Dunn, Winnier et al. 1997).

- Genetic alterations in *Bmp5* result in mouse short ear and defects in the development and repair of skeletal structures and soft tissues (Green 1968; Kingsley, Bland et al. 1992; King, Marker et al. 1994). Knockout of *Bmp6* results in viable and fertile mice and are largely normal

throughout development except for an apparent delay in ossification of the developing sternum. Interestingly, *Bmp5/6* double mutants display more severe defects in sternum ossification (Solloway, Dudley et al. 1998).

- Knockout of *Bmp7* results in defects in kidney (renal dysplasia) and eye (anophthalmia) development (Dudley, Lyons et al. 1995). The kidneys lack a glomerulus due to defects in metanephric mesenchymal differentiation. Additionally, *Bmp7* null mice also exhibit skeletal defects localized to the rib cage, skull, and hindlimbs (Luo, Hofmann et al. 1995). Eye defects in *Bmp7* mutants are exacerbated in the C3H/He background, where it becomes apparent that *Bmp7* is required for the early stages of lens placode formation (Wawersik, Purcell et al. 1999).

- *Bmp8a* null mice display normal development and survive to adulthood. However, *Bmp8a* mice exhibit defects in germ cell maintenance and display higher levels of germ cell degeneration than wild-type control animals. Additionally, *Bmp8a* knockout mice exhibit defects in the epididymal epithelium (Zhao, Liaw et al. 1998).

- Knockout of *Bmp8b* results in failure to form PGCs, and shortening or absence of allantois. Since these phenotypes are similar to *Bmp4*

mutants, *Bmp8a/Bmp4* double mutants were analyzed; however, double mutants did not display an exacerbated phenotype, suggesting that BMP4 and BMP8A may be hetero- and homodimerizing during PGC and allantois specification (Zhao and Hogan 1996).

- Knockout of *Bmp11* (*Gdf11*) disrupts Hox gene expression, resulting in defects in axial skeleton development and improper positioning of the hindlimbs (McPherron, Lawler et al. 1999). Additionally, GDF11 promotes pancreatic beta cell differentiation at least in part by modulating NGN3+ islet cell progenitor numbers in the pancreas (Harmon, Apelqvist et al. 2004)

- Knockout of *Bmp12* (Gdf7) results in loss of class D1A interneurons in the dorsal spinal cord. Over one-third of mutants succumb to hydrocephalus by 14-21 days after birth (Lee, Mendelsohn et al. 1998). BMP12 has also been shown to be a mesenchymal signal that is required for seminal vesicle development (Settle, Marker et al. 2001).

- *Bmp15* null females exhibit reduced fertility due to reduced ovulation and *Bmp15/Gdf9* double mutants display oocyte loss and defects in ovarian follicle development (Yan, Wang et al. 2001).

BMP Receptors

- *Alk1* is expressed in the blood vessels and knockout of *Alk1* results in embryonic lethality (E11.5). Mutants present vascular malformations such as capillary plexus formation defects and large vessel hyperdilation (Oh, Seki et al. 2000).

- ALK2 (ACTRIA) can bind activin and BMP7 in a heteromeric signaling complex with BMPRII. *Alk2* is expressed in the extraembryonic visceral endoderm and *Alk2* knockout results in embryonic lethality (E9.5) due to severe defects in mesoderm formation (Gu, Reynolds et al. 1999; Mishina, Crombie et al. 1999). Mutants also exhibit thickening of the primitive streak (Mishina, Crombie et al. 1999).

- *Bmpr1A* is ubiquitously expressed in most tissues during early murine development. Deletion of *Bmpr1A* results in early embryonic lethality (E9.5) due to defects in mesoderm formation (Mishina, Suzuki et al. 1995). Since a null mutation of *Bmpr1A* results in early embryonic lethality, a conditional allele of *BmpR1A* has been utilized in numerous studies to address the later role of BMPR1A in various tissues during development (Mishina, Hanks et al. 2002). Conditional knockout of *BmpR1a* in the limb bud shows that BMPR1A (ALK3) is required for limb development, specifically proximal-distal and dorsal ventral-axis

formation and agenesis of the hindlimb (Ahn, Mishina et al. 2001). Additionally, studies conducted with floxed *Bmpr1a* have shown the importance of *Bmpr1a* in a number of tissues during development including the cardiovascular system (Yu, Beppu et al. 2005; Park, Lavine et al. 2006; Kaneko, Li et al. 2008), lung (Eblaghie, Reedy et al. 2006), limb (Ovchinnikov, Selever et al. 2006), central nervous system (Zhang, Mehler et al. 1998; Ming, Elkan et al. 2002) and many more.

- Knockout of *Alk4* (*ActrIB*) results in defects in epiblast and extraembryonic ectoderm formation and embryonic lethality before gastrulation (Gu, Nomura et al. 1998).

- *Bmpr1B* mice are viable, but exhibit defects in the appendicular skeleton. *Bmpr1b*$^{-/-}$ mice display defects in the proliferation of prechondrogenic cells and phalangeal differentiation (Yi, Daluiski et al. 2000). *Bmpr1B* null mice are also infertile and display irregular estrus cycles and defects in pseudopregnancy response. Additionally, fertilized eggs display impaired cumulus expansion due to decreased levels of aromatase in granulosa cells. These mice also exhibit defects in endometrial gland formation (Yi, LaPolt et al. 2001). Disruption of *Alk6* (*Bmpr1B*) by insertional mutation results in defects in digit cartilage formation from skeletal blastema (Baur, Mai et al. 2000).

- Knockout of *BmpRII* results in embryonic lethality at the egg cylinder stage due to impaired mesoderm formation similar to the *Bmpr1A* null phenotype. These embryos also displayed defects in epiblast differentiation (Beppu, Kawabata et al. 2000). Surprisingly, epiblast deletion of *BmprII* only leads to heart and smooth muscle defects of the pulmonary artery (Hong, Lee et al. 2008; Yu, Deng et al. 2008).

- Knockout of *ActRIIa* results in a low frequency of skeletal and facial defects, with most mutants developing normally until adulthood when they display defects in follicle-stimulating hormone (FSH) production and reduced fertility. Interestingly, this phenotype was different than that of the activin-deficient mouse, suggesting that a ligand other than activin is required for *ActRIIa* mediated FSH production and fertility in wild type mice (Matzuk, Kumar et al. 1995).

- Knockout of *ActRIIB* display defects in anteroposterior and left-right axes formation. *ActRIIB* null animals exhibit misregulation of several Hox genes and exhibit defects in vertebrae development. These mice die shortly after birth due to defects including randomization of heart position, mispositioning of the arteries as well as septal defects. Additionally, these mice exhibit asplenia (Oh and Li 1997).

Members of the BMP family, including Nodal, Lefty1, and Lefty2, are central players in left-right patterning of the embryo. Interestingly, deletion of BMP effectors *Smad1* and *Smad5* results in symmetrical expression of *Nodal*, a key regulator of left-right (LR) patterning usually expressed on the left side of the embryo.

Left-right Asymmetry During Embryonic and Pancreatic Development

Lateral growth is an important part of pancreatic and splenic development (Hecksher-Sorensen, Watson et al. 2004). As the pancreatic bud evaginates, the pancreatic bud begins to grow towards the left. The mesodermal tissue that envelops the pancreas as it grows is shared with the spleen. Left-right patterning in the embryo begins with expression of *Nodal*, a member of the Bmp family that is required during early LR patterning. After initial Nodal-mediated early LR patterning, much less is known about LR patterning during organogenesis. This section will review both early LR patterning during embryonic turning and late LR patterning during organogenesis, specifically with respect to lateral growth of the pancreas.

Early Embryonic Left-Right (LR) Patterning

All vertebrates require signals that allow left and right axis formation to occur in the developing embryo. A central player in this process is known as Nodal, a member of the BMP family within the TGFB superfamily (Levin 2005; Tian and Meng 2006; Shen

2007). Nodal was first discovered for its requirement for mesodermal differentiation. Nodal is expressed in the primitive streak and then becomes restricted to the node (Zhou, Sasaki et al. 1993). A requirement for Nodal in axis formation was observed by Conlon and colleagues (Conlon, Lyons et al. 1994), based on the observation that 25% of *Nodal* mutants form randomly positioned patches of cells that resemble posterior mesodermal nature and fail to form a primitive streak. *Nodal* becomes asymmetrically expressed during early somite stages with higher levels seen on the left side of the embryo and in twice as many cells as the right side. Misexpression of *Nodal* results in randomized embryo turning, heart situs and polarity of the viscera (Collignon, Varlet et al. 1996). Lefty -1 acts upstream of nodal and lefty-2, acting to localize their expression to the left side of the embryo, where they encode signals for leftness (Meno, Shimono et al. 1998).

Nodal signal transduction occurs through ALK receptors and Activin type II receptors. ALK7 forms a complex with ActRIIB to form a functional receptor signaling unit. Additionally, ALK4 can also form functional receptor complexes in the presence of both nodal and cripto, an EGF-CFC protein that can serve as both and co-receptor and/or co-ligand for Nodal (Reissmann, Jornvall et al. 2001; Yan, Liu et al. 2002). Additionally, ALK4 can couple with ACTRIIB to phosphorylate and activate downstream SMAD2 (Yeo and Whitman 2001). EGF-CFC co-receptor molecules such as Cripto and Cryptic (mammals) are required for type I receptor ALK4 specificity and Nodal activity (Shen and Schier 2000; Reissmann, Jornvall et al. 2001; Yeo and Whitman 2001; Yan, Liu et al. 2002; Chen and Shen 2004).

Nodal function can be down-regulated by Lefty proteins which are also members of the BMP family. Lefty molecules can directly interact with Nodal and EGF-CFC

molecules to act as signaling antagonists (Chen and Shen 2004; Cheng, Olale et al. 2004). *Lefty1* is expressed in the embryonic midline, likely to serve as a barrier to prevent Nodal ligand from crossing to the right side of the embryo since deletion of *Lefty1* results in *Nodal* expression on the right side (LPM) of the embryo, causing left isomerism, (asymmetrically patterned organ adopts left identity). (Meno, Shimono et al. 1998). *Lefty1* and *Lefty2* have a similar phenotype when knocked down, suggesting that both are required to restrict Nodal activity to its proper location (Meno, Gritsman et al. 1999; Meno, Takeuchi et al. 2001). Another antagonist of Nodal is Cerberus, a secreted protein that can bind to Nodal, BMP and Wnt ligands to down-regulate signal transduction through these pathways (Piccolo, Agius et al. 1999).

SMADs mediate much of the signal transduction downstream of Nodal / ALK / ACTRII receptor complexes. SMADs shown to be involved in downstream Nodal signaling include SMAD2, SMAD3, and SMAD4 (Massague, Seoane et al. 2005). The SMAD2/4 complex then shuttles to the nucleus where it interacts with Mixer subclass homeodomain transcription factors to promote transcription of Nodal target genes (Germain, Howell et al. 2000). SMAD2 containing transcriptional complexes can also interact with winged-helix homeodomain transcription factor FoxH1 to promote transcription of Nodal target genes (Randall, Howell et al. 2004). Downstream transcriptional targets of Nodal signaling include *Nodal, Lefty2, Pitx2*, *FoxA2*, and *Lhx1* (Dickmeis, Aanstad et al. 2001; Whitman 2001). Nodal itself is a secreted morphogen and forms a gradient of ligand that signals in paracrine fashion. Since it is highly expressed and restricted on one side of the embryo, Nodal signaling is able to affect just the left side of the embryo to initiate left-right asymmetry (Ashe and Briscoe 2006).

Exactly how Nodal becomes expressed on the left side of the embryo to begin with is a controversial issue. The reigning hypothesis is the 'Nodal flow hypothesis' which states that monocilia located on the cells of the node beat in a uniform fashion which directs Nodal towards the left side of the embryo (Takaoka, Yamamoto et al. 2007; Lee and Anderson 2008), but critics say there is still not enough evidence to support this theory (Tabin 2005).

Left-right (LR) patterning in the mouse requires two steps, initially, *Nodal* expression in the node is concentrated on the left side of the embryo at E7.5, this is required for the second step of asymmetric signals that is passed on to the lateral plate mesoderm (LPM) where *Nodal* also becomes asymmetrically expressed (Brennan, Norris et al. 2002; Saijoh, Oki et al. 2003). Sonic Hedgehog (*Shh*) is also asymmetrically expressed and is required for LR determination in the chick (Levin, Johnson et al. 1995); however, *Shh* is not required for LR determination in other vertebrates. *Shh*$^{-/-}$ mice exhibit symmetrical expression of *Nodal* and left isomerism due to defects in midline function (Meyers and Martin 1999). Indian hedgehog (*Ihh*), like *Shh*, is asymmetrically expressed and *Ihh*$^{-/-}$ animals exhibit symmetrical expression of *Nodal* (Zhang, Ramalho-Santos et al. 2001); however, LR defects in these animals are likely due to lack of *Gdf1*, which regulates *Nodal* expression in the LPM (Rankin, Bunton et al. 2000). These results suggest that hedgehogs are having an indirect effect on LR patterning through disruption of functional midline and node, and are not directly regulating LR patterning (Shiratori and Hamada 2006). Fibroblast growth factor 8 (*Fgf8*) null animals fail to express *Nodal* in the LPM and exhibit right isomerism (asymmetrically patterned organ adopts right identity) (Meyers and Martin 1999); however, this is likely due to failure of the embryo

to secrete nodal vesicular parcels (NVPs) and not due to FGF8 functioning to drive *Nodal* expression (Tanaka, Okada et al. 2005). NVPs are secretions from the node that contain a number of factors required for LR axis formation.

Nodal remains expressed in the LPM atop developing organs such as the lungs, heart, and gut which undergo asymmetric development (Raya and Belmonte 2006; Shiratori and Hamada 2006). The mechanism by which this signal is transferred from the node to the LPM is still under investigation (Shen 2007). Another Bmp family member, *Gdf1* is expressed in the developing node and LPM in a similar fashion to *Nodal* (Wall, Craig et al. 2000). *Gdf1* and *Cryptic* mutant mice exhibit an identical phenotype and *Nodal* expression is attenuated in both mutants (Yan, Gritsman et al. 1999; Rankin, Bunton et al. 2000), suggesting that GDF1 is required for *Nodal* expression and signaling.

Situs-specific organogensis

Little is known about asymmetric LR development that follows embryonic turning, specifically with respect to LR patterning during organogenesis (Shiratori and Hamada 2006). Asymmetric LR patterning is observed in several organs including the brain, the lungs, the heart, the liver, and the pancreas. One key question that remains is how do LR signals induce asymmetric organogenesis? Interestingly, asymmetric development appears well after expression of *Nodal* has attenuated, implying the function of other cell-cell players; however, these remain unknown.

Macroscopically, three mechanisms are observed that typically result in asymmetric organ development (Shiratori and Hamada 2006). One mechanism is

directional looping. This is when an organ that starts out as a tube loops, bends, and rotates to achieve organ asymmetry, as seen in the heart and gut. Another method that is used to achieve asymmetric organogenesis is unequal growth which results in unequal size or number of branches produced by organs that appear on both halves of the body for example, the lungs. The third main process for achieving asymmetry includes a structure that is developed on both sides of the body; however, at some point the tissue is only maintained on one side of the embryo while the lateral counterpart regresses and disappears. For example, the right pulmonary arch artery disappears in humans while the left artery remains (Navaratnam 1963).

Very little is known about morphogenetic mechanisms that drive left-right asymmetry during organogenesis. However, in one study of gut development, ectopic expression of the transcription factor *Pitx2*, a LR gene expressed on one side of the gut tube, resulted in perturbations of LR symmetry during gut tube looping in *Xenopus* (Muller, Prather et al. 2003). Additionally, misregulation of *Pitx2* in *Xenopus* results in defects in heart and gut looping (Campione, Steinbeisser et al. 1999). Knockout of *Pitx2* in the mouse results in body wall closure defects, right pulmonary isomerism, mislocation of the heart, as well as lung, pituitary, duodenum, and tooth defects (Lin, Kioussi et al. 1999; Liu, Liu et al. 2001). *Pitx2* is an interesting LR marker because unlike other genes in the LR specification pathway, *Pitx2* expression does not disappear from the LPM as organs form. Instead, *Pitx2* is expressed in asymmetric patterns within several developing organs (Logan, Pagan-Westphal et al. 1998; Meno, Shimono et al. 1998; Piedra, Icardo et al. 1998; Ryan, Blumberg et al. 1998; St Amand, Ra et al. 1998; Yoshioka, Meno et al. 1998; Campione, Steinbeisser et al. 1999; Essner, Branford et al. 2000; Schweickert,

Campione et al. 2000). After *Nodal* expression disappears from the LPM, Nkx2 has been shown to be responsible for the persistence of *Pitx2* expression (Shiratori, Sakuma et al. 2001). Nonetheless, the cellular mechanisms behind asymmetric organ morphogenesis are not identified and no target gene of *Pitx2* has been identified that plays a role during asymmetric morphogenesis (Shiratori and Hamada 2006).

Recently, more studies have begun to address potential mechanisms by which the gut and pancreas achieve asymmetric growth. Midgut looping is driven by asymmetric growth of the dorsal mesentery, which connects the gut tube to the body wall. Cells on the left side of the dorsal mesentery condense and consist of columnar cells, while cells on the right side of the dorsal mesentery remain cuboidal; this asymmetric growth changes the orientation of the gut tube and results in a trapezoidal shape that tilts the gut tube leftward. Additionally, this process is controlled by Pitx2, Isl1 (expressed on the left side), and Tbx18 (expressed on the right side) (Davis, Kurpios et al. 2008).

Laterality of the pancreas and spleen are driven in part by asymmetric proliferation of the splanchnic mesodermal plate (SMP), a columnar mesodermal-derived cell layer that drives leftward growth of the pancreatic bud (Fig.2.1). The spleen is derived from the spleno-pancreatic mesenchyme (SPM), the mesoderm that is located posterior to the stomach and surrounding the pancreatic bud as it grows laterally. As the pancreatic mesenchyme or SPM goes laterally during pancreas and spleen organogenesis, the expanding SPM provides the tissue that comprises the spleen. Therefore, the pancreas and spleen share a common expanding mesenchyme as they develop and for this reason, splenogenesis and pancreatic lateral growth are very closely linked. The spleen is normally located on the left side of the body. Frequently, defects in spleen development

are linked to laterality defects (Patterson, Drysdale et al. 2000; Hecksher-Sorensen, Watson et al. 2004). Splenic conditions include asplenia and polysplenia which are examples of situs ambiguous (a non-mirror defect in organ sidedness), while the normal arrangement of organs is called situs solitus. Loss of splenic tissue is associated with right isomerism and is linked to a loss of left identity, and gain of spleen tissue is called polysplenia or 'left isomerism' and marks an increase in left-signals. Lateral growth of the pancreas is independent of the endoderm, since *Fgf10* mutant pancreata are missing the endodermal component of the pancreatic bud, but the SMP can still grow laterally. Additionally, *Bapx1* is required for SMP formation and *Bapx1* mutants exhibit down-regulation *Fgf10* and *Fgf9,* which are expressed in the SMP. Therefore, *Bapx1* is upstream *Fgf10* and *Fgf9* and is required for their expression in the SMP (Hecksher-Sorensen, Watson et al. 2004). Mice carrying the dominant hemimelia (Dh) spontaneous mutation which results in situs ambiguous of the pancreas, also exhibit decreased *Fgf10* and *Fgf9* expression via different signaling pathways (Hecksher-Sorensen, Watson et al. 2004). Dh mutant mice exhibit defects in visceral and limb development (Green 1967). Interestingly, Nodal and Lefty have been shown to regulate growth in pancreatic cell lines. Introduction of Nodal to cell culture results in inhibition of cell proliferation, while Lefty1 results in increased proliferation, suggesting that LR molecules play a role during pancreatic development (Zhang, Sterling et al. 2008).

The role of BMPs in the pancreas

Given the vital role BMPs play in several tissues, some work has been done to understand the role of BMPs in the pancreas with some success. The bulk work that has been done on the pancreas does not directly address the role of BMPs during pancreatic development; however, recent work with zebrafish suggests that BMP2 prevents pancreatic differentiation and instead drives liver cytodifferentiation (Chung, Shin et al. 2008). BMPs have been shown to promote proliferation and colony formation in pancreatic cell lines (Jiang and Harrison 2005; Hua, Zhang et al. 2006). Additionally, BMPs are required for the differentiation of pancreatic cell lines into insulin-positive cells (Jiang, Stanley et al. 2002; Yew, Hembree et al. 2005). BMPs regulate anteroposterior endoderm patterning and pancreatic development in the zebrafish (Tiso, Filippi et al. 2002), in part by down-regulating *Vhnf1* (Song, Kim et al. 2007). This may explain tissue recombination experiments done my Kumar and colleagues which indicate that BMP stimulation of foregut endoderm is sufficient to activate ectopic *Pdx1* gene expression in explant experiments (Kumar, Jordan et al. 2003). Additionally, knockout animals of receptors known to respond to BMP ligand (Activin receptor IIA and IIB) result in anteriorization of the midgut and reduced pancreatic proliferation and differentiation (Kim, Hebrok et al. 2000). Pancreatic development in *Xenopus* has been shown to be dependent on down-regulation of BMP signaling in the endoderm (Spagnoli and Brivanlou 2008). Perhaps the strongest and least examined evidence that suggests a role of BMPs during pancreatic development of vertebrates is work by Dichmann et al, demonstrating that overexpression of *Bmp6* in the developing pancreas results in agenesis of the pancreas (Dichmann, Miller et al. 2003). BMPs have also been shown to play

functional roles in the pancreas, deletion of *BmpR1a* in the mouse islets results in reduced glucose stimulated insulin secretion (Goulley, Dahl et al. 2007).

Thesis rationale and significance

The BMP family of signaling molecules has an amazing array of roles during embryonic development, some of which are only now becoming clear. Since their discovery about 40 years ago, BMPs have been shown to play a role in the development of almost every organ in vertebrates, with the notable exception of the pancreas. Recent work however suggests a role for BMPs during pancreatic development in the mouse (Dichmann, Miller et al. 2003) and the focus of this thesis is aimed towards the elucidation of that role. Consequently, the goals of this thesis work are to: 1) Characterize the expression of Bmp ligands and receptors during organogenesis. 2) Characterize the expression of BMP ligands and receptors during midgut development. 3) Determine the role of BMP signaling during lateral growth of the pancreas.

I showed that *Bmps* are expressed in the tissues that are adjacent to the pancreas during pancreatic bud development. Interestingly, I noted that *Bmp4* is expressed asymmetrically in the gut tube during pancreatic bud evagination. This led us to propose that BMPs may be playing a role during lateral development of the pancreas. To test this, we knocked down BMP signals in the developing midgut and saw defects in SMP formation and lateral growth of the pancreas. Conversely, overexpression of *Bmp2* did not affect lateral growth of the pancreas, but did block cellular differentiation to endocrine and exocrine lineages. Lastly, global conditional knockout of *Bmpr1a*, but not

BmprII, resulted in defects in SMP formation and lateral growth of the pancreas as well as reduced *Bapx1* expression, a gene required for SMP formation and lateral growth of the pancreas (Hecksher-Sorensen, Watson et al. 2004). Therefore, defects in lateral growth of the pancreas in *Bmpr1a* mutants are mediated by *Bapx1* and accordingly *Bmpr1a* mutant pancreata exhibit misregulation of genes that require *Bapx1* including *Fgf9* and *Fgf10*. Additionally, deletion of *Bmpr1a* also exhibit misregulation of *Barx1*, another gene with SMP restricted expression. This work concludes that BMPs act as signaling molecules to drive lateral growth of the pancreas. Currently, nothing is known about what drives pancreatic laterality. In fact, no signal has been shown to drive lateral development in any organ. Work in this thesis will present the first evidence of signaling molecules driving lateral development during organogenesis. A better understanding of the role of BMP signaling during pancreatic lateral growth will shed light on mechanisms that may be playing a role in lateral development of other organs, such as the heart, lungs and brain.

CHAPTER THREE

N.B. Oneka Green assisted with cloning of Foxf1 and Hlx1 promoters into Cre transgenic construct.

Methodology

Transgenic constructs

Foxf1 and *Hlx1* promoters (Figure 3.1 and 3.2 respectively) were amplified and cloned into pGEMT-Easy vector using T/A cloning. *Foxf1* and *Hlx1* promoters were then released from pGEMT-Easy vector by NotI digestion and subcloned into the pIGCN21 Cre:eGFP vector. Transgenic constructs were purified using Whatman ELUTIP-d DNA purification columns (Whatman cat no. 10 462 617). Purified Foxf1 Cre:eGFP and Hlx1 Cre:eGFP transgenic constructs were submitted to the UTSW transgenic core (Dr. Robert Hammer) for injection. Genotyping was performed using PCR with primers #794: 5'-CCTGAAGTTCATCTGCACCA -3'and #795: 5' – TGCTCAGGTAGTGGTTGTCG -3' for the presence of the GFP gene at 478bp. GFP PCR reactions were: 95°C-3min; (95°C-45sec, 58°C-45sec, 72°C-1min 10 sec) X 30 cycles; 72°C-10min; 4°C hold.

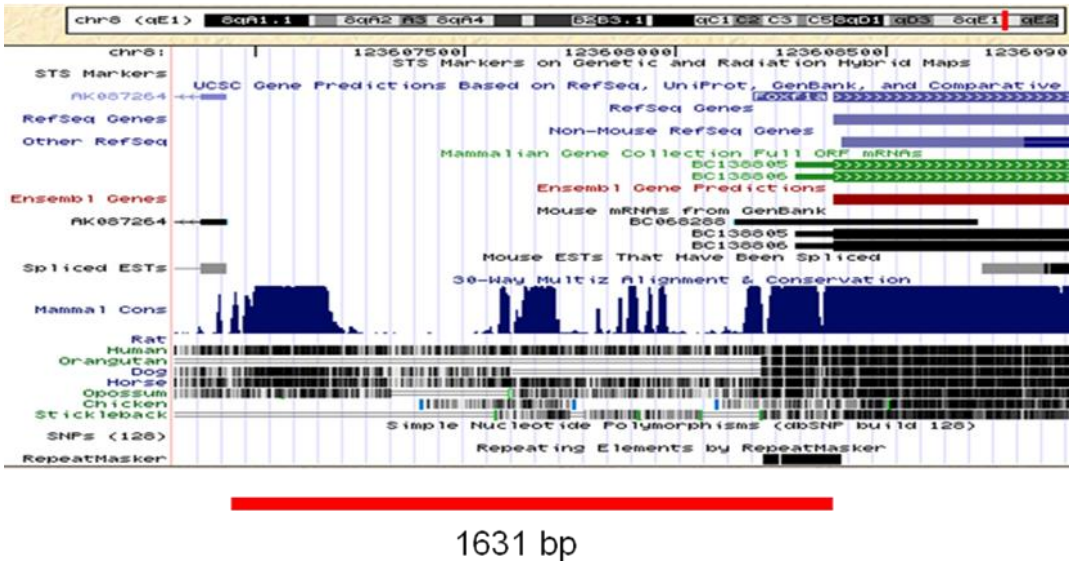

Figure 3.1. Subcloning *FoxF1* promoter. UCSC Genome Browser view of *Foxf1* promoter region. 1,631 bp of the upstream promoter was cloned, including the three conserved regions depicted.

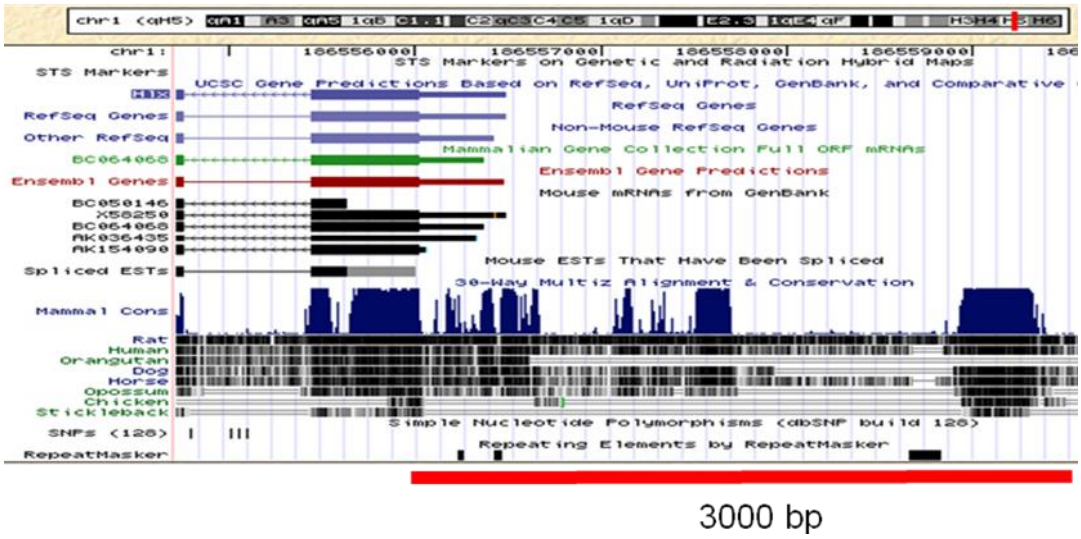

Figure 3.2. Subcloning *Hlx-1* promoter. UCSC Genome Browser view of *Hlx1* promoter region. 3,000 bp of the upstream promoter was cloned, including the four conserved regions depicted.

Immunohistochemistry

Samples were fixed in 4% paraformaldehyde/PBS overnight at 4°C, followed by 3 washes with PBS for 30-60 minutes. Samples were then placed in 10% sucrose for 1 hour, 15%, sucrose for 1 hour and 20% sucrose overnight at 4°C. Samples were then placed in OCT and cooled until samples solidify. Blocks were then placed at -80°C overnight. Frozen samples were then sectioned and slides were washed in PBS twice. Slides were then blocked for 1 hour in 5% normal donkey serum, then exposed to primary antibody for 1 hour at the following ratios (1:1600 for rabbit anti-glucagon (Linco, 4030-01F); 1:1000 for guinea pig anti-insulin (DakoCy, A0564); 1:1000 for rabbit anti-amylase (Sigma A8273); and 1:500 for Hamster Armenian anti-muc1 (Thermo Scientific, HM-1630). Slides were then washed twice in PBS for 5 minutes and then treated with secondary antibody for 1 hour at the following concentrations (1:500 donkey anti-rabbit FITC (Jackson Laboratories); 1:500 goat anti-guinea pig TRITC (Jackson Laboratories); 1:500 goat anti-Armenian Hamster Cy3 (Jackson Laboratories). Samples were washed twice more in PBS for 5 minutes and then mounted (Vectashield).

Animal Husbandry

The *Bmpr1a* floxed mice was provided by Y. Mishina and have been previously described (Mishina, Hanks et al. 2002). The FoxA3-Cre transgenic mouse was provided by K. Kaestner as described (Lee, Sund et al. 2005). The Flk-1 Cre mouse was provided by T.N. Sato and has been described (Motoike, Markham et al. 2003). The Tie2-Cre

transgenic mouse was provided by M. Yanagisawa and has been described (Kisanuki, Hammer et al. 2001). The Cagg-ER-Cre transgenic mouse was provided by A.P. McMahon and has been described (Hayashi and McMahon 2002). Floxed *BmpRII* E15.5 embryos were provided by H. Beppu and have been described (Beppu, Lei et al. 2005). Rosa Cre reporter mice were provided by P. Soriano and have been described (Soriano 1999). BMP response element (BRE) LacZ reporter mice were provided by L. Oxburgh and have been described (Blank, Seto et al. 2008).

To promote tamoxifen induced activation of Cre activity in BmpR1a flox X Cagg ER Cre crosses. 10 mg / ml tamoxifen (Sigma cat no. T5648-1G) was dissolved in corn oil (Sigma cat no. C8267-500ML) by stirring at 80°C for 10-20 minutes and stored at 4°C. For injection 300ul aliquots were warmed to 30C and pregnant mice were weighed at E8.25. 3mg tamoxifen was injected per 40 grams of body weight. A gavage needle was utilized to inject tamoxifen oil (FST cat no. 18060-20). Mouse was immobilized by pinching behind the neck and held upside down. Gavage needle was attached to 1 ml syringe and then gently guided down throat and down esophagus for injection. Mice were then sacked after two days at E10.5.

Genotyping was performed using PCR with primers FX2: 5'-GCAGCTGCTGCCGCAGCCTCC -3'and FX4: 5' – TGGCTACAATTTGTCTCATGC - 3' for the wild type (150bp) and floxed (230bp) *BmpR1a* locus, and with FX4: 5' – TGGCTACAATTTGTCTCATGC - 3' and FX0: 5'-CACCTCCACCATACTGAAAGCATTAG - 3' for the null allele. PCR conditions for *BmpR1a* reactions were: 95°C-5min; (95°C- 1 min, 54°C- 45 sec, 72°C- 1min 30 sec) X 35 cycles; 72°C-10min; 4°C hold. Cre mice were genotyped with primers #838: 5' -

TGCCACGACCAAGTGACAGC - 3'and #839: 5' - CCAGGTTACGGATATAGTTCATG - 3' to give a 572bp band. PCR conditions for Cre reactions were: 95°C- 3 min; (95°C- 1min, 56°C- 45sec, 72°C- 1 min 30 sec) X 35 cycles; 72°C- 5 min; 4°C hold. LacZ mice were genotyped with primers 735#: 5' - GGTGGCGCTGGATGGTAAGC - 3'and 734#: 5' - CGCCATTTGACCACTACC - 3' to give a 630bp band. PCR conditions for LacZ reactions were: 95°C- 3 min; (95°C- 1min, 56°C- 45sec, 72°C- 1 min 30 sec) X 35 cycles; 72°C- 5 min; 4°C hold. Rosa mice were genotyped with primers 638 R1295#: 5' - GCGAAGAGTTTGTCCTCAACC - 3'and 639 R523#: 5' – GGAGCGGGAGAAATGGACATG - 3' 640 R26F2#: 5' – AAAGTCGCTCTGAGTTGTTAT - 3'to give a 650bp WT band and a 340bp mutant band. PCR conditions for LacZ reactions were: 95°C- 5 min; (93°C- 30 sec, 58°C- 30 sec, 65°C- 1 min) X 40 cycles; 72°C- 7 min; 4°C hold.

Tissue preparation

CD1 embryos were collected from pregnant females (E7.5 through E10.5) after dissection in ice-cold 1xPBS buffer, and then fixed in 4% paraformaldehyde in PBS solution overnight at 4°C with gentle rocking. For embryos E8.75 and older, the amnion was removed during dissection for better probe penetration. Embryos were washed three times in 1xPBS for 10 min at RT and dehydrated using a series of ethanol washes. Embryos were then stored in 70% ethanol at -20°C.

Whole mount in situ hybridization

For generation of antisense Dig-labeled RNA probes used for in situ hybridization experiments, the following clones were processed as described: pExpress-1-Bmp2 plasmid was linearized with EcoRV, and anti-sense DIG-RNA probe was made using T7 polymerase. pCMV5Sport6-Bmp4 was linearized by EcoRI, and anti-sense DIG-RNA probe was made by using T7 polymerase. pCMV5Sport6-Bmp7 was linearized with SalI and anti-sense DIG-RNA probe was made by using T7 polymerase. pCMV5Sport6-BmpR1a was linearized with SalI and anti-sense DIG-RNA probe was made by using T7 polymerase. pCRIITopo-BmpR1b was linearized with NotI, and anti-sense DIG-RNA probe was made by using Sp6. pCRIITopo-BmprII and pBSSK^{+}-Noggin were linearized with HindIII and the anti-sense DIG-RNA probe was made by using T7 polymerase. pCMV-Sport6-Bapx1 was linearized with SalI and anti-sense DIG-RNA probe was made by using T7 polymerase. pCR4-Topo-Barx1 was linearized with NotI and anti-sense DIG-RNA probe was made using T3 polymerase. *Fgf9* was linearized with NotI and anti-sense DIG-RNA probe was made by using T3 polymerase. *Fgf10* was linearized with BglII and anti-sense DIG-RNA was made by using SP6 polymerase. pBSK-Nkx2.5 was linearized with XbaI and anti-sense DIG-RNA was made by using T7 polymerase. pBlueScriptII SK-Foxf1 was linearized with NotI and anti-sense DIG-RNA was made by using T3 polymerase. pT7T3D-PacI-Hlx1 was linearized with XhoI and anti-sense probe was made by using T3 polymerase. The reagents were mixed in the following order at room temperature: linearized plasmid (1µg), DIG-RNA labeling mix (Roche

11277073910) 2.0µl, 10x transcription buffer (Roche 1465384) 2µl, Placental ribonuclease inhibitor (Promega N2111) 2µl, RNA polymerase (Roche) 1µl, and double distilled RNase free water to a final volume of 20µl. The mixture was incubated at 37°C for 2 hours. 2µl RQ1 DNase1 was added (Promega M6101), and then incubated again at 37°C for 15 min. The probes were then purified with Micro Bio-spin columns (Bio-RAD 732-6250). Source of clones used to generate probes were as follows: Open biosystems: *Bmp2* CK792068, *Bmp4* BF538517, *Bmp7* BC010771, *BmpR1a* BC042611, *BmpR1b* BC065106, *BmpRII* from Doug Melton lab (Clone #227), *Noggin* from Jill McMahon with permission from Richard Harland, *Bapx1* BC021014 / EMM1002-6886, *Barx1* BC119047 / EMM1002-983660435, *Fgf9* from Tom Carroll, *Fgf10* from Eric Small with permission of Stephane Zaffran, *Nkx2.5* from Eric Olson, *Foxf1* from Tom Carroll, *Hlx1* EMM1002-2013208.

Prehybridization solution (50% Formamide (Fisher BP227-100), 5xSSC, pH 4.5, 50µg/ml Ribonucleic acid from torula yeast, TypeVI (Sigma R6625), 1% SDS, 50µg/ml Heparin (Sigma H4784)) was added to probes to a concentration of 10µg/ml, as a stock solution and kept at -80°C. The stock probe was diluted to 1 µg/ml working solution and kept at -20°C. Whole mount in situ hybridization was carried out based on D. Wilkinson's Method (Wilkinson, 1999). Briefly, embryos were treated with 10µg/ml proteinase K (Roche #03115852001) for different time points (E7.5, 5 minutes; E8.5, 10 minutes; E9.0, 15 minutes; E9.5, 20 minutes; E10.5, 30 minutes), re-fixed in a gluteraldehyde/4% paraformaldehyde (PFA) solution, and later prehybridized at 60°C for 1 hour. The samples were transferred into the Digoxigenin-labeled probes and let hybridized overnight. The washing steps were carried out using a Biolane HTI automated

incubation liquid handler (Holle & Huttner) as previously described (Villasenor, Chong et al. 2008). Images were acquired on an Olympus DP70 camera mounted to a Zeiss NeoLumar microscope.

In situ hybridization on sections

Paraffin sections (on glass slides) were washed 3x3min in PBS, followed by a 10min treatment with 15µg/ml proteinase K. Sections were then rinsed in PBS, fixed in 4% PFA for 5 min, and incubated for 10 min in acetylation solution: mix of 2.66ml Triethanolamine, 350µg HCl, 750µg acetic anhydride and 200ml water. Prehybridization was carried out in plastic slide mailers (Fisher) containing hybridization buffer at RT for 1 hour. Slides were then transferred to a humidified chamber (humidified with 50% formamide/5xSSC) for probe hybridization (probe at 1µg/ml) with 100µl probe/slide (covered with glass coverslips) at 68°C overnight.

Slides were washed post-hybridization in 2xSSC at 72°C just long enough to allow coverslips to separate. Then slides were rinsed in 0.2xSSC at 72°C and RT for 1x1min, respectively, then MBST buffer at RT (100mM Maleic acid, 150mM NaCl, pH7.5, 0.1% Tween20). Slides were incubated in blocking solution (2% blocking reagent (Roche) and 5% heat-inactivated sheep serum in MBST) for 1 hour at RT. Anti-Dig alkaline phosphatase conjugated antibody was applied on slides in a chamber humidified with MBST (250µl of 1/4000 anti-Dig antibody (Roche)), covered with parafilm and incubated at 4°C overnight. Slides were washed for 3x30min in MBST after antibody incubation, and treated in NTMT (100mM NaCl, 100mM Tris, pH9.5, 50mM MgCl2,

0.1% Tween20) for 3x5min. Color reaction was carried out using BM purple as described above. For microscopic examination, slides were sealed and coverslipped using Permount (Fisher).

Histology

For paraplast sectioning of embryos following in situ hybridization, the embryos were fixed and dehydrated as described above. Embryos were rinsed twice in 100% ethanol for 30 minutes, twice in xylene at room temperature for 10 minutes, 1:1 paraplast:xylene at 60°C for 10 minutes, then a series of 100% paraplast at 60°C (McCormick Scientific 502004), including one overnight. The embryos were then embedded and sectioned with a Reichart Jung microtome. For examination, the sections were mounted on slides (Fisher SuperFrost Plus), deparaffinized in xylene twice for 1 minute each and mounted with cover slips using Permount (Fisher SP15500). Images were acquired on an Axiovert 200M Zeiss inverted fluorescence microscope using an Olympus DP70 camera.

Expression analysis

Relative expression levels were assessed by visual inspection of the intensity of the chromogenic reaction in the whole mount in situ hybridization and sections of embryos

and tissues. Timing of chromogenic reactions was standardized, to allow accurate comparisons of relative transcript levels. Whole mount and section observations were used to describe expression of Bmp ligands and receptors in both the text of this report and the tables presented. Strength of expression intensity was arbitrarily assigned visually by four independent observers. Average expression scores in tables were calculated from independently assigned values. Embryonic structures identified in text were assessed using structures previously described in a widely referenced embryonic atlas (Kaufman, 1992).

CHAPTER FOUR

N.B. This chapter has been published in the journal Gene Expression Patterns. Ondine Cleaver, Alethia Villasenor, and Diana Chong contributed manuscript writing, redoing various in-situs for better images and to tables 4.1 and 4.2 by helping to quantitate gene expression intensity.

BMP and BMP receptor expression during murine organogenesis

Abstract

Cell-cell communication is critical for regulating embryonic organ growth and differentiation. The Bone Morphogenetic Protein (BMP) family of transforming growth factor ß (TGFß) molecules represents one class of such cell-cell signaling molecules that regulate the morphogenesis of several organs. Due to high redundancy between the myriad BMP ligands and receptors in certain tissues, it has been challenging to address the role of BMP signaling using targeting of single *Bmp* genes in mouse models. Here, we present a detailed study of the developmental expression profiles of three BMP ligands (*Bmp2*, *Bmp4*, *Bmp7*) and three BMP receptors (*Bmpr1a*, *Bmpr1b*, and *BmprII*), as well as their molecular antagonist (*noggin*), in the early embryo during the initial steps of murine organogenesis. In particular, we focus on the expression of Bmp family members in the first organs and tissues that take shape during embryogenesis, such as the heart, vascular system, lungs, liver, stomach, nervous system, somites and limbs. Using in situ hybridization, we identify domains where ligand(s) and receptor(s) are either singly or co-expressed in specific tissues. In addition, we identify a previously unnoticed asymmetric expression of *Bmp4* in the gut mesogastrium, which initiates just prior to gut turning and the establishment of organ asymmetry in the gastrointestinal tract. Our studies will aid in the future design and/or interpretation of targeted deletion of individual *Bmp* or *Bmpr* genes, since this study identifies organs and tissues where redundant BMP signaling pathways are likely to occur.

Introduction

Morphogenesis of embryonic tissues and initiation of organogenesis begins post-gastrulation around embryonic day 8 (E8.0) in the mouse, with the onset of heart and blood vessel development. Over the next 24 hours, the embryonic endoderm transforms from a single cell layer sheet into an open cylinder which rapidly zippers up into the gastrointestinal tract and associated umbilical cord. Coordinately, the embryo undergoes 'turning', as it twists on its axis and acquires its characteristic fetal shape. In addition, during this time, most organs appear along the anteroposterior axis, including the budding pituitary, thyroid, salivary glands, lung, liver and pancreas. During the next 48hrs these organs continue to develop and take shape, and in the strikingly short span of approximately two days (E8.0-E10.5), the principal embryonic organs have become specified and emerged from relatively simple germ layers (ectoderm, mesoderm and endoderm), and undergone morphogenesis resulting in complex, multi-cellular organs. This dynamic process is ultimately the result of step-wise cell differentiation that involves a busy crosstalk of cell-cell signaling between growing tissues and the interplay of numerous different intrinsic gene pathways. A number of extrinsic factors have been shown to interact and drive organ and tissue formation during embryonic development, including the Wnt, hedgehog (Hh), fibroblast growth factor (Fgf), Notch, and transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) families of signaling molecules. Our studies focus on the BMP growth factor family and the expression of Bmps during organogenesis.

Bone morphogenetic proteins (BMPs) are part of the TGF-β superfamily (Kingsley 1994) and comprise a large, evolutionarily conserved family of secreted signaling molecules that are required for numerous developmental processes. BMPs were originally isolated because of their capacity to promote bone and cartilage formation (Urist 1965). However, they have also been shown to participate in the establishment of the initial vertebrate body plan, somite and neural tube patterning, as well as the development of a large number of structures and organs, such as kidney, lung, liver, limb, amnion, eye, teeth, pituitary, and testes (reviewed in (Hogan 1996; Zhao 2003). The importance of the development function of these BMP factors is highlighted by the fact that deletion of many Bmp genes (including *Bmp2* and *Bmp4*) and their receptors (including *Bmpr1a* and *II*) results in early embryonic lethality (prior to E9.5) when most gastrointestinal organs are just beginning to initiate development (reviewed in (Zhao 2003). Although conditional ablation of *Bmpr1a* has demonstrated its specific requirement in a number of tissues (Eblaghie, Reedy et al. 2006; Park, Lavine et al. 2006), the specific roles of BMP ligands and other BMP receptors have yet to be described.

BMPs, like other TGFβs, are first synthesized and folded in the cytoplasm and subsequently cleaved by proteases during secretion. BMPs form large, dimeric proteins, whose proper conformation is required for their receptor binding and biological action (Eimon and Harland 1999). In mouse, a single BMP type II receptor subunit (BMPRII) has been identified (Beppu, Minowa et al. 1997), while at least three type I receptors have been found (BMPR1a/Alk3, BMPR1b/Alk6 and ActRIa/Alk2) (ten Dijke, Yamashita et

al. 1994). After BMP ligand binding, BMPRII heterodimerizes with a type I receptor, such as BMPR1a or BMPR1b, resulting in type II receptor phosphorylation and activation of the type I receptor. The type I receptor in turn phosphorylates cytoplasmic downstream target proteins, including Smad family proteins (see review (Kretzschmar and Massague 1998) which act as transcription factors and regulate many downstream pathways. Adding to BMP signaling complexity, BMPs can also interact with type II activin receptors ActRIIa and ActRIIb (Yamashita, ten Dijke et al. 1995). Therefore, as BMPs display promiscuity in binding affinities and their receptors function as heterodimers, multiple possible signaling cascades exist and depend on the expression of specific BMP ligands and receptors in given tissues.

Bmp ligand and receptor expression profiles have been described in scattered reports in the literature (Jones, Lyons et al. 1991; Bitgood and McMahon 1995; Dewulf, Verschueren et al. 1995; Lyons, Hogan et al. 1995; Furuta, Piston et al. 1997; Solloway and Robertson 1999), however, a comprehensive comparative gene expression analysis has not been described for the decapentaplegic (*dpp*) subgroup of Bmp genes (*Bmp2, Bmp4*) and their receptors (*Bmpr1a, Bmpr1b* and *BmprII*) throughout organogenesis. Myriad reports have demonstrated the critical importance of both Bmp ligands and their receptors for embryonic organ and tissue development. In particular, elegant conditional deletion studies have shown the requirement for *Bmp4* (Kulessa and Hogan 2002), *BmpRII* (Beppu, Lei et al. 2005) and *BmpR1a* (Mishina, Hanks et al. 2002) in a number of tissues including the cardiovascular system (Yu, Beppu et al. 2005; Park, Lavine et al. 2006; Kaneko, Li et al. 2008), lung (Eblaghie, Reedy et al. 2006), limb (Ovchinnikov, Selever et al. 2006), central nervous system and many more. In this report, we analyze

expression of *Bmp2, Bmp4, Bmp7* and their receptors *Bmpr1a, Bmpr1b*, and *BmprII*, and the BMP antagonist *noggin,* prior to and during organogenesis, with special focus on tissues where multiple ligands and receptors are co-expressed at distinct time points. These studies will help elucidate interpretations of genetic deletion studies that may be complicated by tissue specific BMP signaling redundancy. We employ in situ hybridization to examine and compare expression of transcripts of these genes, in postgastrulation embryos from stages E7.5 to E10.5. We aim to identify sites where single or multiple BMPs may play a role during organogenesis.

Results and discussion

***Bmp2*- E7.25 to E10.5**

We initially assayed expression of Bmp genes using whole mount in situ hybridization. (For all descriptions of gene expression in embryonic organs and tissues refer to structure annotations found in top panels of each figure, either in schematic or overlaid on photographed embryo, and associated organ or tissue name in figure legends). Our analysis of Bmp gene expression began at E7.25, a time in development when embryonic tissues start to form following gastrulation. We find that at this stage, *Bmp2* is primarily expressed in the yolk sac (y) and allantois (a), in the precardiac crescent (cc), just anterior to the anterior intestinal portal, or AIP (aip) (Fig.4.1A1). Expression appears distinctly absent in the medial, open gut region (g) of the embryo, which includes pre-

somitic mesoderm, endoderm, and neural tissues. By E8.25, we observe an increase in *Bmp2* expression in the linear heart tube (h) and allantois, while high levels of expression are observed in the constricting AIP and sinus venosus (sv), regions immediately ventroposterior to the heart (Fig.4.1A2). In addition, strong expression initiates in the dorsal most tip of the rostroanterior neural folds (nf) and the midline fusion point of the neural folds spanning the length of the embryo. Expression also appears robustly in the lateral plate mesoderm (lpm).

Slightly later, at E8.75, expression remains strong in the sinus venosus and in the fusing dorsal neural tube (Fig.4.1A3, Fig.4.2A1 and Fig.4.3A1). Strikingly, rather significant expression is also detected in the heart, shortly after the heart takes shape and starts to loop. Expression is particularly strong in the region that joins the left ventricle and the atria, which will later give rise to the atrioventricular canal region, or AVC (avc) (arrow in Fig.4.1A3), a region previously shown to express both *Bmp2* and *Tbx2* (Christoffels, Hoogaars et al. 2004). Expression is also high in the pericardium, with declining expression in the 'seam' of the dorsal brain, which represents the region of the midline fusion of the anterior neural folds.

Later, at E9.0, *Bmp2* remains strongly expressed in the AVC, but has declined from the midline seam of the telencephalon (ms) (Fig.4.1A4, Fig.4.2A2 and Fig.4.3A2). In addition, expression has decreased in the constricting base of the yolk sac (or the future umbilical cord), and associated lateral plate mesoderm, as embryonic turning proceeds and the gut tube forms (Fig.4.1A4). Expression is low in the head and branchial arches, as well as the dorsal neural tube at this stage of development. Expression remains strong

in the liver diverticulum (l) (Fig.4.1A4 and Fig.4.2A2). *Bmp2* also increases expression in the developing forelimbs (lb) (Fig.4.2A3 and Fig.4.3A3), the rostral dorsal aortae (ao) of the trunk and the anterior tip of the developing mesenephros (m) (arrowheads in Fig.4.1A4).

At E9.5, *Bmp2* has also begun to be expressed in limb bud, including the apical ectodermal ridge (AER), a region long noted for its organizing activity in driving limb development, and the ventral portion of the developing limb bud, as previously noted (Lyons, Hogan et al. 1995; Ahn, Mishina et al. 2001) (Fig.4.1A5, Fig.4.2A3 and Fig.4.3A3). Notably, expression in the heart AVC peaks at this point. In addition, expression is prominent in subpopulations of cells between the branchial arches (Fig.4.1A5). By E10.5, expression levels have generally declined throughout the head and tail, and are low throughout the gut tube (Fig.4.2A4 and Fig.4.3A4-5); however, there is still detectable expression in the constricting yolk sac (ysc) (Fig.4.1A6). In addition, expression remains relatively high in the heart AVC (inset, Fig.4.2A5), otic vesicle (ov), and branchial arches. Expression is robust in the AER at this stage and appears in an ectodermal patch on the posterior aspect of the forelimbs. Additionally, expression in the ventromedial somites (s) increases, especially in the anterior trunk (Fig.4.1A6 and Fig.4.2A6).

***Bmp4* – E7.5 to E10.5**

Bmp4 is expressed in a pattern initially similar to that of *Bmp2*; however, it subsequently varies dynamically throughout early embryogenesis. At E7.25, *Bmp4* like *Bmp2*, is strongly expressed in the yolk sac and allantois, and is concentrated in the anterior part of the embryo, including the cardiac crescent and early neural folds (Fig.4.1B1). At E8.25, *Bmp4* remains expressed in the allantois, and strong expression initiates in the posterior lateral plate mesoderm, the AIP, and the sinus venosus (Fig.4.1B2). However, yolk sac expression is lower than that of *Bmp2*.

After turning, around E8.75, expression continues to increase in the posterior lateral plate and remains relatively strong in the sinus venosus, the allantois and the caudal most mesoderm of the tail (t) (Fig.4.1B3). Again similar to *Bmp2*, *Bmp4* is detected in the dorsal most tips of the rostral neural tube, albeit at significantly lower levels (Fig.4.2B1 and Fig.4.3B1), and expression appears in the seam of the diencephalon (d) (Fig.4.1B3). Thus, *Bmp2* and *Bmp4* are co-expressed in the AIP, the lateral plate and the neural folds of the head during these earliest stages of embryogenesis (E7.25-E8.75). *Bmp4* has been reported to be more strongly expressed in the myocardium at this time, however using our whole mount method, we detect only low *Bmp4* expression in the heart (Jones, Lyons et al. 1991). *Bmp4* is expressed very strongly in the posterior lateral plate mesoderm, allantois, and yolk sac.

At E9.0, additional domains of *Bmp4* expression appear in many different regions of the embryo (Fig.4.1B4 and Fig.4.2B2). These domains include the midline seam of the

telencephalon, the pituitary infundibulum (pi), the otic vesicle, the rostral somites, the tip and cleft of the branchial arches (b), the thyroid primordium (th), the heart sinoatrial region, the emerging limb buds, the liver, the dorsal optic cup (oc), and the posterior lateral plate mesoderm (Fig.4.1B4, Fig.4.2B3 and Fig.4.3B2-3). Additionally, *Bmp4* is expressed in the distal portion of the first branchial arch. Most of these domains are maintained in the E9.5 embryo with marked increase in the rostral dorsal somites, seam of the telencephalon, branchial arches, and in populations of cells likely to be neural crest derived neurons near the branchial arches (Fig.4.1B5) (and as shown by (Grotewold, Plum et al. 2001). Expression increases in the dorsal portion of the optic cup.

At E10.5, expression of *Bmp4* increases in all these domains, particularly in the telencephalon, the first branchial arch, and around the nasal placode (n), as assessed by intensity of staining by in situ hybridization (Fig.4.1B6). Additionally, expression of *Bmp4* is strong in the dorsal somites spanning the length of the embryo, the dorsal gut tube mesoderm, the dorsal aspect of the limb, the outflow tract of the heart (ot), the lung buds (lu) and the AER (Fig.4.1B6, Fig.4.2B4-6 and Fig.4.3B5).

***Bmp7* – E7.5 to E10.5**

Similar to *Bmp2* and *4*, *Bmp7* expression varies dynamically throughout early embryogenesis. *Bmp7* expression can be observed in many of the same developing tissues as *Bmp2* and *4*, although its levels appear relatively lower during early development. *Bmp7* expression is evident at E7.5 broadly expressed in the cardiac

crescent, the allantois, anterior embryonic endoderm, yolk sac, and the anterior primitive streak (Fig.4.1C1), as previously described (Solloway and Robertson 1999). By E8.25, however, expression remains strong in the yolk sac, but is significantly lower in the AIP, sinus venosus and allantois than either *Bmp2* or *4* (Fig.4.1C2). Expression is evident in the dorsalmost portion of the rostral neural folds including strong staining in the ectoderm of the future otic vesicle. In contrast to previous reports, we observe only low levels in the developing heart and allantois (Solloway and Robertson 1999). By the end of turning, at E8.75, *Bmp7* is prominently expressed in the otic vesicle, telencephalon, the dorsal tip of the neural folds, the future pituitary within the head and a portion of the first branchial arch, while it is present at lower levels in the notochord, heart, somites and the lateral plate mesoderm of the trunk (Fig.4.1C3, Fig.4.2C1 and Fig.4.3C1). Overall, these observations are in agreement with previous reports, except that we detect lower expression in foregut endoderm (Solloway and Robertson 1999).

By E9.0, *Bmp7* expression continues in these same tissues, however intensifies in the roof of the midbrain, the otic vesicle, telencephalon and branchial arches, with marked expression along the entire length of the developing kidney (mesonephros), while it remains low in the heart (Fig.4.1C4, Fig.4.2C2 and Fig.4.3C2). Expression in the limb buds also initiates at this stage (Fig.4.2C3 and Fig.4.3C3). By E9.5, expression levels continue to increase in the telencephalon, the dorsalmost neural tube and the heart (Fig.4.1C5). New domains of expression also appear, such as the dorsal somites and the gut endoderm (Fig.4.1C5 and data not shown). *Bmp7* continues to intensify in the dorsal forebrain, neuroectoderm, lateral plate mesoderm, mesonephros, forelimb bud ectoderm and the neural tube, as previously shown (Solloway and Robertson 1999). By E10.5,

Bmp7 is relatively strongly expressed in portions of the branchial arches, mesonephros, telencephalon, both fore- and hindlimbs, otic vesicles, dorsal optic cup, and throughout the dorsal somites (Fig.4.1C6 and Fig.4.2C6). In addition, it is expressed in the gut tube, around both lung buds and posterior stomach (st), as well as in the myocardium of the heart (Fig.4.2C4-5 and Fig.4.3C2,4-5).

***Noggin* – E7.0 to E10.5**

To assess regions where BMP activity may be regulated by BMP antagonists, we examined the expression of the high affinity BMP inhibitor *noggin*. Similar to Bmp ligand expression patterns, *noggin* transcription occurs in highly restricted embryonic domains. Interestingly, it displays only limited overlap with that of early *Bmp2*, *4* and *7* expression.

At E7.0 *noggin* levels are undetectable in most embryonic tissues, except for strong expression in the node and emerging notochord (Fig.4.1D1). Tissues like the allantois, AIP and yolk sac, which co-express Bmp ligands, show no expression of their antagonist *noggin*. At E8.25, *noggin* continues to be expressed in node derivatives and is observed throughout the developing notochord (Fig.4.1D2). In fact, we find that at these stages, *noggin* is a robust marker of the early notochordal plate and early notochord. In addition, *noggin* becomes expressed in the dorsal tips of the neural folds, in a pattern that overlaps that of *Bmp2* and *4* (compare Fig.4.1A2, B2, D2). At E8.75, following turning, *noggin* expression expands to encompass the entire dorsalmost neural tube, from the tip of the

tail to the rostral telencephalon (Fig.4.1D3, Fig.4.2D1 and Fig.4.3D1). In addition, expression begins in the dorsalmost edge of the somites (as previously shown in (Reshef, Maroto et al. 1998). *Noggin* expression at this stage strongly marks the fusing and fused neural folds, along the entire length of the embryonic axis, and it continues to be maintained in the notochord, although it soon declines in the anterior portion of the embryo.

As development proceeds, at E9.0, *noggin* expression appears at low levels in the dorsal optic cup, the dorsal rostral portion of the somites and more generally throughout the dorsalmost region of the fore- and midbrain, while lower levels are detectable in the liver, as well as the myocardium of the heart (Fig.4.1D4, Fig.4.2D2-3 and Fig.4.3D2). This is in contrast to the high level of *noggin* expression previously reported in the early developing heart (Yuasa, Itabashi et al. 2005). We observe only low levels of *noggin* in the heart, starting around E8.75. Strikingly, while expression intensifies in the seam of the head and neural tube, it is always absent from the isthmus, the region between the mid- and hindbrain (arrowheads in Fig.4.1D3-6 and Fig.4.2D1). At E9.5, *noggin* continues to be expressed in the posterior notochord (no), the telencephalon, and the dorsal neural tube, however overall levels in the notochord and neural tube begin to decrease (Fig.4.1D6). Expression within the dorsal tip of the somites, however, peaks at this stage. At E10.5, overall *noggin* expression declines slightly from that seen at E9.5. It is expressed in the mesoderm of the lung buds, but is absent from most of the gut tube and heart (Fig.4.2D4-5). It also declines in the somites, but remains in the dorsal neural tube (nt) (Fig.4.2D6).

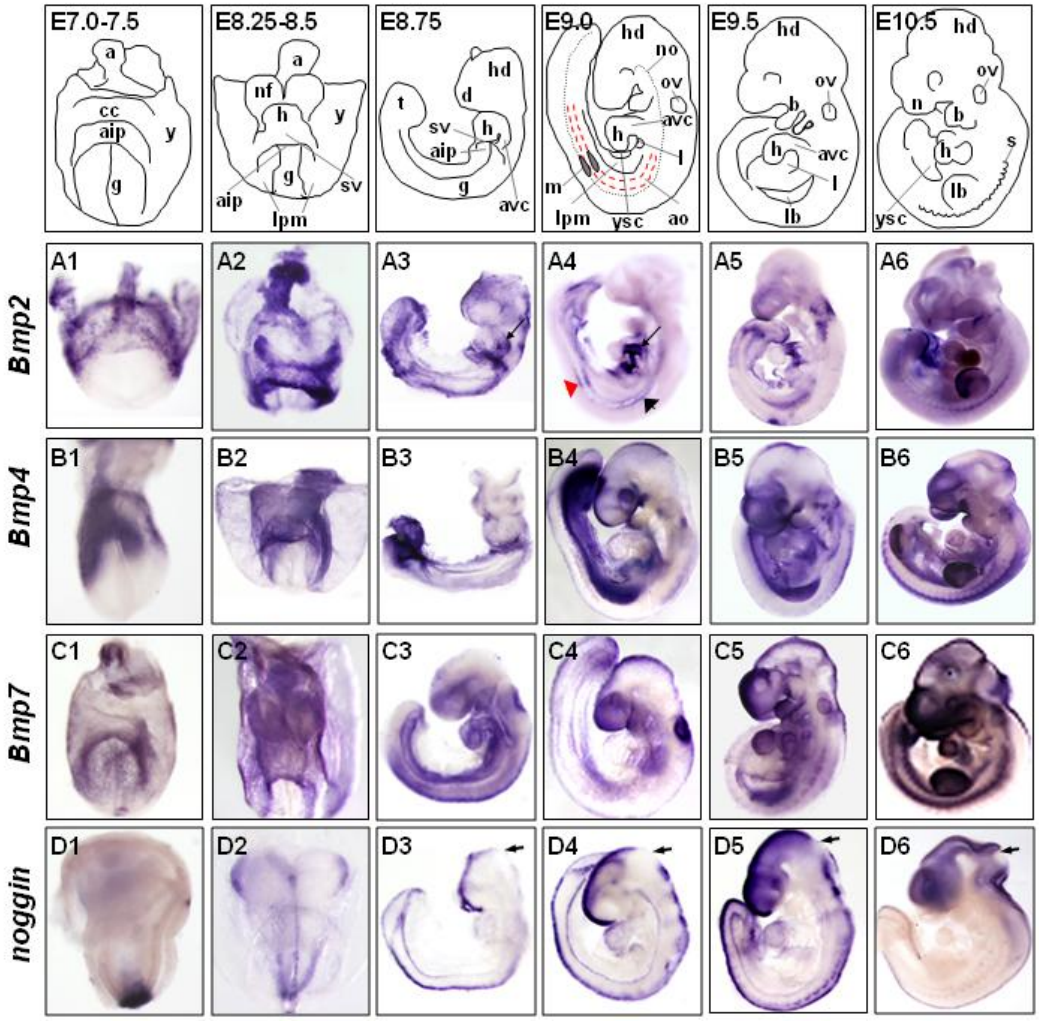
E7.0-7.5
E8.25-8.5
E8.75
E9.0
E9.5
E10.5
a
cc
aip
y
g
nf
h
lpm
sv
t
d
hd
avc
no
ov
l
m
ao
ysc
lb
n
b
s
Bmp2
Bmp4
Bmp7
noggin
A1
A2
A3
A4
A5
A6
B1
B2
B3
B4
B5
B6
C1
C2
C3
C4
C5
C6
D1
D2
D3
D4
D5
D6

Fig.4.1. Expression of Bmp ligands and the BMP antagonist *noggin* during embryogenesis. Whole mount in situ hybridization of embryos E7.5-E10.5 for the following transcripts: Panels A) *Bmp2*; Panels B) *Bmp4*; Panels C) *Bmp7;* and Panels D) *noggin*. Panels 1) E7.0-7.5 embryos are frontal views with head facing forward, allantois in back, pointing up, and yolk sac still attached, except panel B1 which is slightly tilted to the left. Panel A1 and B1 are E7.25, C1 is E7.5 and panel D1 is E7.0. Panels 2) E8.25-E8.5 unturned embryos are shown in frontal views, with head and open AIP facing forward; yolk sac is still attached. Panels 3) E8.75 are shown in process of turning, or shortly after turning. Panels 4-6) E9.0-E10.5 embryos have completed turning and are shown in lateral view facing left. Red dashed lines outline dorsal aorta. All embryos are shown anterior up, dorsal on right. Arrow in panels A3 and A4 indicates atrioventricular canal (avc); in panel A4, red arrowhead shows mesonephros and black arrowhead points to aorta; small black arrowheads in panels D3-D6 indicate isthmus. Embryonic structures and tissues are annotated in top panel schematics, which represent embryos shown in photographs below. Annotations as follows: a, allantois; aip, anterior intestinal portal; ao, aorta; avc, atrioventricular canal; b, branchial arches; cc, pre-cardiac crescent; d, diencephalon; g, pre-gut endoderm; h, heart; hd, head; l, liver diverticulum; lb, limb bud; lpm, lateral plate mesoderm; m, mesonephros; n, nasal placode; no, notochord; nf, neural folds; ov, otic vesicle; s, somites; sv, sinus venosus; t, tail; y, yolk sac; ysc, yolk sac constriction.

Expression of Bmp ligands and *noggin* in developing organs and tissues

Notable overlaps in expression of Bmp ligands and their antagonist *noggin* are observed in developing organs and tissues during organogenesis. The first embryonic regions with overlap of multiple Bmp ligands in post-gastrulation tissues are the allantois, yolk sac and cardiac crescent (compare Fig.4.1A1, B1, C1). Shortly thereafter, a similar direct overlap of *Bmp2, 4, 7* and *noggin* are all observed in the fusing seam of the telencephalon (with *Bmp2* being weakest and *noggin* being strongest) and in the dorsal most tips of the neural folds, both before and after their fusion into the neural tube (compare Fig.4.1A2-4, B2-4, C2-4 and Fig.4.2A1-D1).

Bmp co-expression is also evident in the early developing heart. Expression in the heart is initially distinct: *Bmp2* is strongly expressed in the AVC (Fig.4.2A2,5 inset and Fig.4.3A2); *Bmp4* is localized to both the inflow, or sinus venosus, and the outflow tracts (Fig.4.2B2,5 and Fig.4.3B2); *Bmp7*, in contrast, is found throughout the heart at low levels (Fig.4.2C2,5 and Fig.4.3C2). However, later during embryogenesis, *Bmp2* continues to be highly localized and robustly expressed within the AVC, while *Bmp4* transcripts increase in the outflow tract and *Bmp7* become more ubiquitous throughout the myocardium (compare Fig.4.1A5-6, B5-6, C5-6, Fig.4.2 panels 5 and Fig.4.3 panels 2). Interestingly, *noggin* is notably absent from the heart at most of these early stages, with only slight and transient expression observed around E9.0 (Fig.4.2D2 and Fig.4.3D2).

Another tissue showing evident overlap of the Bmp ligands is the limb bud. *Bmp2*, *4*, and *7* are all expressed in the limb bud as previously described (Fig.4.2A3, B3, C3 and Fig.4.3A3, B3, C3) (Francis, Richardson et al. 1994; Ahn, Mishina et al. 2001). *Bmp2* initiates around E9.0 in the ventral ectoderm of the limb bud, as previously noted (Lyons, Hogan et al. 1995), and can be found shortly thereafter strongly expressed along the AER (Fig.4.2A3 and Fig.4.3A3). *Bmp4* and *7* are initiated slightly earlier, as the limb bud emerges, and they are more broadly and more strongly expressed throughout both the epithelium and the underlying mesenchyme of the limb bud (Fig.4.2B3, C3 and Fig.4.3B3, C3). Notably, *noggin* is absent from this domain of Bmp ligand co-expression (Fig.4.2D3 and Fig.4.3D3).

In somites, all three ligands and their antagonists show dynamic patterns of expression. While they are all expressed at low levels during early somite development, by E9.0, *noggin* and *Bmp4* are expressed robustly in the dorsal portions of the somites, while *Bmp2* and *7* are just initiating low levels of expression (Fig.4.1 panels 4 and 5; Fig.4.2 panels 3). By E9.5, *Bmp7* and *noggin* become evident in the ventral- and dorsalmost portions of the somites (Fig.4.1C6,D6 and Fig.4.2C3,D3). Then at E10.5, all three ligands and their antagonist are strongly expressed in nested and overlapping patterns, as follows (Fig.4.2 panels 6): *Bmp2*, mid-somite region, stronger in anterior trunk of embryo; *Bmp4*, low ventral somite expression in anterior trunk, robust dorsal somite expression along entire embryonic axis; *Bmp7*, high expression in both the dorsal and ventral domains of the somites, along entire embryonic axis; *noggin*, low expression in ventral portion of somites, stronger expression in dorsal edge of somites, especially in anterior trunk (Fig.4.1 panels 6, and Fig.4.2 panels 6).

Bmp ligand expression is also striking in the budding organs of the developing gastrointestinal tract. While *Bmp2* is almost undetectable in the foregut (Fig.4.2A4 and Fig.4.3A4-5), *Bmp4* is expressed in a strikingly unique pattern around the developing midgut (Fig.4.2B4 and Fig.4.3B4-5). Strong asymmetric expression is observed on the right dorsal side of the gut tube, along the entire length of the dorsal mesogastrium, along the stomach and pancreas (Fig.4.2B4 and Fig.4.3B4), which winds around the lateral right edge of the developing anterior gut tube. This asymmetric expression is particularly interesting, because it is associated with the coincident breaking of embryonic symmetry (Hecksher-Sorensen, Watson et al. 2004), when the pancreas (located posterior to stomach) begins to swing left and the gut tube initiates ‘turning’. Expression of *Bmp4* in

this region is interesting in that BMPs are known to modulate other extrinsic signals, such as Fgfs, which have been reported in this region (Hecksher-Sorensen, Watson et al. 2004) and have been linked to pancreatic development (Bhushan, Itoh et al. 2001). *Bmp2* is notably absent in this midgut region, while *Bmp7* is strong but symmetrical in the posterior stomach and pancreatic domain (Fig.4.2A4, C4 and Fig.4.3A4, C4).

During this time, while *Bmp2* is completely absent from the developing lungs, *Bmp4* is strongly expressed in the lung bud mesenchyme and *Bmp7* initiates in the tips of the lung epithelium (Fig.4.2A4, C4 and Fig.4.3A5, C5). *Noggin*, although expressed at low levels in the distal lung bud mesenchyme, is absent from the epithelium of the lung buds and from the midgut tube (Fig.4.2D4 and Fig.4.3D4,5). Thus, *Bmp4*, *Bmp7*, and *noggin* are all three expressed in a restricted manner in the lung buds, as previously shown for *Bmp4* (Bellusci, Henderson et al. 1996), with *Bmp4* and *noggin* primarily in the mesoderm (m), and *Bmp7* in the epithelium (e) (Fig.4.3B5, C5, D5).

Relative levels of Bmp and *noggin* expression in different tissues at different stages are compared in Table 4.1. Overall, we can summarize Bmp ligand expression during early organogenesis as dynamic and often highly localized to distinct tissues and organs during development. As expected, we observe that Bmp ligand expression is particularly enriched in areas where bone growth is required, such as the proximal region of the limb buds, where in addition *noggin* is completely absent. However, we note that *noggin* overlaps in a few domains where multiple individual Bmp ligands are expressed, including the neural tube and somites during early embryogenesis, but is absent from

other tissues high in Bmp ligand expression, such as the limb buds, otic vesicle, or most other mesodermal and endodermal derived tissues.

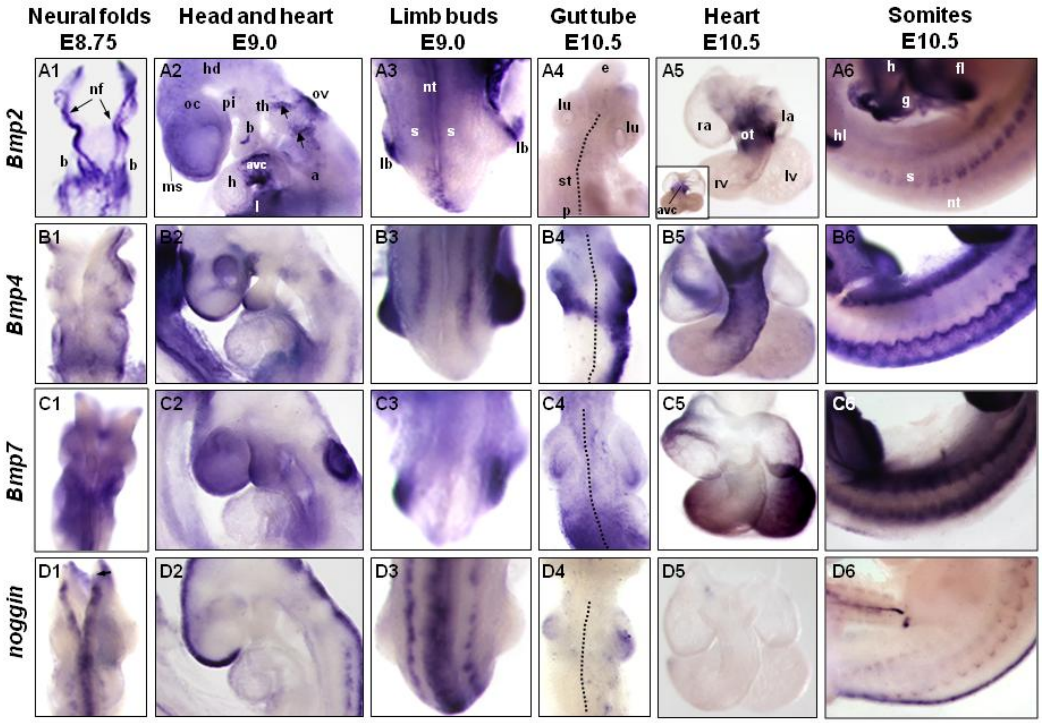

Fig.4.2. Expression of Bmp ligands and the BMP antagonist *noggin* in developing tissues. Whole mount in situ hybridization of embryos for the following transcripts: Panels A) *Bmp2*; Panels B) *Bmp4*; Panels C) *Bmp7;* and Panels D) *noggin*. Panels 1) Close up view of embryonic head, showing open neural tube and fusing headfolds at E8.75. Anterior is up, dorsal view. Panels 2) Close up view of anterior portion of E9.0 embryo, highlighting head, heart, sinus venosus, branchial arches, otic vesicle, liver diverticulum and anterior somite expression. Anterior is up, dorsal is on right. Arrows indicate neuron populations. Panels 3) Close up on dorsal midsection of embryo, showing neural tube, anterior somites and developing limb buds at E9.0. View of dorsal surface of neural tube, head is up, tail points away from viewer. Panels 4) Close up view of E10.5 developing gut tube dissected away from the embryo, highlighting expression in esophagus/trachea, lungs, stomach and anterior edge of pancreas. Dotted line indicates dorsal midline of the gut tube. At this stage, the gut tube is beginning to break symmetry and turn left. Anterior is up, dorsal view. Panels 5) Close up views of dissected E10.5 hearts. Ventroanterior view, with outflow tract in front and AVC behind, except inset in A5 where view is dorsoposterior showing *Bmp2* expression in AVC. Note that strong expression in AVC can be seen through transparent outflow tract in main panel A5. Panels 6) Close up view of E10.5 somites (numbers 12-25). Ventral is up, anterior to right, lateral view. Small black arrowhead in panel D1 indicates isthmus. Annotations as follows: a, aorta; avc, atrioventricular canal; b, branchial arches; e, esophagus; fl, forelimb; g, gut; hd, head; h, heart; hl, hindlimb; l, liver diverticulum; la, left atrium; lb, limb bud; lu, lung; lv, left ventricle; ms, midline seam of neural tube; nf, neural folds; nt, neural tube; oc, optic cup; ot, outflow tract; ov, otic vesicle; p, pancreas; pi, pituitary; ra, right atrium; rv, right ventricle; s, somite; st, stomach; th, thyroid primordium.

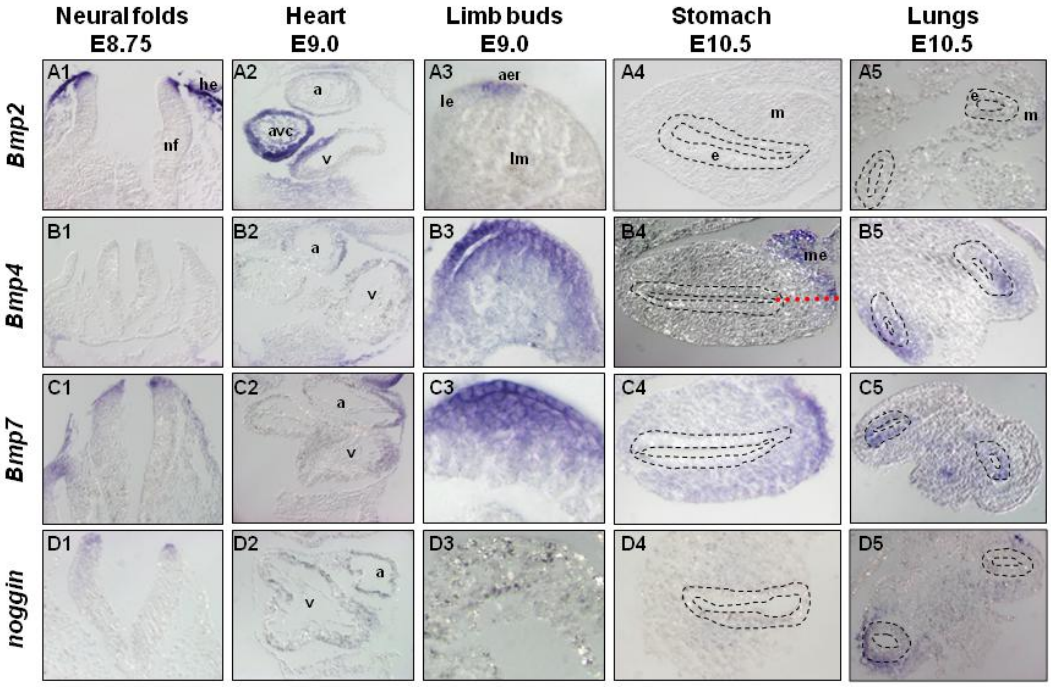

Fig.4.3. Sections showing Bmp ligand and *noggin* expression. Sections of whole mount in situ hybridization of embryonic tissues for the following transcripts: Panels A) *Bmp2*; Panels B) *Bmp4*; Panels C) *Bmp7;* and Panels D) *noggin*. Panels 1) Transverse sections of neural tubes and headfolds at E8.75. Dorsal is up. Panels 2) Transverse sections of heart at E9.0 showing both atria and ventricle. Panels 3) Transverse sections through developing limb buds at E9.0, showing outer epidermis (top) and underlying limb mesenchyme. Panels 4) Transverse sections of stomach at E10.5, showing both gut epithelium and gut mesoderm. Stomach epithelium outlined with dashed lines; dorsal is to the right. Red dotted line in panel B4 shows embryonic midline. Panels 5) Transverse sections through developing lung buds. Lung bud epithelium outlined with dashed lines. Anterior is up, dorsal view. Annotations as follows: a, atria; aer, apical ectodermal ridge; avc, atrioventricular canal; e, epithelium; he, head epidermis; le, limb epidermis; lm, limb mesenchyme; m, mesenchyme; me, mesogastrium; nf, neural folds; v, ventricle.

Table 1
Summary of Bmp ligand and *noggin* expression

	Bmp2			*Bmp4*			*Bmp7*			*noggin*		
	E8.75	E9.0	E10.5	E8.75	E9.0	E10.5	E8.75	E9.0	E10.5	E8.75	E9.0	E10.5
Neural folds	++++	N/A	N/A	+	N/A	N/A	++	N/A	N/A	+++	N/A	N/A
Head												
Telencephalon	+	++	++	+	++	+++	++	+++	++++	-	++	+++
Seam	++	++	+	+	+++	++	+	+++	++++	++++	++++	++++
Midbrain	++	++	+	+	+	+	+	+	++	+	++	+++
Hindbrain	++	+	+	-	-	++	-	+	+++	+	++	+++
Heart	++	++	+++	+	+	+	+++	++	++++	+	+	-
Lung	N/A	N/A	-	N/A	N/A	++++	N/A	N/A	++	N/A	N/A	+
Stomach	N/A	N/A	-	N/A	N/A	++	N/A	N/A	+++	N/A	N/A	-
Limb buds	N/A	+++	++++	N/A	+++++	++++	N/A	+++	+++++	N/A	-	-
Somites	+	+	++	++	+	++++	+	+	++++	+	++	+

Table 4.1. Relative expression of Bmp ligands and *noggin* during embryogenesis. Relative in situ intensities were determined from whole mount in situs of *Bmp2*, *Bmp4*, *Bmp7* and *noggin* for various tissues during organogenesis (embryonic stages E8.75, E9.0, E10.5). (-) absent, (+) weak, (+++) medium, and (+++++) strong expression. (N/A refers to absence of structure at early embryonic stage, i.e. not developed yet).

***BmpR1a* – E7.5 to E10.5**

In contrast to the localized and distinct expression patterns of *Bmp2*, *4*, and *7* during early mouse embryogenesis, the BMP receptor *Bmpr1a* is expressed more ubiquitously throughout most embryonic tissues. Expression can be detected at E7.25, albeit at low levels (Fig.4.4A1). Around E8.25, slightly increased levels of transcripts can be detected in the neural folds, as well as the lateral plate mesoderm (Fig.4.4A2). However, moderate levels of transcripts can be observed throughout the embryo, when compared to controls (Fig.4.4A2 and data not shown). By the end of embryonic turning (E8.75), *BmpR1a* is detected in all tissues examined, with the notable exception of the heart atria and ventricle, which have either low or undetectable levels (arrowhead in Fig.4.4A3).

Increased expression can be seen in the telencephalon, the dorsal neural tube, the pharyngeal endoderm and the first two branchial arches (Fig.4.5A1-2 and Fig.4.6A1).

At E9.0, expression is still widespread, but has increased to moderate to high levels, with more intense expression detected in the telencephalon, the first branchial arch and the emerging limb bud (Fig.4.4A4, Fig.4.5A2 and as previously shown in (Dewulf, Verschueren et al. 1995). In addition, higher levels of expression are detected throughout the neural tube, lateral plate mesoderm and limb buds (Fig.4.4A4, Fig.4.5A3 and Fig.4.6A3). Expression continues to appear either low, or absent in the heart myocardium during early heart development (Fig.4.4A4, Fig.4.5A2 and Fig.4.6A2). The increase in limb bud, pharyngeal and neural tube expression intensifies in the following days, from E9.5 to E10.5 (Fig.4.4A5-6). In particular, the anteriormost portion of the head expresses high levels of *BmpR1a* at E10.5. The posterior portion of the tail and most somites also increase expression (Fig.4.5A6). As the hindlimbs (hl) appear, expression of *BmpR1a* appears relatively high in this emerging tissue (Fig.4.4A6). At these later stages, *BmpR1a* is ubiquitously expressed at lower levels in the stomach, mesogastrium of the stomach, and lung buds (Fig.4.4A5, Fig.4.5A4 and Fig.4.6A4-5).

***BmpR1b* – E7.5 to E10.5**

In contrast to the relatively ubiquitous expression of *BmpR1a*, *BmpR1b* expression is more restricted to specific tissues throughout most of early development. At E7.5 expression is present at low levels throughout the early embryo, but is particularly

evident in the forming AIP and the distal yolk sac (Fig.4.4B1). This is in contrast to previous findings that *Bmpr1b* expression does not appear until E9.5 (Dewulf, Verschueren et al. 1995). By E8.5, expression is either low or undetectable in most tissues, however localized expression becomes detectable in the developing neural folds (Fig.4.4B2), including two distinct patches in the hindbrain, in the region of rhombomere 3 (r3) (Fig.4.4B2 inset and Fig.4.5B1). Yolk sac, neural tube, rostral neural folds, somites and lateral plate mesoderm are notably devoid of detectable expression. At E8.75, expression continues in the hindbrain and developing otic vesicle and begins to increase in the telencephalon and in the lateral plate mesoderm (Fig.4.4B3, Fig.4.5B1 and Fig.4.6B1).

By E9.0, *BmpR1b* becomes strongly expressed in the anteriormost telencephalon, the optic cup, the first branchial arch and the hindbrain, while expression has appeared at low levels in the dorsal neural tube, the endoderm, the limb buds, the liver and throughout the somites (Fig.4.4B4, Fig.4.5B2-3 and Fig.4.6B2-3). At E9.5, expression increases in the somites, but declines in most of the gastrointestinal tract (Fig.4.4B5 and Fig.4.5B4). Expression is also observed in the dorsal neural tube, head (hd) and branchial arches as previously shown (Dewulf, Verschueren et al. 1995). This same pattern is mostly maintained through E10.5, when expression is still evident in the head, branchial arches, somites, dorsal neural tube, and with low levels of expression maintained in the limb buds (Fig.4.4B6 and previously shown by (Dewulf, Verschueren et al. 1995). Expression in the gut tube and heart is either low or undetectable (Fig.4.4B3-6, Fig.4.5B4-5 and Fig.4.6B4-5). However, expression continues in both dorsal neural tube and somites (Fig.4.5B6).

***BmpRII* – E7.5 to E10.5**

BmpRII expression resembles *BmpR1a* expression in its widespread embryonic distribution. From E7.25 to E8.25, expression is almost negligible, notably lower than either *BmpR1a* or *BmpR1b* (Fig.4.4C1-2). However, ubiquitous expression begins after turning, at E8.75 (Fig.4.4C3 and previously shown in Bernard et al., 1997). At this stage, *BmpRII* can be found at moderate levels in most tissues, with increased expression in the dorsal rostral neural tube, head, tail, and dorsal region of the somites (Fig.4.4C3, Fig.4.5C1 and Fig.4.6C1). However, like *BmpR1a* and *BmpR1b*, relatively lower levels are observed in the early heart than those observed for Bmp ligands.

At E9.0, expression continues to increase in all tissues, with a marked increase in the anteriormost telencephalon, branchial arches, limb bud and tail tip mesoderm (Fig.4.4C4, Fig.4.5C2-3 and Fig.4.6C2-3). Continued low to negligible levels are observed in the heart (Fig.4.5C2 and Fig.4.6C2). This expression distribution remains constant through E9.5, with levels increasing in the posterior tail, first branchial arch, developing liver and sinus venosus region (Fig.4.4C5). At E10.5, expression is now evident at low levels in the heart myocardium and gut tube (Fig.4.4C6, Fig.4.5C4-5 and Fig.4.6C4-5). Expression in limb buds, both forelimbs and hindlimbs becomes strong by this stage (Fig.4.4C6 and Fig.4. 5C6). In addition, expression in somites intensifies, albeit diffusely, throughout both the dorsal and ventral portions, along entire embryonic axis (Fig.4.5C6).

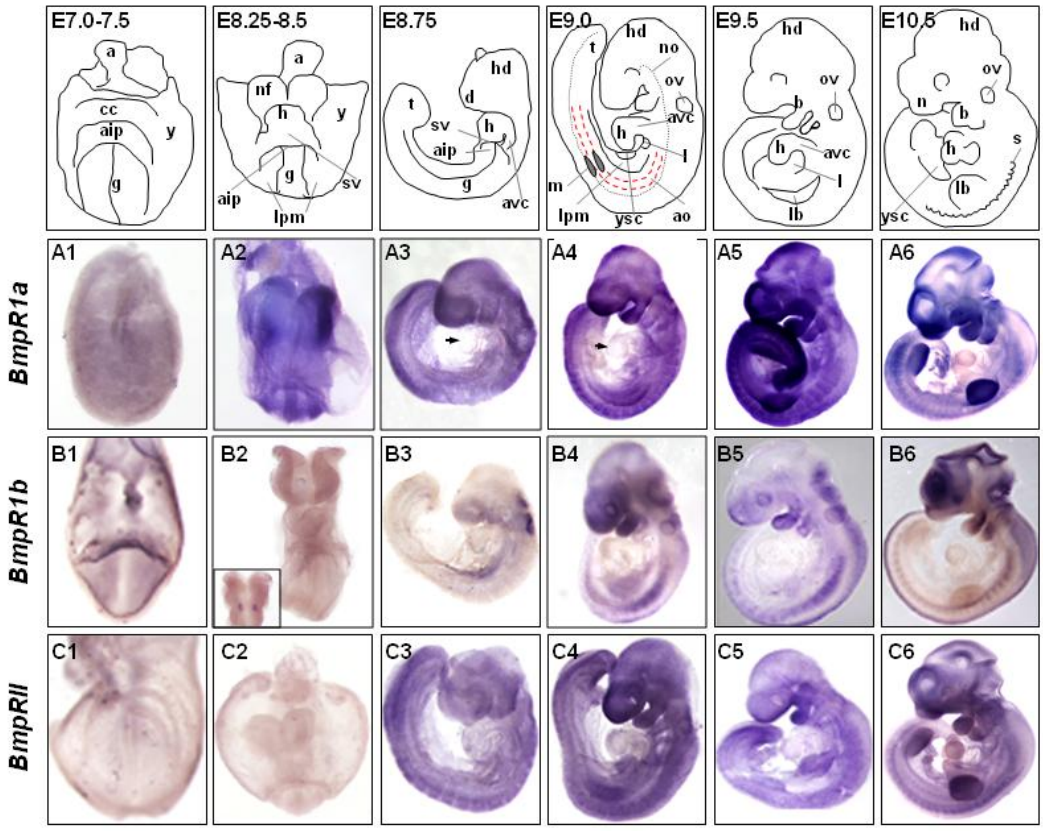

Fig.4.4. Expression of Bmp receptors during embryogenesis. Whole mount in situ hybridization of embryos E7.5-E10.5 for the following transcripts: Panels A) *Bmpr1a*; Panels B) *Bmpr1b*; and Panels C) *BmprII*. Panels 1) E7.25-7.0 embryos are frontal views with head facing forward, allantois in back, pointing up, and yolk sac still attached. A1 and C1 are E7.25; B1 is E7.5. Panels 2) E8.25-E8.5 unturned embryos are shown in frontal views, with head and open AIP facing forward: and yolk sac is still attached, except for panel B2 where yolk sac has been removed. In panel B2, inset shows dorsal view and expression in hindbrain rhombomere 3. Panels 3) E8.75 are shown in process of turning, or shortly after turning. Panels 4-6) E9.0-E10.5 embryos have completed turning and are shown in lateral view facing left. All embryos are shown anterior up, dorsal on right. Embryonic structures and tissues are annotated in top panel schematics, which represent embryos shown in photographs below. Arrowheads in A3 and A4 show low levels of expression in heart. Annotations as follows: a, allantois; aip, anterior intestinal portal; ao, aorta; avc, atrioventricular canal; cc, pre-cardiac crescent; d, diencephalon; g, pre-gut endoderm; h, heart; hd, head; l, liver diverticulum; lb, limb bud; lpm, lateral plate mesoderm; m, mesonephros; n, nasal placode; nf, neural folds; ov, otic vesicle; s, somites; sv, sinus venosus; t, tail; y, yolk sac; ysc, yolk sac constriction.

Expression of Bmp receptors in developing organs and tissues

Overall, expression of the Bmp receptors is relatively widespread in organs and tissues during early embryonic mouse development. *BmpR1a* and *BmpRII* are expressed ubiquitously in most tissues throughout development, whereas expression of *BmpR1b* displays more restriction to specific tissues. At E8.75, however, all three Bmp receptors are expressed in the fusing neural folds, with each receptor having its own distinct pattern (Fig.4.5A1, B1, C1, and Fig.4.6A1, B1, C1). For example, *BmpR1a* is enriched in the cephalic region of the neural folds (absent or low in midbrain region), while *BmpR1b* is only observed in a set of small patches within rhombomere 3 of the hindbrain region, and *BmpRII* is expressed throughout the neural folds with increased expression in the dorsalmost neural tube along its length. Similarly, later between E9.5 and E10.5, all three receptors are found in the telencephalon and branchial arches (Fig.4.4 panels 5-6 and Fig.4.5 panels 1,2). However, *BmpR1b* and *BmpRII* are strongly expressed in neuroectoderm of the roof of the mouth anlagen, while *BmpR1a* is distinctly absent from this region (Fig.4.5 panels 2 and data not shown).

Differences in receptor expression can also be observed in the E10.5 gut tube (Fig.4.5 panels 4, and Fig.4.6 panels 4-5). *BmpR1a* is expressed ubiquitously in all budding organs examined, including the developing lungs and stomach, while *BmpR1b* is completely absent from the lung buds and only slightly expressed in the stomach. Like *BmpR1a*, *BmpRII* expression is widespread in this region, with higher levels near the tips of the lung bud mesoderm and the mesogastrium of the developing gut tube.

Interestingly, all three receptors are expressed at notably low to absent levels in the developing heart (Fig.4.4A3-6, B3-6, C3-6, Fig.4.5 panels 2 and 5, and Fig.4.6 panels 2).

Patterns of Bmp receptor gene expression are also distinct, but overlapping in the developing somites. *BmpR1a* is most strongly expressed throughout the somites, from E9.0 to E10.5 (Fig.4.5A3, A6). While expression of *BmpR1b* is lower and localized to the medial region of the somites (Fig.4.5B3, B6), that of *BmpRII* is more widespread in the somites at E9.0, but becomes slightly enriched in the dorsal portion of the anterior trunk somites by E10.5 (Fig.4.5C3, C6).

Nonetheless, Bmp receptors *1a* and *II* display overlaps in expression in many tissues and organs. Both receptors have markedly strong expression in the branchial arches at E9.0 (Fig.4.4A4, C4 and Fig.4.5A2, C2) and are weakly expressed in the heart throughout development from E8.75 to E10.5 (Fig.4.4A3-6, C3-6 and Fig.4.5A2, A5, C2, C5). Interestingly, in the limb buds, while *BmpR1a* and *BmpRII* are strongly expressed in the limb mesenchyme, they are notably absent from the ectoderm of the forelimb, including the AER (Fig.4.5A3, C3 and Fig.4.6A3, C3). This is surprising given reports that describe inactivation of *BmpR1a* in the AER or ventral limb ectoderm, which show that *BmpR1a* is required in this tissue (Ahn, Mishina et al. 2001; Pajni-Underwood, Wilson et al. 2007). However, *BmpR1a* possibly plays a greater role in the hindlimbs as these are more severely affected in Msx2-cre;BmpR1a$^{flox/null}$ mutants (Pajni-Underwood, Wilson et al. 2007). Overall, we could generalize that Bmp receptors are expressed in a more widespread manner than their ligands and display more overlap. When we analyze the expression patterns of both ligand and receptors, we find that ligand expression is not

confined to regions that completely overlap with domains of receptor expression, suggesting the possibility that other receptors, such as activin receptors, function to transmit BMP signaling in those regions. Relative levels of Bmp receptor co-expression are summarized in Table 4.2.

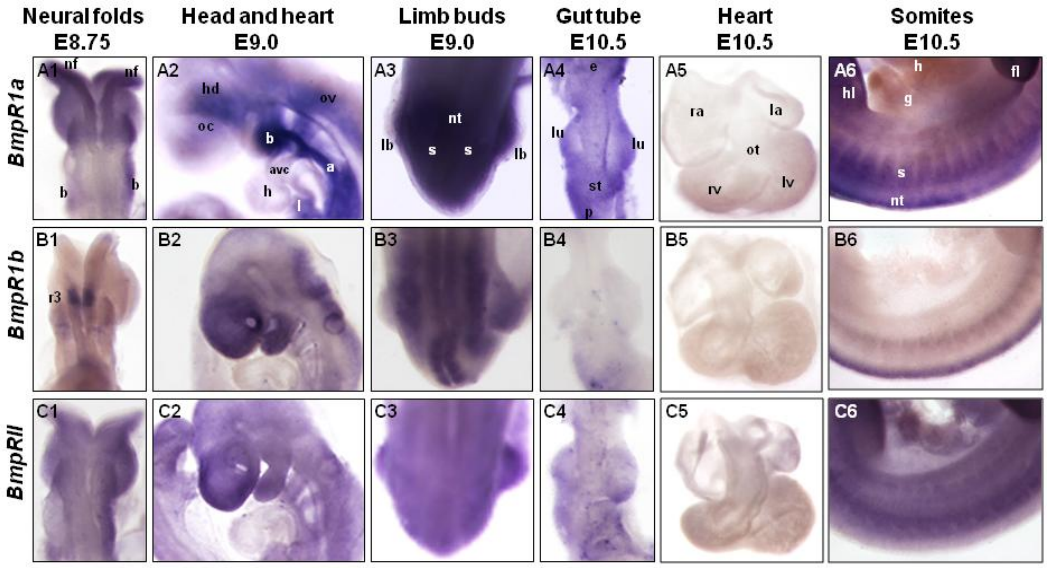

Fig.4.5. Expression of Bmp receptors in developing tissues. Whole mount in situ hybridization of embryos for the following transcripts: Panels A) *Bmpr1a*; Panels B) *Bmpr1b*; and Panels C) *BmprII*. Panels 1) Close up view of embryonic head, showing open neural tube and fusing headfolds at E8.75. Anterior is up, dorsal view. In panel B1, r3 indicates rhombomere 3. Panels 2) Close up view of anterior portion of E9.0 embryo, highlighting head, heart, sinus venosus, branchial arches, otic vesicle, liver and anterior somite expression. Anterior is up, dorsal is on right. Panels 3) Close up on dorsal midsection of embryo, showing neural tube, anterior somites and developing limb buds at E9.0. View of dorsal surface of neural tube, head is up, tail points away from viewer. Panels 4) Close up view of E10.5 developing gut tube dissected away from the embryo, highlighting expression in esophagus/trachea, lungs, stomach and anterior edge of pancreas. At this stage, the gut tube is beginning to break symmetry and turn left. Anterior is up, dorsal view. Panels 5) Close up views of dissected E10.5 hearts. Ventroanterior view, with outflow tract in front and AVC behind. Panels 6) Close up view of E10.5 somites (numbers 12-25). Ventral is up, anterior to right, lateral view. Annotations as follows: a, aorta; avc, atrioventricular canal; b, branchial arches; e, esophagus; fl, forelimb; g, gut; hd, head; h, heart; hl, hindlimb; l, liver diverticulum; la, left atrium; lb, limb bud; lu, lung; lv, left ventricle; nf, neural folds; nt, neural tube; oc, optic cup; ot, outflow tract; ov, otic vesicle; p, pancreas; ra, right atrium; rv, right ventricle; s, somite; st, stomach.

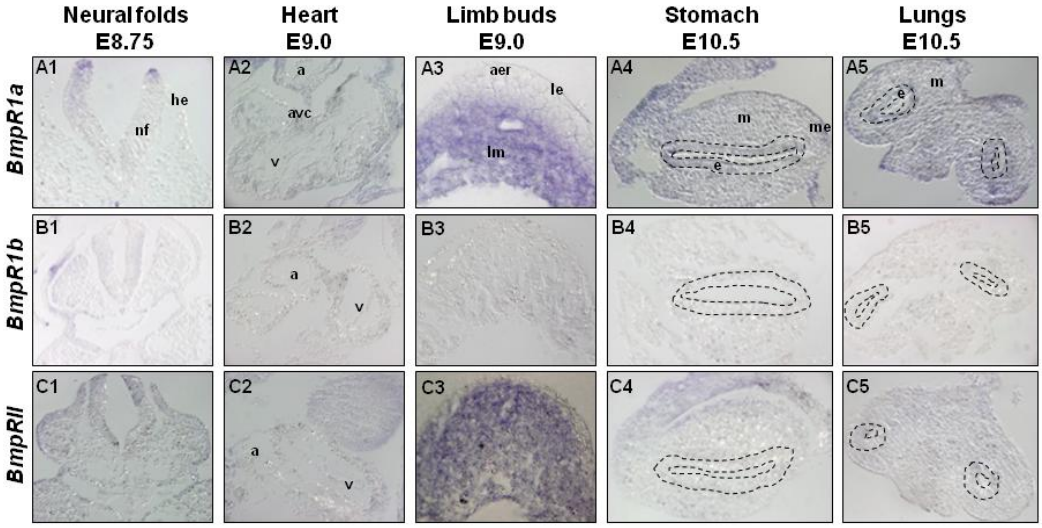

Fig.4.6. Sections showing Bmp receptor expression. Sections of whole mount in situ hybridization of embryonic tissues for the following transcripts: Panels A) *Bmpr1a*; Panels B) *Bmpr1b*; and Panels C) *BmprII*. Panels 1) Transverse sections of neural tubes and headfolds at E8.75. Dorsal is up. Panels 2) Transverse sections of heart at E9.0 showing both atria and ventricle. Panels 3) Transverse sections through developing limb buds at E9.0, showing outer epidermis and underlying limb mesenchyme. Panels 4) Transverse sections of stomach at E10.5, showing both gut epithelium and gut mesoderm. Stomach epithelium outlined with dashed lines; dorsal is to the right. Panels 5) Transverse sections through developing lung buds. Lung bud epithelium outlined with dashed lines. Anterior is up, dorsal view. Annotations as follows: a, atria; aer, apical ectodermal ridge; avc, atrioventricular canal; e, epithelium; he, head epidermis; le, limb epidermis; lm, limb mesenchyme; m, mesenchyme; me, mesogastrium; nf, neural folds; v, ventricle.

Table 2
Summary of Bmp receptor expression

	BmpR1a			*BmpR1b*			*BmpRII*		
	E8.75	E9.0	E10.5	E8.75	E9.0	E10.5	E8.75	E9.0	E10.5
Neural folds	+++	N/A	N/A	++	N/A	N/A	++	N/A	N/A
Head									
Telencephalon	++++	+++	++++	+	+++	++++	++	+++	+++
Seam	+++	+	++	+++	-	+	++	++	++
Midbrain	+++	++	+	-	+	++++	+	++	++
Hindbrain	++	++	++	++	++	++	+	+	+
Heart	-	+	-	-	-	-	+	+	+
Lung	N/A	N/A	+++	N/A	N/A	-	N/A	N/A	++
Stomach	N/A	N/A	+++	N/A	N/A	+	N/A	N/A	++
Limb buds	N/A	++++	++++	N/A	++	+	N/A	+++	++++
Somites	++	+++	+++	-	+++	++	++	++	+++

Table 4.2. Relative expression of Bmp receptors during embryogenesis. Relative in situ intensities were determined from whole mount in situs of *BmpR1a*, *BmpR1b*, and *BmpRII* for various tissues during organogenesis (embryonic stages E8.75, E9.0, E10.5). (-) absent, (+) weak, (+++) medium, and (+++++) strong expression. (N/A refers to absence of structure at early embryonic stage, i.e. not developed yet).

Summary

In this report, we have detailed the expression pattern of several Bmp ligands and their receptor in multiple tissues during murine organogenesis. The Bmp receptor genes, *BmpRII* and *BmpR1a* display largely ubiquitous expression in almost all tissues during embryogenesis; while in contrast, *BmpR1b* expression is more restricted to specific developing tissues such as patches within the hindbrain, otic placode, telencephalon, dorsal neural tube, the first and second branchial arches, limb buds and somites. Bmp ligands, on the other hand, exhibit often highly restricted expression domains that dynamically change patterns in embryonic tissues during their development and are often co-expressed. Generally, *Bmp2* can be summarized to be expressed most strongly in

head, heart AVC, limbs, dorsal aorta, and lateral plate mesoderm. *Bmp4* is found in limb buds, lateral plate mesoderm, somites and telencephalon, with a striking asymmetrical expression in the gut mesenchyme. Lastly, *Bmp7* displays robust expression in the limbs, otic vesicle, telencephalon and somites.

Comparison of the spatial expression patterns of ligands and their receptors demonstrates that ligands are often found in tissues where their accepted receptors are either absent or expressed at low levels, such as the heart. This suggests that perhaps ligands in these tissues bind more than one type of receptor, possibly activin receptors. In addition, the limited overlap between Bmp ligands, and *noggin* expression suggests that the few regions of overlap are likely to represent regions of active extracellular regulation by BMP signaling. The number of receptors and ligands that are co-expressed throughout development suggest that several different BMPs, or combinations thereof, are required for the development of various organs during early embryogenesis. This study should help elucidate single Bmp deletion strategies involving Bmp receptors given the observed redundancy of both ligands and other receptors present in regions of expression.

CHAPTER FIVE

N.B. This chapter has been prepared for submission to the journal Gene Expression Patterns

BMP Ligand and Receptor Expression in the Developing Murine Midgut

Abstract

Here, we report expression of members of the bone morphogenetic protein (Bmp) family and their receptors (Bmprs) in the developing midgut, including the pancreas, stomach, pylorus, and spleen. During pancreatic outgrowth, *Bmp2* is expressed in the dorsal aorta adjacent to the nascent pancreatic bud. *Bmp7* is expressed in the pancreatic epithelium. Additionally, *BmpR1a* and *BmpRII* are expressed ubiquitously in all tissues whereas *BmpR1b* is restricted to the pancreatic mesenchyme. As the pancreas grows and branches, *Bmp7* expression is restricted to the tips of the pancreas located on the left side of the pancreas, and *BmpR1a* and *BmpRII* continue to be expressed ubiquitously. The BMP antagonist *Noggin* is notably absent throughout pancreatic development. Interestingly, *Bmp4* is expressed in an asymmetric pattern along the developing gut tube on the right side of the stomach and pancreas, specifically in the mesogastrium. This study represents the first detailed analysis of expression of Bmps and their cognate receptors during the early development of the pancreas, stomach and spleen. This work will help identify tissues where BMP signaling plays a role in the developing midgut and aide in knockout strategies.

Results and discussion

The pancreas initially forms as dorsal and ventral evaginations of the midgut endoderm beginning on embryonic day 9.0 (E9.0) (reviewed in (Kim and MacDonald 2002). Proliferation of pancreatic bud epithelium and its surrounding mesenchyme results

in the growth and fusion of the dorsal and ventral buds, and the elaboration of a complex branching organ. During growth of the pancreatic epithelium, endocrine cells delaminate from the epithelium, while the tips of branches in the periphery give rise to exocrine clusters of acinar cells and a system of ducts that connect the acinar cells to the duodenum. Despite decades of anatomical, cellular and molecular studies, we are only beginning to understand the complex events that drive endocrine and exocrine specification and differentiation. Little is known about the role of secreted growth factors during pancreatic cell fate progression.

Bone morphogenetic proteins (BMPs) comprise a large, evolutionarily conserved family of secreted signaling molecules, which together form a subgroup of the transforming growth factor (TGF-b) superfamily (Kingsley, 1994). BMPs were originally isolated because of their capacity to promote bone and cartilage formation (Urist, 1965). However, they have since been shown to participate in numerous developmental processes, including the establishment of the initial vertebrate body plan, somite and neural tube patterning, as well as the development of a large number of structures and organs, such as kidney, lung, liver, limb, amnion, eye, teeth, testes and more (reviewed in Hogan, 1996; Zhao, 2003). Deletion of many BMPs (including *Bmp2, 4*) and their receptors (including *Bmpr1a* and *II*) results in early embryonic lethality, with all listed here dying prior to E9.5 when most gastrointestinal organs are just beginning to initiate development (reviewed in (Zhao 2003).

BMPs, like other TGFbs, are first synthesized and folded in the cytoplasm and subsequently cleaved by proteases during secretion. BMPs form large, dimeric proteins, linked by a single interchain disulfide bond. This dimeric conformation is required for

their receptor binding and biological action (Eimond and Harland, 1999). In mouse, a single type II receptor subunit (BMPRII) has been identified (Bepu et al., 1997), while at least three type I receptors have been found (BMPR1a/Alk3, BMPR1b/Alk6 and ActR1a/Alk2) (ten Dijke et al., 1994). Once bound to ligand, type I and II receptors heterodimerize, resulting in type II receptor phosphorylation and activation of the type I receptor. The type I receptor in turn phosphorylates cytoplasmic downstream target proteins, including Smad family proteins (Kretzschmar and Massague 1998).

BMP ligands are expressed in, and required by, multiple tissues during development. Detailed studies of BMP expression during early embryogenesis have been carried out (Lyons, Hogan et al. 1995; Furuta, Piston et al. 1997); however, few studies have explored Bmp expression during midgut development. A detailed study of Bmp expression during pancreatic development is available but limited to reverse transcription studies and does not detail spatial expression of Bmp genes (Dichmann, Miller et al. 2003). Dichmann and colleagues also showed that overexpression of BMPs in the pancreatic milieu results in disrupted stomach, spleen, and pancreas development, suggesting a potential role for BMPs during midgut development. BMPs have also been shown to play a functional role in the pancreatic beta cells during glucose stimulated insulin secretion (GSIS) (Goulley, Dahl et al. 2007).

In this report, we analyze expression of Bmps and their receptors prior to and during gastrointestinal organogenesis, with a special focus on midgut development. In particular, we aim to assess whether BMP signaling occurs in and around the developing pancreas, stomach and spleen. We therefore carry out in situ hybridization for transcripts of *Bmp2*, *4* and *7*, *BmpR1a*, *1b* and *II*, and *Noggin*, on postgastrulation embryos at stages

E9.5, to assess expression in and around the pre-organ endoderm, as well as on dissected midgut organs from E10.5 to E14.5.

Bmp2

As the pancreatic bud proliferates and begins to evaginate at E9.5, *Bmp2* is expressed in the dorsal aorta which immediately overlies the pancreatic bud (Fig.5.1B1). The dorsal aorta at this stage lies in contact with the growing pancreatic epilithlia and is required for the pancreatic development (Lammert, Cleaver et al. 2001). *Bmp2* is also faintly expressed in the liver at this stage. Liver expression continues through to E10.5 and then disappears (Fig.5.1B2); however, *Bmp2* is notably absent from the rest of the stomach and pancreas from this point on (Fig.5.1B3-6).

Bmp4

At E9.5 *Bmp4* is expressed strongly in the lateral plate mesenchyme adjacent to the pancreatic bud (Fig.5.1C1). By E10.5, *Bmp4* expression appears on the right side of the dorsal stomach mesoderm, pancreas and duodenum (Fig.5.1C2). Sections reveal that expression is in mesogastrium , which constricts on the right side. At E11.5 *Bmp4* expression is strongest in the fundal stomach and ridge of the stomach (Fig.5.1C3). *Bmp4* expression remains strong in the fundal part of the stomach by E12.5 with faint expression in the mesothelium of the pancreas (Fig.5.1C4). Expression decreases in the

pancreas and fundal part of the stomach decreases between E12.5-E14.5 (Fig.5.1.C4-C6); however, expression appears in a band just below the pyloric sphincter (Fig.5.1C5).

Bmp7

Bmp7 expression appears in the posteriormost lateral plate and pancreatic endoderm at E9.5 (Fig.5.1D1). By E10.5, *Bmp7* expression appears in the pancreas, stomach, and spleen mesoderm and mesothelium (Fig.5.1D2). Later, at E11.5, *Bmp7* expression is strongest in the posterior part of the stomach and proximal pancreas, with weaker expression in the dorsoanterior region of the stomach (Fig.5.1D3). At E12.5 strong *Bmp7* expression can be seen on the tips of the growing branches of the pancreas (Fig.5.1D4). Interestingly, as the pancreas matures (E13.5-E14.5), *Bmp7* expression decreases from the proximal and right side of the pancreas and expression intensifies on the tips of the branches located on the left side of the pancreas (Fig.5.1D5,D6).

Noggin

Noggin expression is negligible in the midgut and surrounding tissues throughout pancreatic development (Fig.5.1E1-6) with the exception of strong expression in the portal vein at E10.5 (Fig.5.1E2).

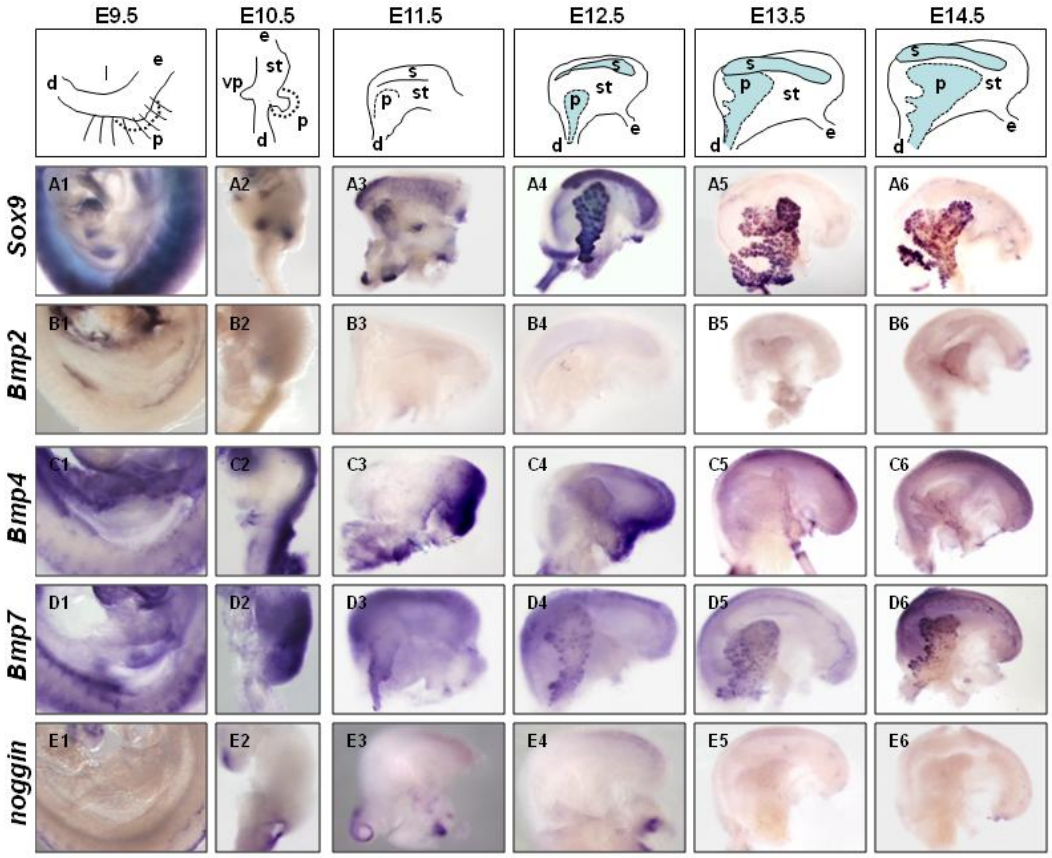

Figure 5.1. Expression of Bmp ligands and the BMP antagonist *noggin* during midgut development. Whole mount in situ hybridization of midgut region E9.5-E14.5 for the following transcripts: Panels A) *Sox9*; B) *Bmp2*; Panels C) *Bmp4*; Panels D) *Bmp7;* and Panels E) *noggin*. Panels 1) E9.5 embryos are lateral views with head facing left, body wall has been dissected for visualization of midgut. Panels 2) E10.5 dissected midgut region is shown in lateral view, with dorsal pancreas at right and ventral pancreas at left. Esophagus at top, intestine at bottom. Panels 3-6) E11.5-E14.5 dissected midgut including stomach, spleen, and pancreas, shown with stomach in background with spleen and pancreas towards viewer. Dotted line denotes dorsal pancreas. d, duodenum; e, esophagus; l, liver; p, dorsal pancreas; s, spleen; st, stomach; vp, ventral pancreas.

Expression of Bmp ligands and *Noggin* in the developing midgut

Bmp ligands are both coexpressed and exhibit differing patterns of expression throughout midgut development (summarized in Table 5.1). The pancreas emerges as a thickening and outpouching (or evagination) of the endoderm epithelia between E8.5 and E9.5 known as the pancreatic bud. By E9.5 the pancreatic bud is in direct contact with two adjacent tissues which are required for pancreatic development; the pancreatic mesenchyme and the dorsal aorta (Fig.5.2A,2B). At this time, *Bmp2* is expressed in the dorsal aorta endothelium (Fig.5.2C), whereas *Bmp4* is restricted to the dorsal gut mesenchyme, or mesogastrium, an area of mesoderm located between the pancreas and the aorta (Fig.5.2D). *Bmp4* expression continues to be expressed in the mesogastrium in an asymmetric manner; away from the developing pancreas as midgut looping occurs (Fig.5.2F,2G). Additionally, *Bmp7* is expressed in both the pancreatic epithelia and mesenchyme (Fig.5.2E).

Bmp4 and *Bmp7* are also expressed in the stomach, especially in the fundal region of the stomach (Fig.5.2H,2I). Conversely, *Bmp2* and *Noggin* are notably absent from the stomach throughout the development of the midgut. Interestingly, all three Bmp ligands and *Noggin* expression is absent from the spleen during this time.

Bmp2, *Bmp4*, and *Noggin* are largely absent from the pancreas during midgut mid to late gestation development. However, following midgut looping, *Bmp7* expression is initiated in the proximal pancreas then becomes restricted to the left side of the pancreas in the epithelial tips of the growing branches (Fig.5.2J-L). Interestingly, these

branches located on the left side of the pancreas have been shown to be the most rapidly growing region during pancreatic development (Villasenor and Cleaver, unpublished).

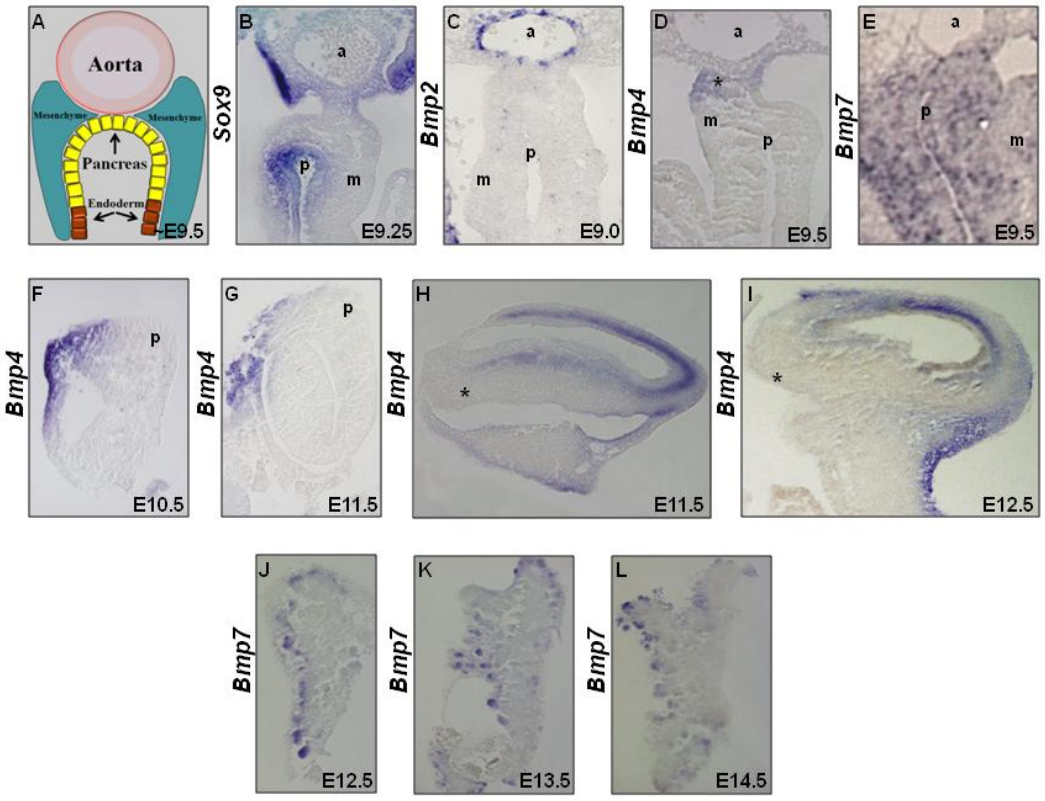

Figure 5.2. Expression of Bmp ligands and the BMP antagonist *noggin* in developing midgut. Sections of whole mount in situ hybridization of developing midgut. A-D) Cross sections of E9.5 pancreatic bud for transcripts: *Sox9*, *Bmp2*, *Bmp4*, *Bmp7* respectively. Dorsal is up. E) Cross section of E10.5 pancreatic bud stained for *Bmp4*. Dorsal is up. F) Cross section of E11.5 stomach and pancreas stained for *Bmp4*. Dorsal is up. G-H) Transverse sections of stomach at E11.5 stained for *Bmp4*, showing both gut epithelium and gut mesoderm. Rostral is left. I-K) Transverse sections through developing pancreas for *Bmp7* E12.5, E13.5, and E14.5 respectively. Pancreatic head is up, tail is down. * denotes mesogastrium and/or fundal stomach; a, aorta; m, pancreatic mesoderm; p, dorsal pancreas.

Summary of Bmp ligand and *noggin* expression in the developing midgut

	Bmp2	Bmp4	Bmp7	Noggin
Liver	+/-	-	-	-
Fundal	-	+/+	+/-	+/-
Gastric	-	+	+/-	-
Pyloric Sphincter	-	+	-	+/-*
Spleen	-	-	-	-
Pancreas	-	+/-	+/+#	-

Table 5.1. Relative expression of Bmp ligands and *noggin* during midgut development. Relative in situ intensities were determined from whole mount in situs of *Bmp2*, *Bmp4*, *Bmp7* and *noggin* for various tissues during midgut development. (-) absent, (+/-) weak, (+) medium, and (+/+) strong expression. #, mostly at tips; *, posterior to pyloric sphincter intestine.

BmpR1a

BmpR1a is initially expressed ubiquitously in the gut epithelia, mesenchyme and dorsal aorta at E9.5 (Fig.5.3A1). At E10.5 *BmpR1a* is enriched in the dorsal portion of the gut tube (Fig.5.3A2). By E11.5 *BmpR1a* expression appears strong in the portal vein, and remains ubiquitously expressed in the stomach, pancreas, and spleen throughout midgut development from E12.5 to E14.5 (Fig.5.3A3-6).

BmpR1b

BmpR1b expression appears in the pancreatic mesenchyme and stomach region at E9.5, and continues to be expressed in the posterior stomach throughout midgut

development from E10.5 to E14.5 (Fig.5.3B2-6). However, *BmpR1b* is notably absent from the pancreas and spleen following gut turning (E11.5-14.5).

BmpRII

BmpRII is ubiquitously expressed in the pancreatic epithelium, pancreatic mesenchyme, dorsal aorta, and throughout the midgut at E9.5 but then lowers in expression between E10.5 to E14.5(Fig.5.3C1-C6). *BmpRII* expression is enriched in a band below the pyloric sphincter (Fig.5.3C5,C6 and Fig5.4F).

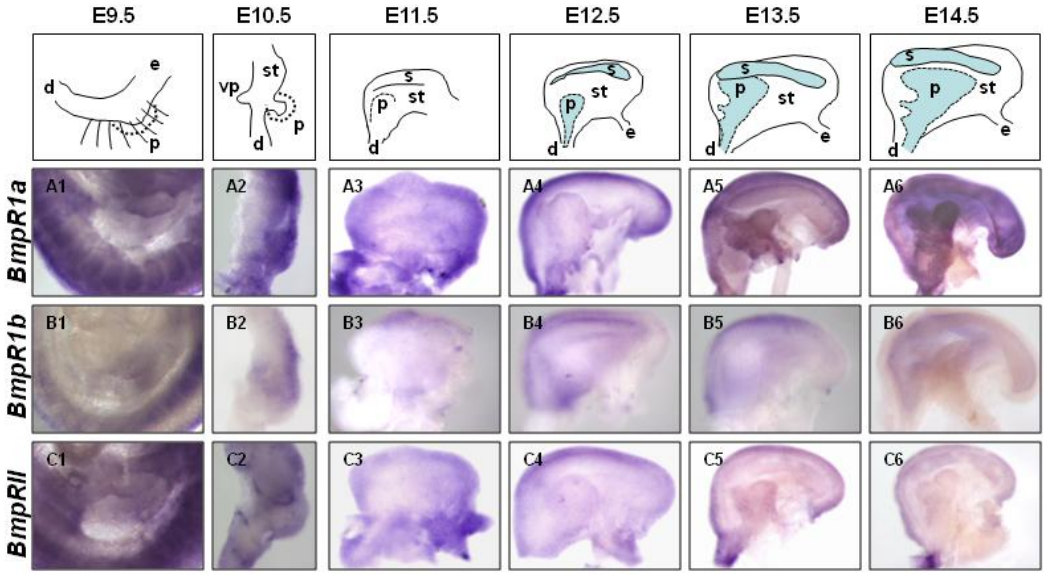

Figure 5.3. Expression of Bmp receptors during embryogenesis. Whole mount in situ hybridization of midgut region E9.5-E14.5 for the following transcripts: Panels A) *BmpR1a*; B) *BmpR1b*; and Panels C) *BmpRII*. Panels 1) E9.5 embryos are lateral views with head facing left; body wall has been dissected away for visualization of midgut. Panels 2) E10.5 dissected midgut region is shown in lateral view, with dorsal pancreas at right and ventral pancreas at left. Esophagus at top, intestine at bottom. Panels 3-6) E11.5-E14.5 dissected midgut including stomach, spleen, and pancreas, shown with stomach in background with spleen and pancreas towards viewer. Dotted line denotes dorsal pancreas; d, duodenum; e, esophagus; p, dorsal pancreas; s, spleen; st, stomach; vp, ventral pancreas.

Expression of Bmp receptors in the developing midgut

BmpR1a and *BmpRII* are expressed in all midgut tissues during early development (summarized in Table 5.2). *BmpR1b* on the other hand, is expressed in the earlypancreatic mesoderm and later in the stomach. During pancreatic bud evagination, the pancreatic epithelium, mesenchyme, and aorta all ubiquitously express *BmpR1a* and

BmpRII (Fig.5.4B,D). *BmpR1b* however, is only expressed in the pancreatic mesenchyme and is notably absent from the pancreatic endoderm and the aorta (Fig.5.4C).

BmpR1a, *BmpR1b* and *BmpRII* are all ubiquitously expressed in the stomach during midgut development (Fig.5.3A2-A6, Fig.5.3B2-B6 and C2-C6). In particular, *BmpR1a* has striking expression along the seam of the stomach (the region between left and right halves of the stomach) at embryonic day 12.5 (Fig.5.3A4 and Fig.5.4E), while *BmpRII* is strongest in a band located directly posterior to the pyloric sphincter (Fig.5.3C5-C6 and Fig.5.4F).

As the pancreas branches and grows after gut turning (E11.5-E14.5), *BmpR1a* and *BmpRII* are ubiquitously expressed throughout the pancreas (Fig.5.3A3-A6, and C3-C6). However, *BmpR1b* is notably absent in expression (Fig.5.3B3-B6). Similarly, the spleen also expresses both *BmpR1a* and *BmpR1b* throughout midgut development, and once again *BmpR1a* is notably absent.

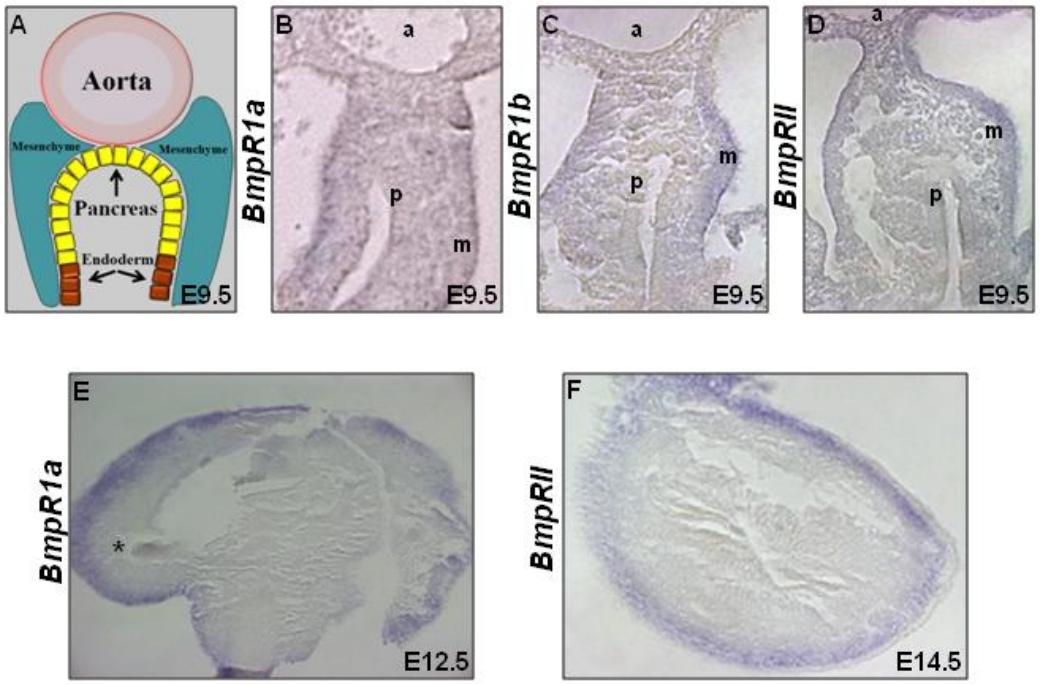

Figure 5.4. Sections showing Bmp receptor expression. Sections of whole mount in situ hybridization of developing midgut. A-C) Cross sections of E9.5 pancreatic bud for transcripts: *BmpR1a*, *BmpR1b*, and *BmpRII* respectively. Dorsal is up. D) Transverse section of E12.5 pancreatic bud stained for *BmpR1a* showing both gut epithelium and gut mesoderm. Rostral is left. E) Cross section through duodenum at E14.5 stained for *BmpRII*. *, denotes fundal stomach; a, aorta; m, pancreatic mesoderm; p, dorsal pancreas.

Summary of Bmp receptor expression in the developing midgut

	BmpR1a	*BmpR1b*	*BmpRII*
Liver	+/-	-	+/-
Fundal	+/-	+/-	+/-
Gastric	+/-	+/-	-
Pyloric Sphincter	+/-	-	+/-
Spleen	+/-	-	+/-
Pancreas	+/-	-	+/-

Table 5.2. Relative expression of Bmp receptors during midgut development. Relative in situ intensities were determined from whole mount in situs of *BmpR1a*, *BmpR1b*, and *BmpRII* for various tissues during organogenesis. (-) absent, (+/-) weak, (+) medium, and (++) strong expression.

Summary

This study has shown that various Bmp ligands and Bmp receptors are expressed in the developing midgut in dynamic patterns. It has been shown that overexpression of Bmps in the murine pancreatic milieu can block pancreatic development (Dichmann, Miller et al. 2003), implying tissues are receptive to BMP signaling most likely due to their expression of Bmp ligands. This study tracks the expression of the Bmp ligands, *Bmp2, 4*, and *7*, as well as the Bmp receptors throughout pancreatic development. At the beginning of pancreatic development at around E9.0-9.5, Bmp ligands and receptors are expressed in all three of the tissues that are required for pancreatic development, which include the pancreatic epithelium, the pancreatic mesenchyme and the dorsal aorta endotheilium (Summarized in Fig.5.5). Later in development, after the gut has completed

turning, *Bmp7* is strikingly expressed in the tips of the growing branches located on the left side of the pancreas when viewed laterally, an area known for rapid proliferation (Villasenor and Cleaver, unpublished). Various Bmp ligands and receptors are also expressed in the forming spleen and stomach as well, another area shown to have developmental defects upon overexpression of BMPs in the midgut milieu (Dichmann, Miller et al. 2003).

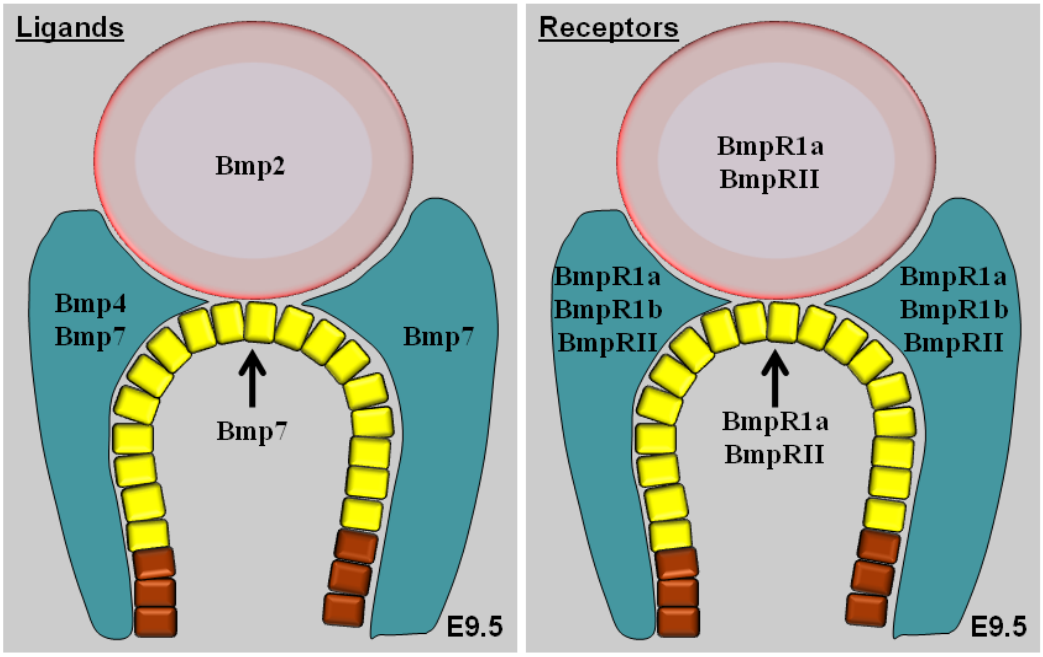

Figure 5.5. Summary of Bmp ligand and receptor expression during pancreatic bud evagination. Model denotes Bmp ligand and receptor expression in dorsal pancreatic bud cross section with pancreatic epithelia in yellow, pancreatic mesenchyme in blue, aorta in red, and endoderm in brown.

CHAPTER SIX

N.B. Ondine Cleaver provided pictures for wt vs. Pdx Noggin pancreata shown in figure 6.1 A, B and performed section immunohistochemistry for analysis of the Pdx Bmp2 images in figure 6.4

BMP signaling through BMPR1A is required for establishment of pancreatic laterality

Abstract

Signals that drive left-right (LR) asymmetric morphogenesis during organogenesis have not been identified. Bone morphogenetic proteins (BMPs) play important roles in the development of numerous organs (Hogan 1996). A role for BMPs during pancreatic development has been suggested by others; however, due to the inherent redundancy of BMP ligands and receptors, a developmental role for BMPs during murine pancreatic development has not been elucidated. In this study, we address redundancy issues by utilizing a transgenic system to knockdown several BMP ligands simultaneously to show that BMP signaling is indeed required for lateral growth of the pancreas. Additionally, we show that signaling through BMPR1A, but not BMPRII, is essential for expression of *Bapx1*, a gene required for SMP formation and lateral growth of the pancreas (Hecksher-Sorensen, Watson et al. 2004). Therefore, defects in lateral growth of the pancreas in *Bmpr1a* mutants are mediated by *Bapx1* and accordingly *Bmpr1a* mutant pancreata exhibit misregulation of genes that require *Bapx1* including *Fgf9*, and *Fgf10*. *Bmpr1a* null pancreata also exhibit misregulation of *Barx1*, another gene with SMP restricted expression. Interestingly, *Bmp4* is asymmetrically expressed in the developing midgut, and BMPs are also asymmetrically signaling through blood vessels in the pancreas. Therefore, pancreatic laterality is driven by *Bapx1*-mediated BMP signaling through BMPR1A to promote SMP formation, likely through endotheilial signaling, which depends itself on remodeling of the pancreatic vasculature. Here we present the first evidence of a cell-cell signaling molecule required for left-right patterning in organogenesis.

Introduction

Pancreatic development begins at embryonic day 8 (E8.0) in the mouse (Kim and MacDonald 2002). The pancreas originates from two patches of endoderm located at the juncture of the future stomach and duodenum. These patches begin to proliferate and evaginate to form the pancreatic bud by E9.0. As the pancreatic bud grows and differentiates, the pancreas and stomach begin to turn clockwise (Hecksher-Sorensen, Watson et al. 2004). In addition, inductive signals from the adjacent notochord (Kim, Hebrok et al. 1997), aorta (Lammert, Cleaver et al. 2001; Jacquemin, Yoshitomi et al. 2006) and pancreatic mesenchyme (Golosow and Grobstein 1962; Duvillie, Attali et al. 2006), are required for pancreatic development; however, the identity of these signaling molecules remains largely unknown. The identification of these molecules is essential for understanding the underlying mechanism, by which the pancreas develops and grows laterally.

During development of the mammalian embryo. In addition left-right (LR) asymmetric growth occur during embryonic turning and continue throughout embryogenesis to pattern organs (Capdevila and Belmonte 2000). Several organs undergo asymmetric LR morphogenesis during organogenesis including the heart, brain, lungs, pancreas and more (Shiratori and Hamada 2006). Organs that are either present only on one side of the body, or exhibit differences between left and right have undergone asymmetric growth have undergone lateral growth.

LR patterning requires two steps, initially, *Nodal* is expressed in the node and concentrated on the left side of the embryo at E7.5 where it functions in axis formation (Levin 2005; Tian and Meng 2006; Shen 2007). Next, asymmetric signaling is transmitted to the lateral plate mesoderm (LPM) by asymmetrical expression of Nodal (Brennan, Norris et al. 2002; Saijoh, Oki et al. 2003). *Nodal* is expressed in the early LPM, but becomes attenuated before organogensis (~E9.0). It is unknown what other signals drive asymmetric patterning during organogenesis. Currently, a few transcription factors have been shown to affect LR patterning during organogenesis (Logan, Pagan-Westphal et al. 1998; Hecksher-Sorensen, Watson et al. 2004; Davis, Kurpios et al. 2008); however, nothing is yet known about the extrinsic signaling molecules upstream of these transcription factors that drive LR patterning in the embryo (Shiratori and Hamada 2006).

One transcription factor that has been shown to be required for the development of the pancreas is Bapx1 (Nkx3.2) (Hecksher-Sorensen, Watson et al. 2004). The authors of this work showed that a layer of mesothelial cells on the left side of the developing pancreas, known as the splanchnic mesodermal plate (SMP), begins to thicken and ultimately is required for proper lateral growth of the pancreas. This mesothelial layer normally expresses *Bapx1, Fgf9,* and *Fgf10* and fails to thicken in *Bapx1* mutant pancreata, resulting in disruption in lateral growth. Additionally, *Bapx1* mutant pancreata exhibit misregulation of *Fgf9* and *Fgf10* (Hecksher-Sorensen, Watson et al. 2004).

Bone morphogenetic proteins (BMPs) are a family of secreted signaling proteins. BMPs signal through type II and type I Activin and BMP receptors. Upon binding of BMP ligands to at least one type II and one type I receptor, downstream effectors known

as SMADs are phosphorylated. Once phosphorylated, SMADs shuttle to the nucleus where they help transcribe inhibitor of differentiation (Id) genes. *Id* genes bind promoters and prevent transcription of various genes, which can promote cellular differentiation.

During development, BMPs function as morphogens and have been shown to be required for the development of several tissues and organs (Hogan 1996; Zhao 2003) such as: lungs (Eblaghie, Reedy et al. 2006), heart (Zhang and Bradley 1996), kidney (Dudley, Godin et al. 1999), limbs (Ovchinnikov, Selever et al. 2006), bone, cartilage, blood vessels (Park, Lavine et al. 2006) and pyloric sphincter. Bmp ligands and receptors are known to be expressed in the developing pancreas (Dichmann, Miller et al. 2003), and several studies have suggested a requirement for BMPs during cellular proliferation (Hua, Zhang et al. 2006) and differentiation (Jiang, Stanley et al. 2002; Kumar, Jordan et al. 2003; Jiang and Harrison 2005; Yew, Hembree et al. 2005) of pancreatic progenitor explants and cells. BMP signaling has been shown to be important for programming of the prepancreatic endoderm in *Xenopus* (Pan, Chen et al. 2007; Spagnoli and Brivanlou 2008) and Zebrafish (Tiso, Filippi et al. 2002). BMP signaling has also been shown to be required for endocrine development in zebrafish (Song, Kim et al. 2007). In vertebrates, overexpression of *Bmp2* in the midgut results in reduced pancreas (Dichmann, Miller et al. 2003), and BMP signaling has been shown to play a role in glucose stimulated insulin secretion (Goulley, Dahl et al. 2007); however, a direct link between BMP function and pancreatic development has not been established in vertebrates.

Here, we demonstrate that mutants that disrupt BMP signaling display defects in lateral growth of the pancreas. This study provides the first evidence of signaling molecules playing a role in asymmetric organ growth during organogensesis.

Specifically, BMP signaling through BMPR1A is required for *Bapx1* mediated SMP formation and lateral growth of the pancreas.

Results

BMP signaling is required for lateral growth of the pancreas

Knockdown of BMPs in the pancreatic anlage results in lateral growth defects

We have previously shown that several Bmp ligands and receptors are expressed in the developing murine midgut during pancreatic development (Danesh, Villasenor et al. 2009). To account for potential functional redundancy between various BMPs and BMP receptors, we employed a strategy to knockdown all BMP ligands by overexpression of NOGGIN, a secreted antagonist of BMP ligands. *Noggin* cDNA was placed in a transgenic construct under the control of the pancreas and duodenum homeobox 1 (*Pdx-1*) promoter; this transgenic construct also contains an internal ribosome entry site (IRES) sequence for co-expression eGFP. The *Pdx-1* gene is expressed during early pancreatic differentiation (E8.5), prior to pancreatic budding and lateral growth. Transgenic mice survive until about P25, but die likely due to malformation of the pyloric sphincter and associated digestive problems. For this reason, we could not maintain lines of Pdx-Noggin-ires-eGFP transgenics but instead studied founder embryos that resulted from transgenic injection and in-vitro re-implantation. Transgenic embryos were identified based on expression of eGFP. The Pdx-Noggin-ires-

eGFP midgut displayed reduced pancreas (Fig.6.1A, B), reduced spleen (Fig.6.1B, C), and reduced stomach (Fig.6.1E, F) when compared to Pdx-eGFP. Additionally, the pyloric sphincter failed to form (Fig.6.1E, F and as previously reported in chicks when BMP signaling was blocked using dominant negative constructs (Smith and Tabin 1999)). Currently, these tissues are being sectioned and cellular architecture is being analyzed. Previous data from Carrie Soukup has shown that Pdx-Noggin-ires-eGFP stomach, pyloric sphincter, and duodenum contain increased levels smooth muscle as demonstrated by increased levels of smooth muscle actin staining (data not shown).

Of particular interest, I noted that sections of Pdx-Noggin-ires-eGFP transgenic pancreata displayed defective SMP formation and lateral growth of the pancreas (Fig.6.2A, B). As a control, Pdx-ires-eGFP transgenics were constructed to mark the epithelium of the pancreas and denote the region of expression of the Pdx promoter. Following normal lateral growth, the pancreas is positioned on the left side of the gastric stomach by E15.5 (Fig.6.3A, B); however, knockdown of BMP signaling in the pancreatic milieu via overexpression of Noggin results in mislocalization of the pancreas to a midline position along the dorsal portion of the stomach (Fig.6.3C, D). Pdx-Noggin transgenics also displayed reduced spleen (right isomerism), usually indicative of LR patterning defects. The pancreas and spleen share a common mesenchyme which expands during lateral growth of the midgut. Therefore, defects in lateral growth of this region often result in situs defects in both the spleen and the pancreas. These results suggest that Pdx-Noggin transgenics display defects in LR patterning of the pancreas and spleen.

BMP2 overexpression does not affect pancreatic laterality, but blocks pancreatic growth and differentiation

Previously, overexpression of BMP6 in the pancreatic milieu have been shown to block pancreatic development (Dichmann, Miller et al. 2003) strongly suggesting that pancreatic tissues can respond to BMP signaling and depend on proper dosage of BMP signals for proper development. To determine whether overexpression of BMPs in the pancreas affects lateral growth of the pancreas, we overexpressed *Bmp2* in the midgut. A transgenic construct was made with Pdx1 promoter driving expression of *Bmp2*. Similar to Pdx-Noggin ires eGFP transgenics, we were unable to maintain stable lines of Pdx-Bmp2 ires eGFP transgenics and individual transiently transgenic embryos were dissected and identified by the expression of GFP in the developing pancreas. Transgenic embryos display highly reduced pancreata that fail to differentiate into endocrine (Fig.6.4 A, B, C) or exocrine (Fig.6.4A, D) lineages. Overexpression of BMP2 appears to block both the proliferation and differentiation of the pancreas. However, sections of E10.5 (35 somite) Pdx-Bmp2-ires-eGFP pancreata did not display defects in lateral growth of the pancreas and exhibited normal thickening of the SMP and leftward growth (Fig6.2A, C), suggesting that defects in differentiation and growth seen with overexpression of *Bmp2* in the pancreas are not caused by defects in laterality. These results show that BMP signaling is required for lateral growth of the pancreas and spleen, but excess levels of BMP signals does not impair asymmetric growth.

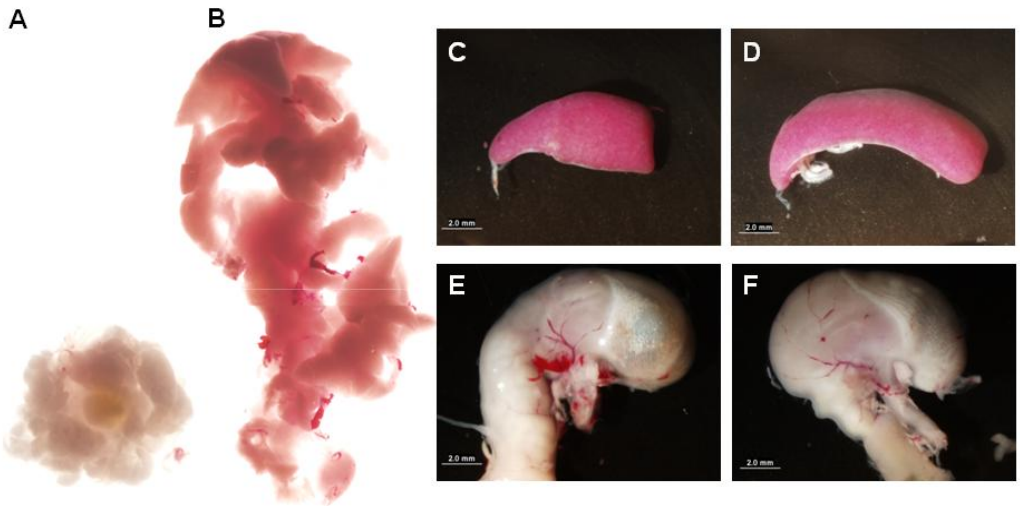

Figure 6.1. Pdx Noggin ires eGFP transgenics exhibit reduced pancreas, spleen, and stomach. A.) Adult Pdx-Noggin ires eGFP pancreas. B.) Adult wild type pancreas. C.) Adult Pdx-Noggin-ires-eGFP spleen. Spleen development is perturbed on the gastric end of the spleen, closest to area of Pdx Noggin expression D.) Adult wild type spleen. E.) Adult Pdx-Noggin-ires-eGFP stomach. F.) Adult wild type stomach.

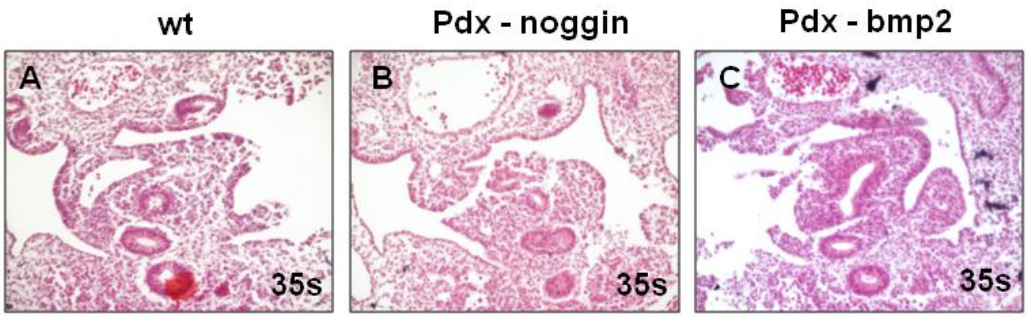

Figure 6.2. BMP signaling is required for lateral growth of the pancreas. A.) Section through wild type pancreatic bud at E10.5 (35 somites). SMP is beginning to thicken and pancreas is growing to the left. Dorsal is up left is right. B.) Section through Pdx Noggin ires eGFP pancreatic bud at E10.5 (35 somites). SMP fails to thicken and pancreas fails to grow laterally. Dorsal is up left is right. C.) Section through Pdx Bmp2 ires eGFP pancreatic bud at E10.5 (35 somites). SMP is beginning to thicken and pancreas is growing to the left. Dorsal is up left is right.

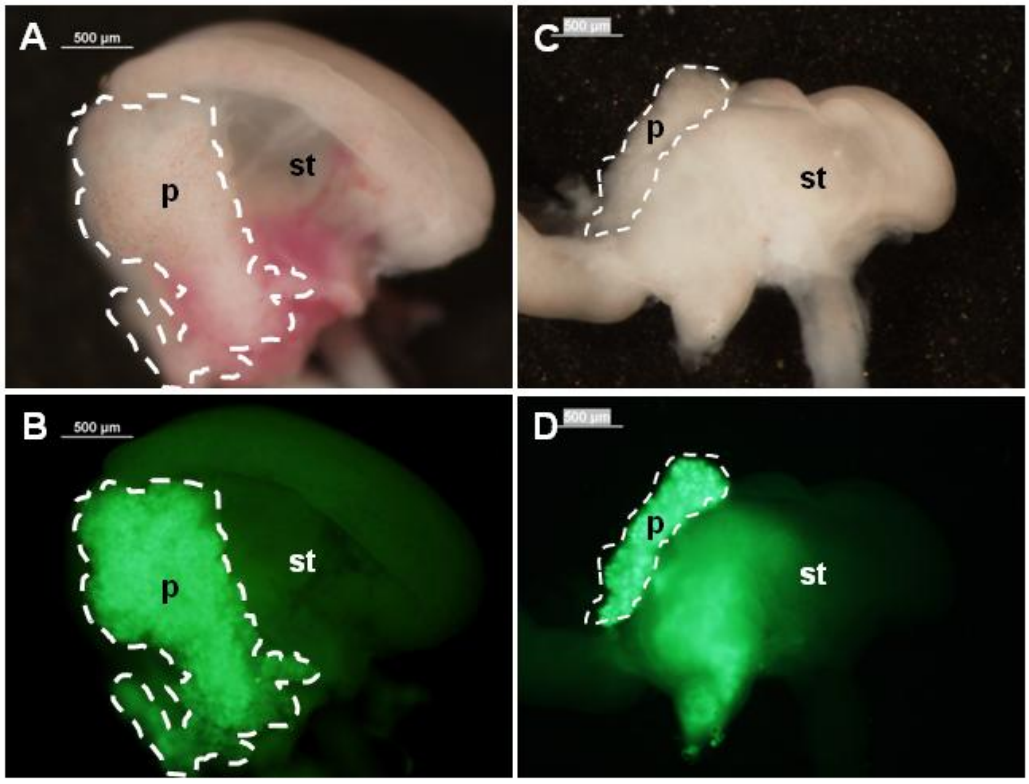

Figure 6.3. Knockdown of BMP signaling in the embryonic midgut results in defective lateral growth of the pancreas. A.) Pdx-ires-eGFP control midgut showing pancreas (outlined) on left side of gastric stomach. B.) GFP expression for Pdx-ires-eGFP control midgut showing pancreatic epithelia (outlined) on left side of stomach. C.) Pdx-Noggin-ires-eGFP transgenic midgut showing reduced pancreas (outlined) mislocated to dorsal side of gastric stomach. D.) GFP expression for Pdx-Noggin-ires-eGFP transgenic midgut showing reduced pancreas (outlined) mislocated to dorsal side of gastric stomach.

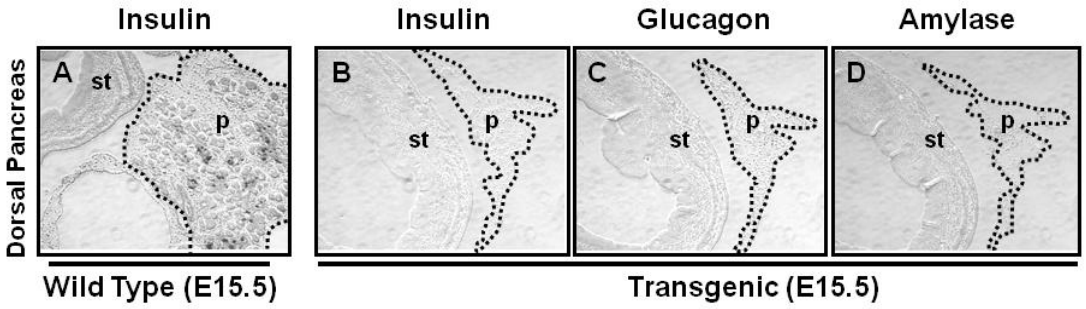

Figure 6.4. Overexpression of *Bmp2* in the pancreas blocks cellular differentiation. A.) Section in immunohistochemistry of wild type pancreas for insulin. B.) Antibody stain of Pdx Bmp2 ires eGFP pancreas for insulin. C.) Antibody stain of Pdx Bmp2 ires eGFP for glucagon. D.) Antibody stain of Pdx Bmp2 ires eGFP for amylase.

BMP signaling through BMPR1A is required for lateral growth of the pancreas and expression of laterally expressed genes in the SMP

BmprII is dispensable for pancreatic development

I have previously shown that both *BmprII* and *Bmpr1a* are widely expressed in the budding pancreas (Fig.5.4 B, C). To determine which of these two BMP receptors is required for pancreatic laterality, I employed conditional knockout strategies to disrupt *BmprII* and *Bmpr1a*. *BmprII* null animals arrest during the egg cylinder stage (Beppu, Kawabata et al. 2000), so a conditional floxed allele of *BmprII* was developed by Beppu and colleagues (Beppu, Lei et al. 2005) and crossed with Mox2-Cre, a ubiquitously expressed Cre line that is not expressed until after gastrulation, thereby avoiding the early lethality seen with *BmprII* null animals. Mutant embryos were provided by Beppu for analysis. Pancreata from wild type and conditional knockout of *BmprII* showed no defects in laterality, morphology, or size (Fig.6.5A, B). Sections revealed no discernable

defect in structure (Fig.6.5.C, D, E) or expression of pancreatic markers for endocrine (Fig.6.6A, B, E, F, I, J), acinar (Fig.6.6C, G, K), or duct lineages (Fig.6.6D, H, L). Therefore, *BmprII* is not required for lateral growth or development of the pancreas and its different cell lineages.

Bmpr1a is required for lateral growth of the pancreas, expression of Bapx1, and regulation of laterally expressed genes in the SMP

Bmpr1a mutant embryos arrest before E9.5 and are unable to form a mesodermal layer (Mishina, Suzuki et al. 1995). To circumvent early lethality of *Bmpr1a* mutants, a floxed allele of *Bmpr1a* was constructed by Mishina and colleagues (Mishina, Hanks et al. 2002). I crossed *Bmpr1a* floxed mice with a Cagg-ER-Cre mouse line, a ubiquitously expressed inducible Cre, developed by Andy McMahon and colleagues (Hayashi and McMahon 2002). Cre activity is induced upon tamoxifen injection. Pregnant females were injected at E8.5 and dissected at E10.5, resulting in efficient ubiquitous expression of Cre as shown by Rosa reporter (Fig.6.7). Bmpr1a $^{flox/flox}$, Cagg-ER-Cre embryos exhibited defects in lateral growth of the pancreas similar to those seen in Pdx-Noggin ires-eGFP transgenics (Fig.6.9). At E10.5, the SMP failed to thicken and leftward growth of the pancreas was blocked.

To determine whether BMP signaling through BMPR1A is required for expression of laterally expressed markers of the pancreas, I first characterized the expression dynamics of a number of genes known to be expressed in the developing SMP during lateral growth, including *Bapx1*, *Barx1*, *Fgf9*, and *Fgf10* (Fig.6.8). *Bapx1* has

previously been shown to be expressed in the SMP and required for lateral growth of the pancreas (Hecksher-Sorensen, Watson et al. 2004). Here, I show that *Bapx1* expression is symmetrical at E9.0-E9.75 (17-27 somites) (Fig.6.8A-C); however, expression becomes restricted to the mesothelium by E10.0 (28-30 somites)(Fig.6.8D) and subsequently becomes most strong in the SMP by E10.5 (33-35 somites)(Fig.6.8E). *Fgf10* and *Fgf9* have also been shown to be expressed in the SMP and their expression is dependent on *Bapx1* (Hecksher-Sorensen, Watson et al. 2004). I found that *Fgf10* is symmetrically expressed in the pancreatic mesenchyme and mesothelium at E9.5 (26-27 somites)(Fig.6.8G), slightly later than expression of *Bapx1*. Also, like *Bapx1, Fgf10* expression becomes restricted to the developing mesothelium and ventral SMP by E10.0 (28-30 somites) (Fig.6.8I). Interestingly, *Fgf9* expression is first observed in the pancreatic mesenchyme between the aorta and the pancreatic bud (mesogastrium)(Fig.6.8M). Unlike *Bapx1* and *Fgf10*, *Fgf9* expression becomes restricted to the dorsal SMP with no expression at all in the mesothelium on the right side of the pancreatic bud by E10.5 (33-35 somites) (Fig.6.8O). *Barx1* is symmetrically expressed at E9.0 (17-19 somites) in both the mesoderm and the mesothelium (Fig.6.8P). Expression intensifies in the SMP and attenuates in the mesenchyme and the mesothelium on the right side of the gut tube by E10.5 (Fig.6.8Q-T). Like *Fgf9*, *Barx1* is only expressed in the SMP at E10.5; however, unlike *Fgf9*, *Barx1* is expressed both dorsally and ventrally along the SMP (Fig.6.8T).

To determine whether BMPR1A signaling is required for expression of these laterally expressed genes, E10.5-E10.75 Bmpr1a $^{flox / flox}$; x Cagg-ER-Cre embryos were sectioned and expression of the aforementioned laterally expressed genes was analyzed.

Bapx1 expression was greatly reduced in mutant pancreata, suggesting that BMP signaling through BMPR1A is required for expression of *Bapx1* (Fig.6.9A, B). *Bapx1* has previously been shown to be required for both the lateral growth of the pancreas and transcriptional regulation of *Fgf10* and *Fgf9* (Hecksher-Sorensen, Watson et al. 2004). In *Bapx1* mutants, it is reported that *Fgf10* expression is reduced in the SMP and increased in the pancreatic mesenchyme. *Fgf10* expression mislocalization in *Bmpr1a* mutant pancreata was consistent with those observations (Fig.6.9C, D). Additionally, *Bapx1* mutant pancreata have been shown to have reduced expression of *Fgf9* in the SMP (Hecksher-Sorensen, Watson et al. 2004). Consistently, I see reduced expression of *Fgf9* in the SMP of *Bmpr1a* mutant embryos (Fig.6.9E, F). Interestingly, although *Bapx1* mutant pancreata did not exhibit defects in *Barx1* expression (Hecksher-Sorensen, Watson et al. 2004), *Barx1* appears to be restricted to the dorsal SMP in *Bmpr1a* mutant pancreata (Fig.6.9G, H), while wild type *Barx1* expression is seen in both dorsal and ventral SMP (Fig6.9G). These results show that BMP signaling through BMPR1A is required for *Bapx1* mediated lateral growth of the pancreas, as well as regulation of the proper localization of *Fgf9*, *Fgf10*, and *Barx1* expression.

Bmpr1a expression in the pancreatic epithelium is dispensable for lateral growth and differentiation of the pancreas

To determine the BMP target tissue required for BMPR1A mediated lateral growth of the pancreas, I employed conditional knockout analysis on embryos in which *Bmpr1a* is specifically deleted from the pancreatic endoderm. To do this I crossed

Bmpr1a floxed mice with *FoxA3 Cre*, an endodermal specific Cre line developed by Klaus Kaestner (Lee, Sund et al. 2005). I conducted Rosa expression analysis of *FoxA3 Cre* to show that Cre expression is restricted to the endoderm throughout the liver, stomach, ventral pancreas, dorsal pancreas, duodenum, and intestine (Fig.6.10A, B). Sections reveal that FoxA3-cre is expressed throughout the dorsal pancreas in at least 90% of the cells counted (Fig.6.10C). To test the efficiency of *Bmpr1a* deletion in *Bmpr1a* $^{flox/-}$; *FoxA3 Cre* embryos, I dissected pancreatic buds from E10.5 embryos, treated buds with dispase, and separated the pancreatic endoderm from the pancreatic mesoderm. Pancreatic endoderm and mesoderm were collected from wild type and mutant embryos and the presence of *Bmpr1a* wild type allele was determined by PCR analysis. Bands appeared for *Bmpr1a* in wild type pancreatic endoderm and mesoderm; however, mutant pancreatic buds only showed a band for mesoderm. As expected, no *Bmpr1a* band appeared in the endoderm of the mutant pancreatic bud (Fig.6.11), validating strong FoxA3-Cre mediated deletion of *Bmpr1a* knockout efficiency.

Deletion of *Bmpr1a* in the pancreatic endoderm does not affect gross anatomy, size or shape of the pancreas (Fig.6.12A, B). Additionally, sections of *Bmpr1a* $^{flox/flox}$*; FoxA3 Cre* pancreata exhibit no discernable defects in cellular architecture (Fig.6.12C, D, E). Deletion of *Bmpr1a* in the endoderm did not appear to affect expression of cellular markers in the pancreas, including insulin, or glucagon (endocrine), amylase, or muc1 (exocrine) (Fig.6.13). To account for potential penetrance issues, *Bmpr1a* $^{f/-}$; *FoxA3 Cre* mutant pancreata were also analyzed. *Bmpr1a* $^{f/-}$*; FoxA3 Cre* mutant pancreata also exhibit no detectable difference in pancreatic growth, or endocrine and exocrine cellular differentiation (Fig.6.14).

Although *Bmpr1a* $^{f/f}$ *FoxA3 Cre* animals survive until adulthood, they begin to exhibit defects in limb growth (data not shown) and body weight (Fig.6.15A, B) shortly after birth. Mutant littermates averaged half the weight of their wild-type counterparts. Growth retardation in these mutant animals is likely due to decreased glucose stimulated insulin secretion (GSIS) as described by Goulley and colleagues when they studied mutant mice with deletion of *Bmpr1a* in beta cells (Goulley, Dahl et al. 2007).

Surprisingly, 15% (n=36) of Bmpr1a ^{flox} FoxA3 Cre animals exhibited ubiquitous expression of Cre as seen in Rosa reporter assays (Fig.6.16). Occurring at the same ratio of 16% (n=19), Bmpr1a ^{flox} FoxA3 Cre embryos exhibit extraendodermal defects such as facial malformaties and defects in body wall closure (Fig.6.17). Interestingly, these rare mutant embryos also exhibit situs inversus of the pancreas. Situs inversus is a condition where the normal arrangement of an organ (situ solitus) is inverted. In the case of extraendodermal expressing *FoxA3 Cre*, the pancreas was located on the right side of the stomach and displayed asplenia (Fig.6.18). Sections of mutant pancreata displaying situs inversus reveal that differentiation into insulin and glucagon producing cells is not blocked (Fig.6.19). These reveal a propensity for the FoxA3-Cre line to exhibit extraendodermal expression. This is likely due to known expression of *FoxA3* in the testes (Behr, Sackett et al. 2007). As expected, extraendodermal deletion of *Bmpr1a* resulted in defects in pancreas and spleen LR patterning.

Bmpr1a in endothelial cells is dispensable for initial differentiation lateral growth of the pancreatic anlagen; however, is required for SMP formation and vascularization of the pancreas.

To test whether BMP signaling through BMPR1A to endothelial cells flanking the pancreatic bud are required for lateral growth of the pancreas, I crossed *Bmpr1a* floxed mice with *Flk*-1 (produced by (Motoike, Markham et al. 2003)) and *Tie-2* (produced by (Kisanuki, Hammer et al. 2001)) endothelial-specific Cre lines. Rosa reporter analysis revealed that both Cre lines are expressed in the developing endothelium throughout the embryo including the aorta located near the pancreatic bud at E9.5 (Fig.6.20A, B). Both Bmpr1a ^{flox} Flk Cre (Park, Lavine et al. 2006) and Bmpr1a ^{flox} Tie2 Cre (Kaneko, Li et al. 2008) phenotypes have been previously described to have defects in vascular remodeling and maturation (Fig.6.20C). Due to early lethality due to vascular defects, I analyzed the pancreatic bud in these mutants at E9.5. Mutant pancreatic buds did not exhibit defects with respect to early pancreatic budding or differentiation (Fig.6.21). Additionally, mutants exhibited defective SMP formation, indicating that early lateral growth of the pancreas was disrupted (Fig.6.22A-D). To account for penetrance issues, Bmpr1a $^{f/-}$ Tie2 Cre and Bmpr1a $^{f/-}$ Flk1 Cre animals were also studied and did not appear to have defects in initial differentiation of the pancreas (Fig.6.22A-D). Bmpr1a $^{f/-}$ Tie2 Cre animals exhibited defective vascularization of the pancreas. Defective vascularization of the dorsal pancreas may be responsible for the block in SMP formation. These results suggest that BMP signaling to endothelial cells is not required

for early differentiation of the pancreas, but is required for SMP formation and vascularization of the pancreatic bud.

Construction of Foxf1 and Hlx Cre transgenics for expression of Cre in the pancreatic mesenchyme

For this sub aim we planned to delete various genes in the pancreatic mesenchyme; however, no mesodermal specific cre line is known, so we screened candidate genes including Nkx2.5, Foxf1, Hlx1, Osr1, and Capsulin by in situ hybridization. To determine the role of BMPR1A in the pancreatic mesenchyme, Nkx2.5 Cre animals were obtained from E. Olson and analyzed for early mesodermal expression in the pancreas. I performed in situ hybridization analysis of E9.5 and E10.5 embryos for *Nkx2*.5 and determined that *Nkx2*.5 transcript is present as early as E9.5 (Fig.6.23A, D). However, Rosa reporter analysis of Nkx2.5 Cre animals did not show any staining in the pancreas until around E12.5 (Fig.6.23E, F). These results indicate that Nkx2.5 Cre exhibits delayed mesodermal expression in the pancreas. *Rarb2* is also known to be expressed in the mesoderm and Rarb2 Cre animals were also crossed with Rosa reporter mice. This Cre line was also not expressed in the pancreatic bud between E9.5 and E10.5 (Data not shown). I concluded that a mesodermal-specific Cre line that is expressed at E9.5 in the pancreatic milieu to our knowledge does not exist.

I screened a number of candidate genes by in situ for expression in the pancreatic mesoderm at E9.5-E10.5 as potential candidates for future Cre transgenic construction. Of the genes I analyzed, (*Capsulin*, *Osr1*, *Foxf1*, *Hlx1*), two fit my criteria for a potential

mesodermal specific Cre line, *Foxf1* and *Hlx1*. The criteria that I used were the gene had to be expressed in the pancreatic mesoderm at E9.5 and E10.5 and also be absent from the pancreatic endoderm and from the endothelium. Both *Foxf1* and *Hlx* are expressed specifically in the pancreatic mesoderm and the SMP but are absent from the endoderm and the endothelium (Fig.6.24, 6.25). I chose to construct transgenic Cre animals driven by both the *Foxf1* and *Hlx1* promoters. Promoter regions of the *Foxf1* and *Hlx1* gene were amplified and placed upstream of a Cre:eGFP fusion gene construct as described (Fig.2.1, 2.2). I then purified these constructs and submitted them to the UTSW transgenic core (Bob Hammer) for injection. I have genotyped these transgenic founder mice and in preparation for crossing these mesodermal Cre lines with Rosa reporters to detail pancreatic gene expression patterns. Next, validated mesodermal specific Cre expressing transgenic founders will be crossed with *Bmpr1a* floxed mice to study the role of BMP signaling through BMPR1A in the pancreatic mesoderm. Additionally, I plan to cross these mesodermal Cre lines with *Bmp4* floxed allele to determine the role of BMP4 during pancreatic development since *Bmp4* exhibits lateral expression in the pancreas during development (Fig.6.26A-F).

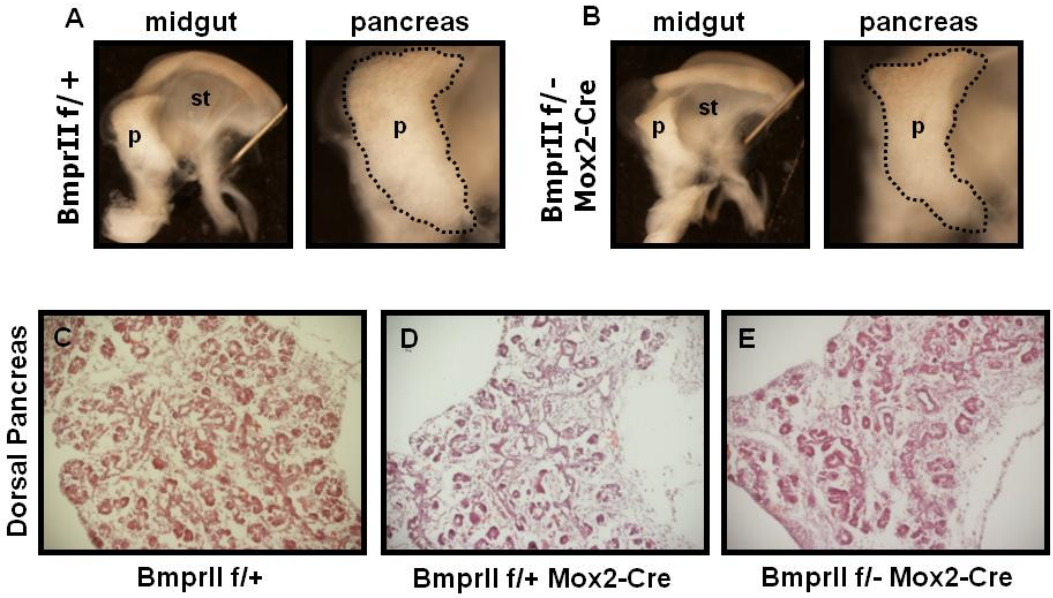

Figure 6.5. Global deletion of BmprII does not affect pancreatic growth or cellular architecture. A.) Whole midgut dissected from wild type E15.5 mouse. Pancreas is outlined with dotted lines. B.) Whole midgut dissected from BmprII f/- Mox2 Cre embryo. Pancreas is outlined with dotted lines. C.) Section through E15.5 wild type pancreas showing normal architecture. D.) Section though BmprII f/+ Mox2 Cre heterozygous mutant showing normal architecture. E.) Section through BmprII f/- Mox2 Cre pancreas showing normal architecture.

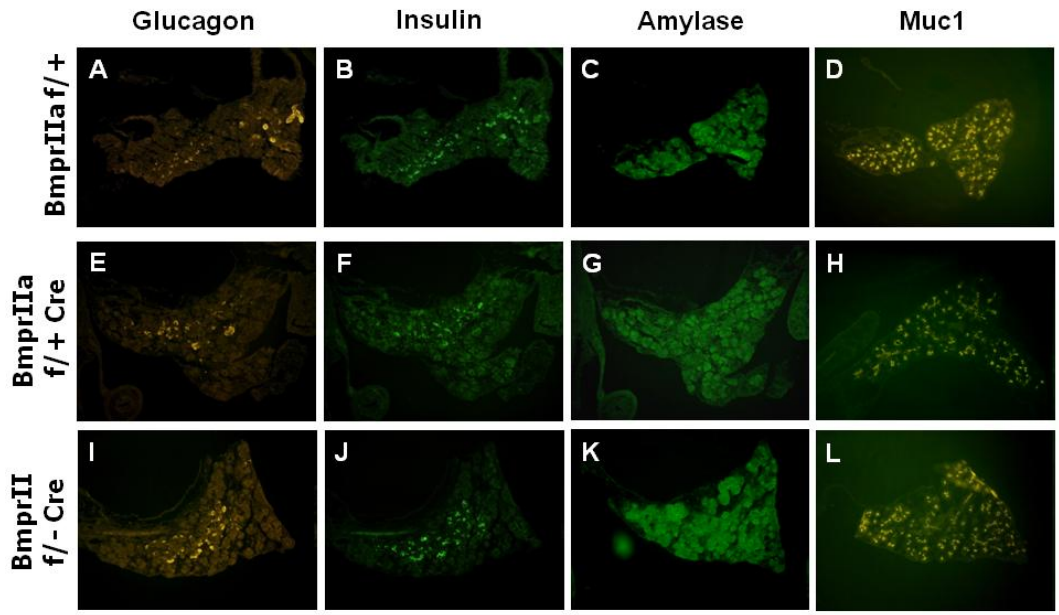

Figure 6.6. Expression of pancreatic cell markers is normal in *BmprII* mutant pancreata. Top panels: Wild type immunostaining on E15.5 pancreatic sections for A.) Glucagon; B.) Insulin; C.) Amylase; D.) Muc1. Middle panels: BmprII f/+ Mox2 Cre heterozygous immunostaining on E15.5 pancreatic sections for E) Glucagon; F.) Insulin; G.) Amylase; H.) Muc1. Bottom panels: BmprII f/- Mox2 Cre immunostaining on E15.5 pancreatic sections for I.) Glucagon; J.) Insulin; K.) Amylase; L.) Muc1.

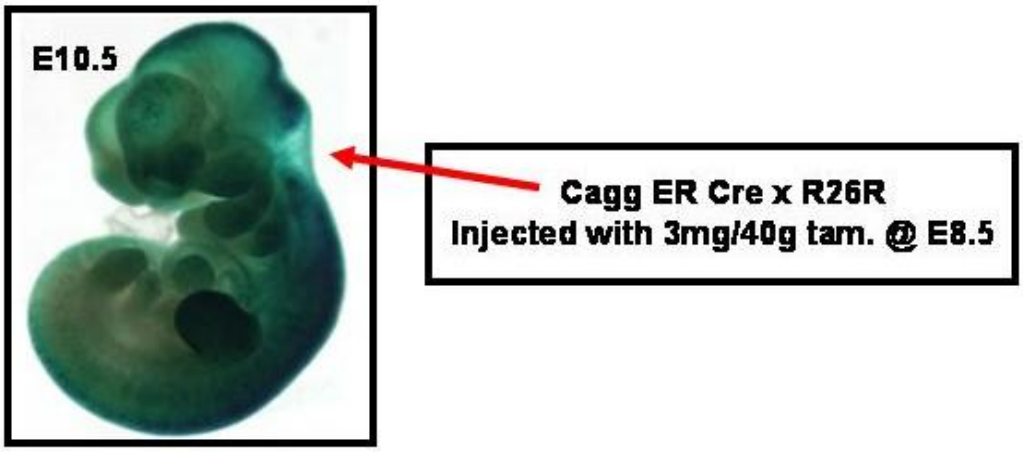

Figure 6.7. Cagg ER Cre is expressed ubiquitously in the embryo. Pregnant Rosa reporter female was plugged by Cagg ER Cre male then injected at 8.5 dpc with 3 mg/40g body weight with tamoxifen and ubiquitous embryonic LacZ expression analyzed at E10.5 as described (Hayashi and McMahon 2002).

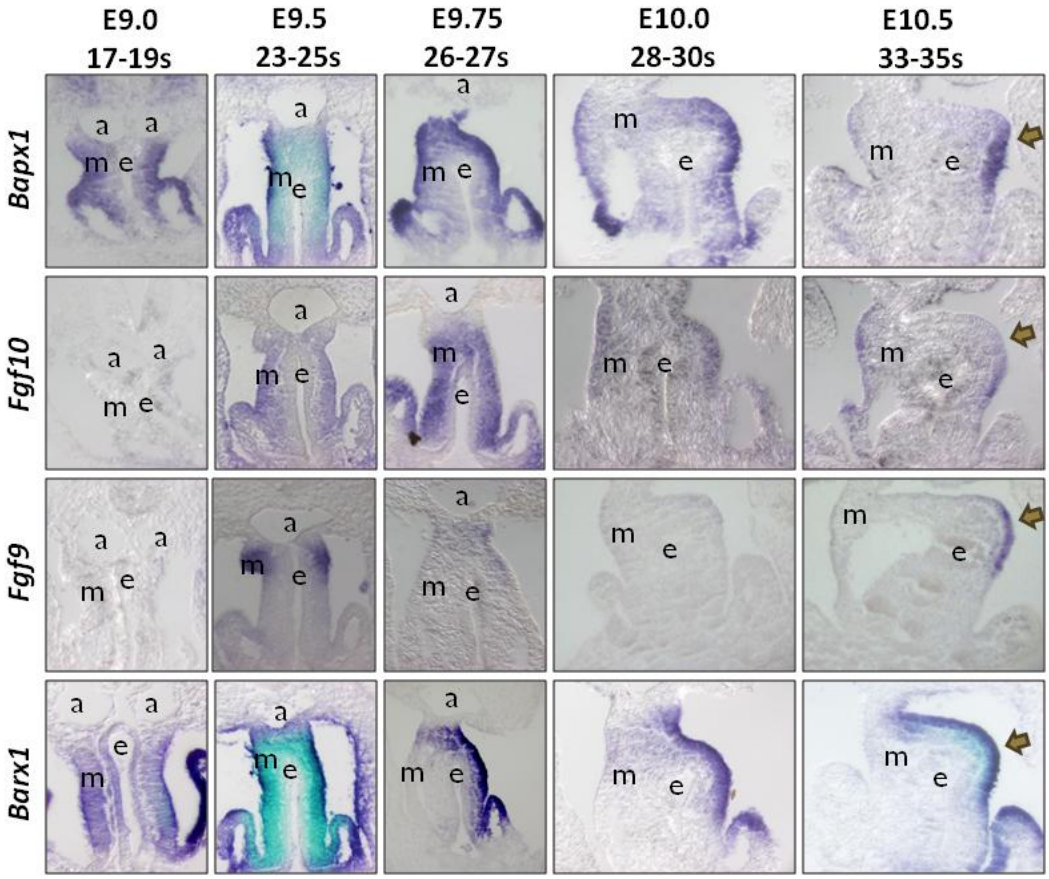

Figure 6.8. Expression of laterally expressed genes in the developing pancreas and SMP. Cross sections of whole mount in situ hybridization of pancreas region showing dorsal aorta at top, pancreatic endoderm and pancreatic mesoderm at E9.0-E10.5 (17-35 somites) for the following transcripts: *Bapx1* (A-E); *Fgf10* (F-J); *Fgf9* (K-O); and *Barx1* (P-T). Dorsal is up left is right. a, dorsal aorta; e, dorsal pancreatic endoderm; m, dorsal pancreatic mesoderm; arrowheads indicate SMP.

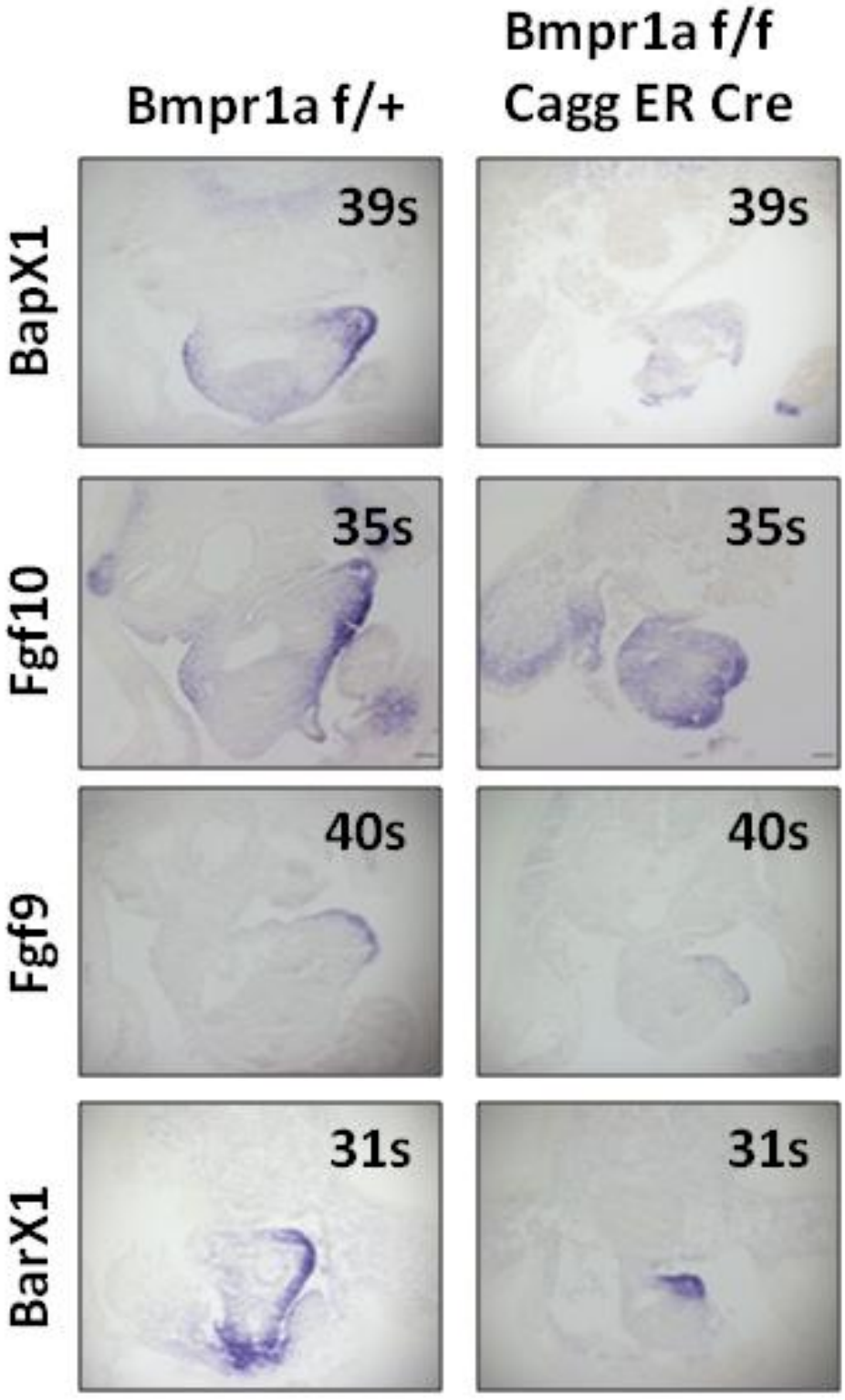
Bmpr1a f/+
Bmpr1a f/f
Cagg ER Cre
BapX1
39s
39s
Fgf10
35s
35s
Fgf9
40s
40s
BarX1
31s
31s

Figure 6.9. Lateral growth of the pancreas and expression of laterally expressed genes are disrupted in *Bmpr1a* mutant pancreata. A.) Wild type expression of *Bapx1* in the pancreatic SMP at E10.75 (38 somites). B.) *Bapx1* expression is reduced in Bmpr1a $^{flox / flox}$; Cagg ER Cre mutant pancreas. Additionally, SMP thickening and lateral growth of the pancreas is disrupted at E10.75 (38 somites) C.) Wild type expression of *Fgf10* in the pancreatic SMP at E10.5 (35 somites). D.) *Fgf10* expression in Bmpr1a flox / flox Cagg ER Cre mutant pancreas is decreased in the SMP and ectopically increased in the pancreatic mesenchyme in a similar fashion to that reported in *Bapx1* mutants (Hecksher-Sorensen, Watson et al. 2004) at E10.5 (35 somites). E.) Wild type expression of *Fgf9* in the SMP at E10.75 (40 somites). F.) *Fgf9* expression in Bmpr1a $^{flox / flox}$ Cagg-ER-Cre mutant pancreas is reduced at E10.75 (40 somites). G.) *Barx1* is expressed in both the dorsal and ventral portions of the SMP on the left side of the pancreas in wild type pancreas at E10.0 (31 somites). *Barx1* expression is restricted to the dorsal portion of the pancreas in Bmpr1a $^{flox / flox}$; Cagg-ER-Cre mutant pancreas at E10.0 (31 somites). Dorsal is up left is right.

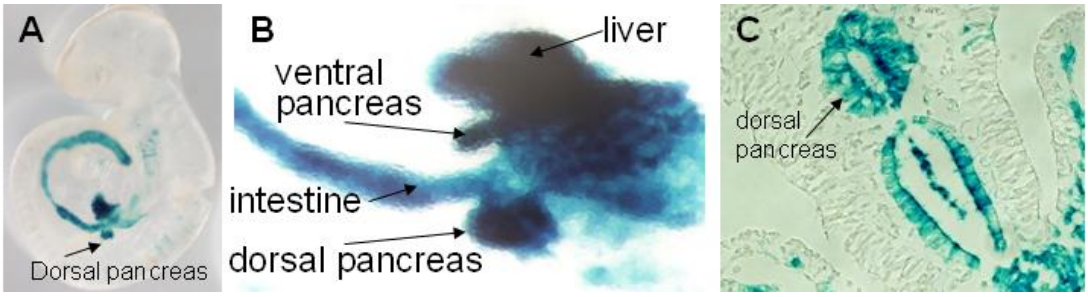

Figure 6.10. FoxA3 Cre is expressed specifically in the embryonic endoderm. A.) Endodermal specific Rosa reporter expression of FoxA3 Cre activity at E9.5. B.) Close-up of FoxA3 Cre Rosa LacZ expression at E9.5 in the liver, ventral pancreas, intestine, and dorsal pancreas. Lower and patchy expression can be seen in the anterior gut tube. C.) Cross section of E9.5 Rosa reporter for FoxA3 Cre activity showing expression in most cells in the dorsal pancreatic bud.

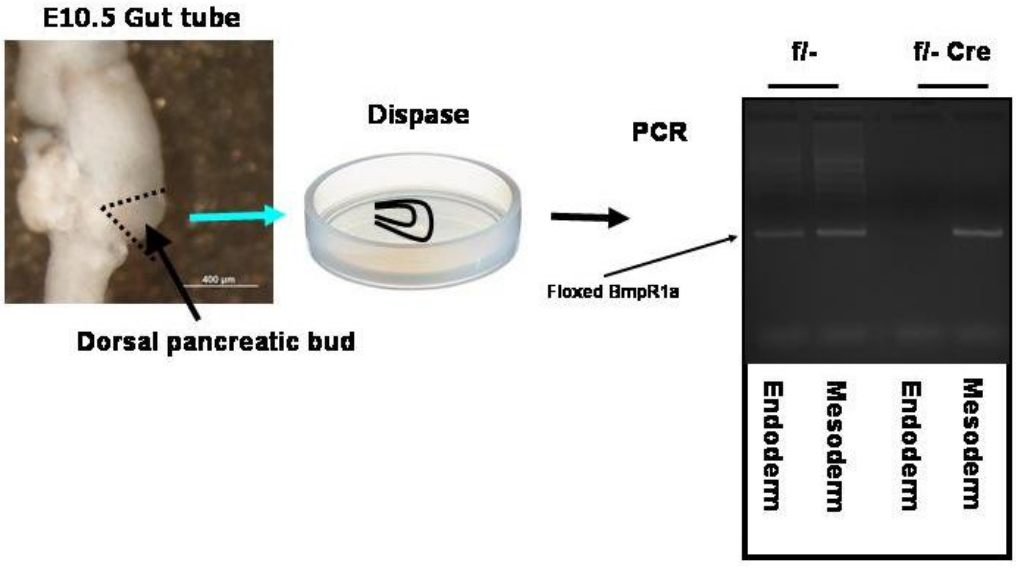

Figure 6.11. *Bmpr1a* floxed allele is efficiently deleted in the pancreatic endoderm by FoxA3 Cre. Dorsal pancreata were dissected from E10.5 gut tube and treated with dispase to separate pancreatic endoderm from mesoderm. Subsequent PCR analysis shows that *Bmpr1a* is efficiently deleted in mutant pancreatic endoderm.

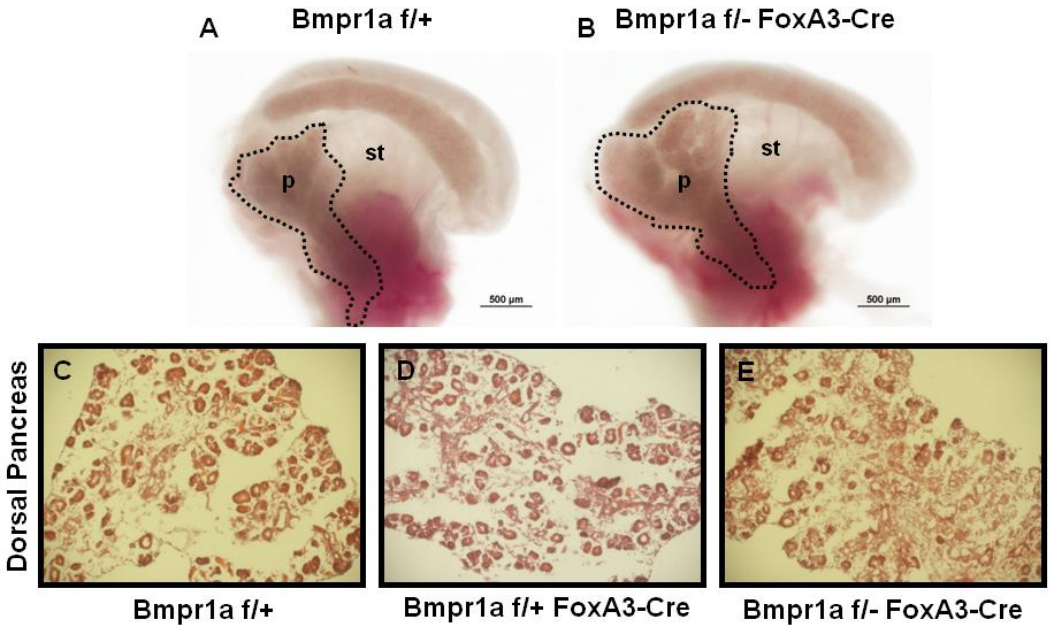

Figure 6.12. Deletion of *Bmpr1a* in the pancreatic endoderm does not affect pancreatic growth or cellular architecture. A.) Whole midgut dissected from wild type E15.5 mouse. Pancreas is outlined with dotted lines. B.) Whole midgut dissected from Bmpr1a $^{f/-}$; FoxA3 Cre embryo. Pancreas is outlined with dotted lines. C.) Section through E15.5 wild type pancreas showing normal cellular architecture. D.) Section though Bmpr1a $^{f/+}$; FoxA3 Cre heterozygous mutant showing normal cellular architecture. E.) Section through Bmpr1a $^{f/-}$; FoxA3 Cre pancreas showing normal cellular architecture.

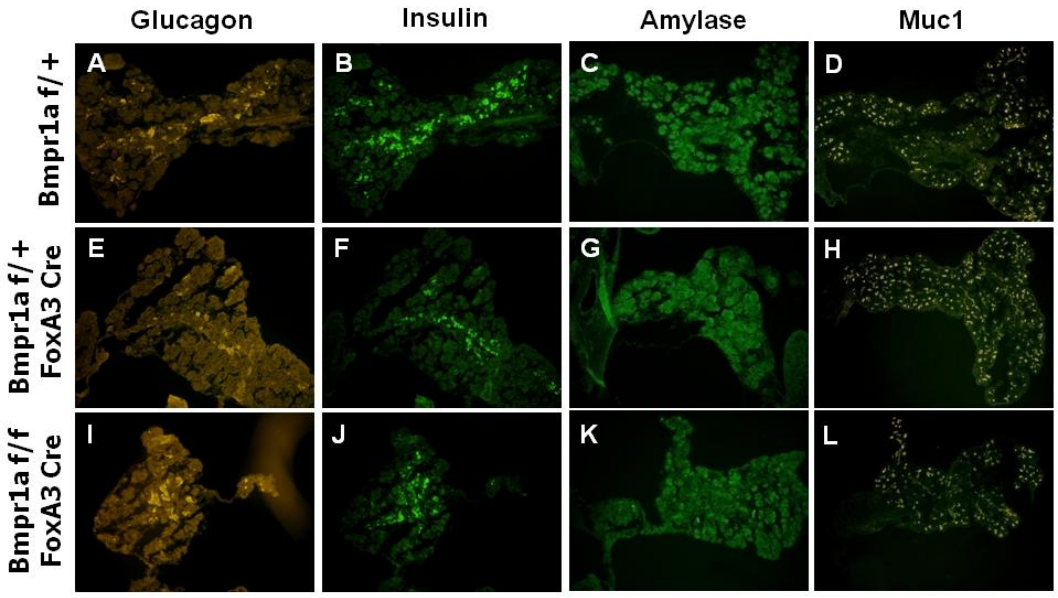

Figure 6.13. Expression of pancreatic cell markers is normal when *Bmpr1a* is deleted from the pancreatic endoderm. Top panels: Wild type immunostaining on E15.5 pancreatic sections for A.) Glucagon; B.) Insulin; C.) Amylase; D.) Muc1. Middle panels: Bmpr1a $^{f/+}$ FoxA3 Cre heterozygous immunostaining on E15.5 pancreatic sections for E) Glucagon; F.) Insulin; G.) Amylase; H.) Muc1. Bottom panels: Bmpr1a $^{f/f}$ FoxA3 Cre immunostaining on E15.5 pancreatic sections for I.) Glucagon; J.) Insulin; K.) Amylase; L.) Muc1.

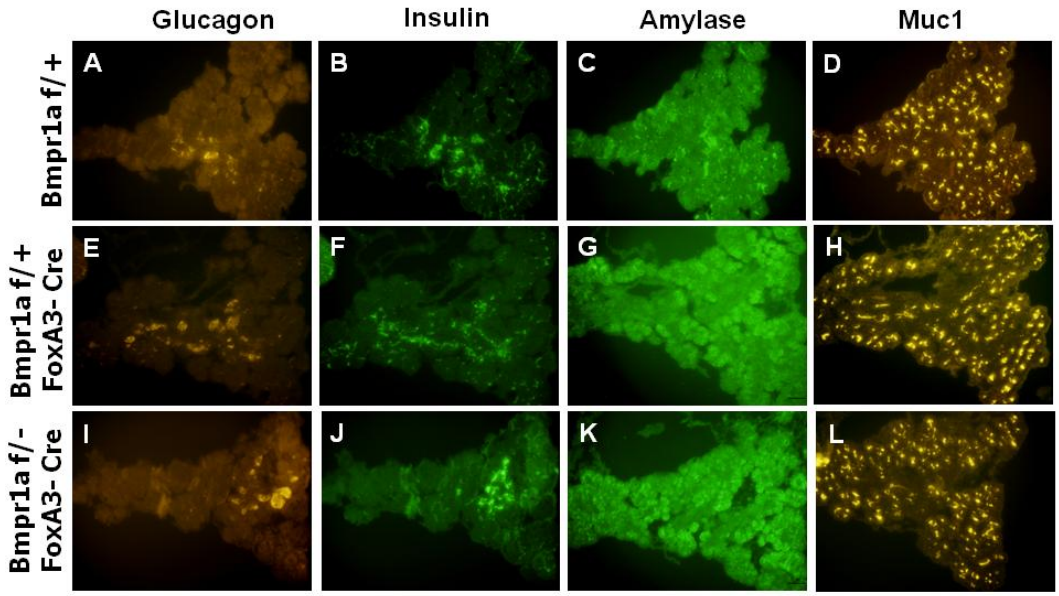

Figure 6.14. Expression of pancreatic cell markers is normal when *Bmpr1a* is deleted from the pancreatic endoderm. Top panels: Wild type immunostaining on E15.5 pancreatic sections for A.) Glucagon; B.) Insulin; C.) Amylase; D.) Muc1. Middle panels: Bmpr1a f/+ FoxA3 Cre heterozygous immunostaining on E15.5 pancreatic sections for E) Glucagon; F.) Insulin; G.) Amylase; H.) Muc1. Bottom panels: Bmpr1a f/- FoxA3 Cre immunostaining on E15.5 pancreatic sections for I.) Glucagon; J.) Insulin; K.) Amylase; L.) Muc1.

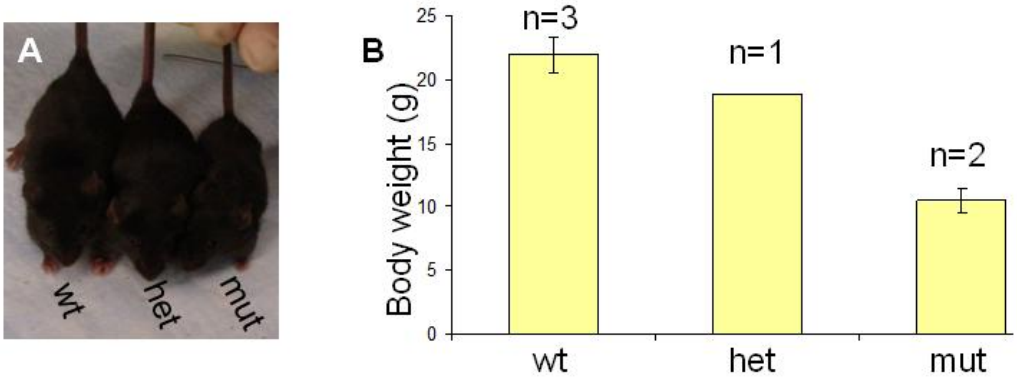

Figure 6.15. Endodermal knockout of Bmpr1a results in reduced body size and weight. A.) Picture of wild type, Bmpr1a f/+ FoxA3 Cre and Bmpr1a f/- FoxA3 Cre littermates at P24. B.) Average body weights for wild type, Bmpr1a f/+ FoxA3 Cre and Bmpr1a f/- FoxA3 Cre littermates at P24.

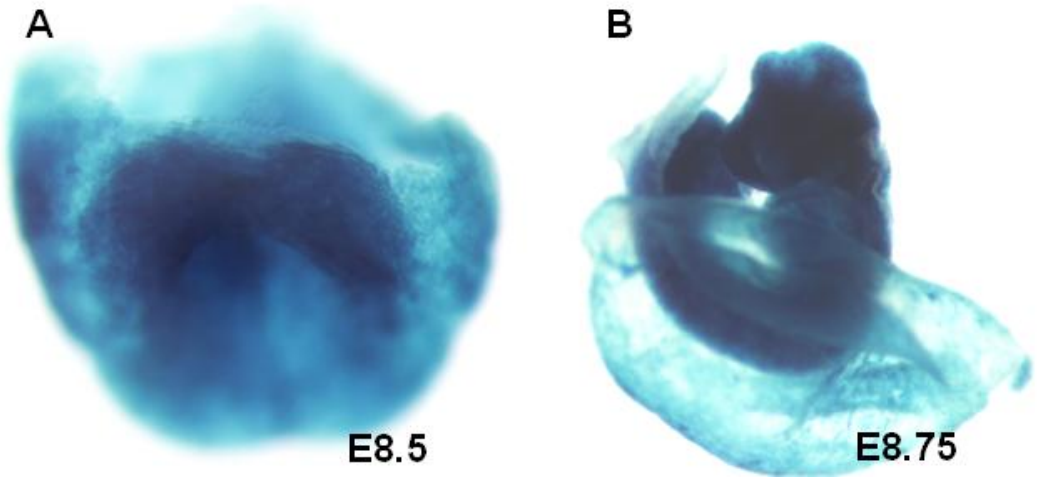

Figure 6.16. Extraendodermal expression in rare FoxA3 Cre embryos. Rosa reporter LacZ staining of FoxA3 Cre embryos observed in A.) E8.5 and B.) E8.75 embryos.

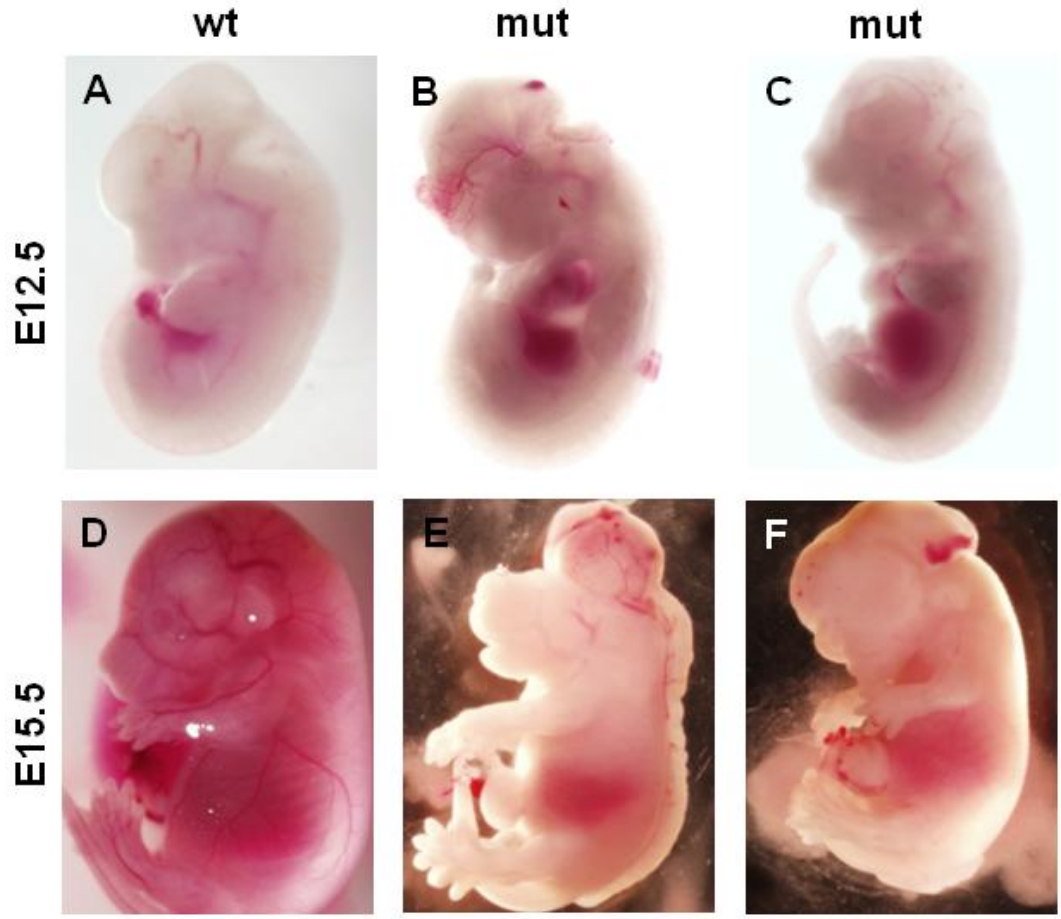

Figure 6.17. Extraendodermal deletion of *Bmpr1a* by FoxA3 Cre results in severe craniofacial malformaties. A.) wild type embryo at E12.5. B,C.) Extraendodermal deletion of *Bmpr1a* by FoxA3 Cre results in severe facial malformaties at E12.5. D.) wild type embryo at E15.5. E,F.) Extraendodermal deletion of *Bmpr1a* by FoxA3 Cre results in severe craniofacial malformaties at E15.5.

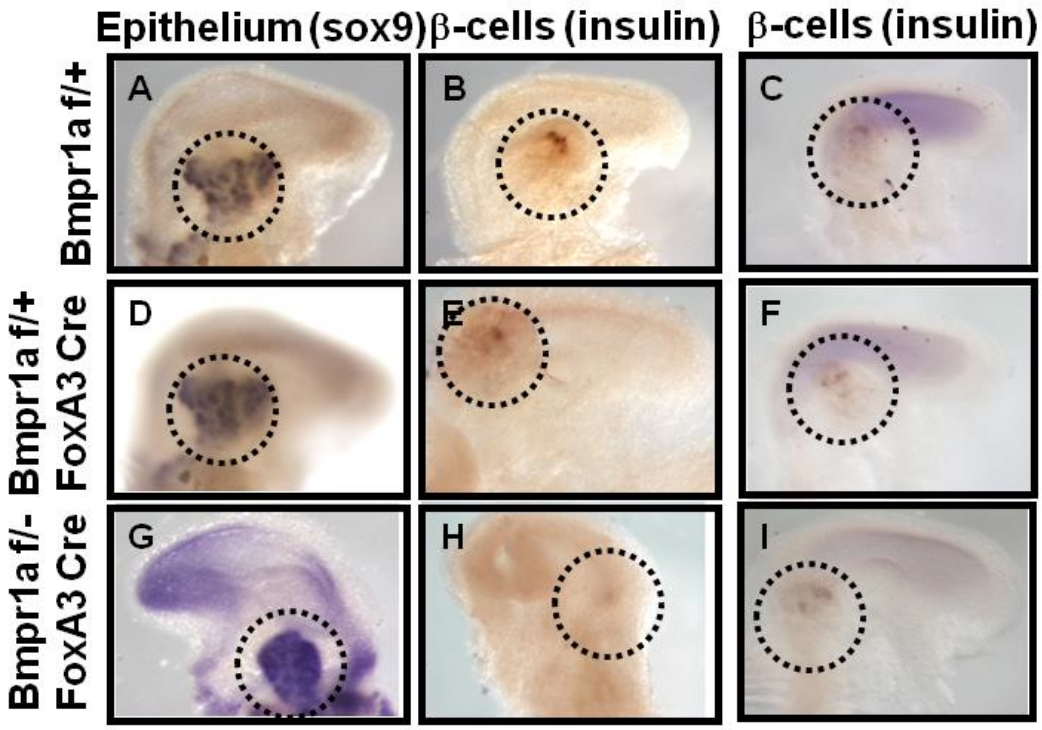

Figure 6.18. Extraendodermal expression of *Bmpr1a* results in situs inversus of the pancreas. Whole mount in situ hybridization of midgut region at E12.5. A.) Wild type expression of epithelial marker Sox9. B,C.) Wild type expression of β cell marker, insulin. D.) Bmpr1a $^{f/+}$; FoxA3 Cre heterozygous expression of epithelial marker Sox9. E,F.) Bmpr1a $^{f/+}$; FoxA3 Cre heterozygous expression of insulin. G.) Bmpr1a $^{f/-}$; FoxA3 Cre extraendodermal expression of FoxA3 Cre mutant expression of epithelial marker Sox9. H.) Bmpr1a $^{f/-}$; FoxA3 Cre extraendodermal expression of FoxA3 Cre expression of insulin. I.) Typical Bmpr1a $^{f/-}$; FoxA3 Cre expression of insulin

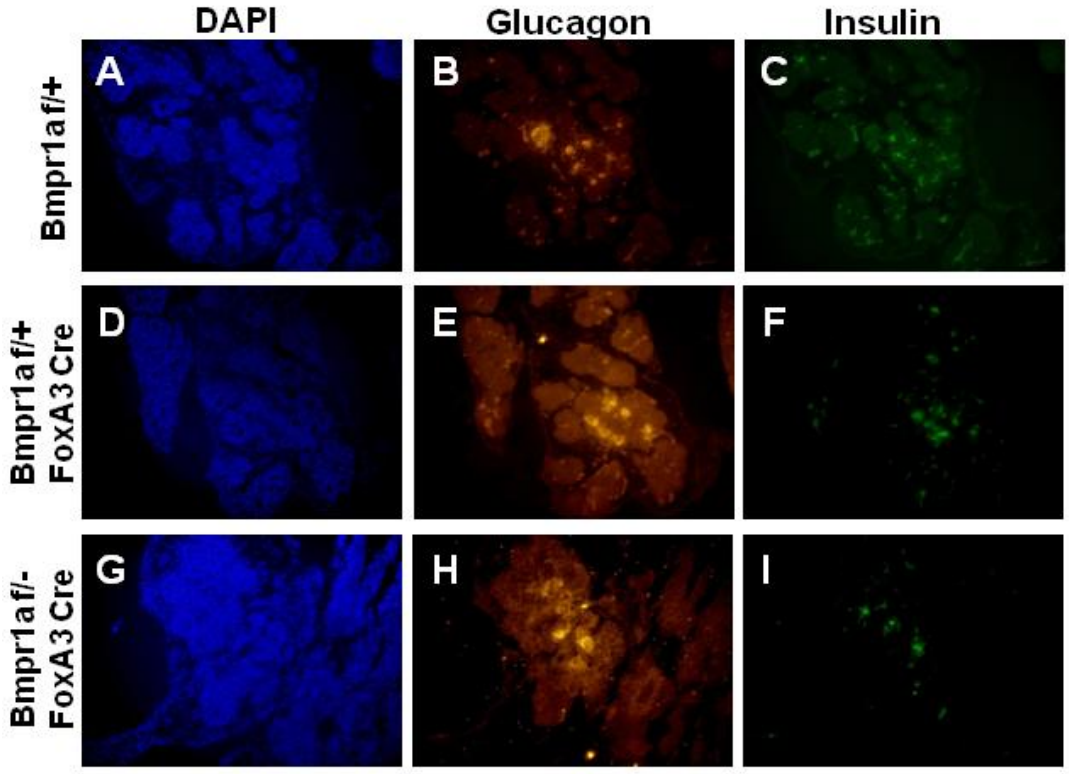

Figure 6.19. Insulin and glucagon are expressed in extraendodermal deletion of *Bmpr1a* pancreata. Top panels: Wild type immunostaining on E12.5 pancreatic sections for A.) Dapi; B.) Glucagon; C.) Insulin. Middle panels: Bmpr1a f/+ FoxA3 Cre heterozygous immunostaining on E12.5 pancreatic sections for D) Dapi; E.) Glucagon; F.) Insulin. Bottom panels: Bmpr1a f/- FoxA3 Cre immunostaining on E15.5 pancreatic sections for G.) Dapi; H.) Glucagon; I.) Insulin.

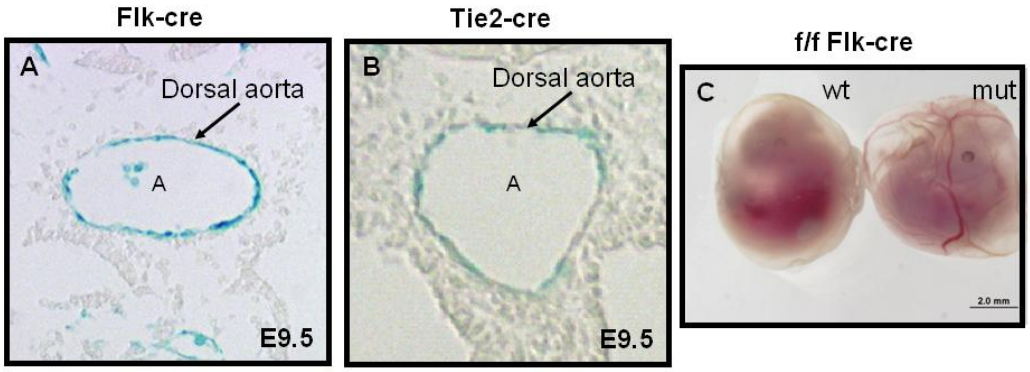

Figure 6.20. Flk1 Cre and Tie2 Cre are expressed specifically in the embryonic endothelium and *Bmpr1a* expression in endothelial cells is required for vascular remodeling and maturation. A,B.) Cross sections of E9.5 embryo showing dorsal aorta flanking the growing pancreatic bud stained for LacZ in Flk1 Cre and Tie2 Cre crossed to a Rosa reporter. Deletion of Bmpr1a with Flk1 Cre results in defects in vascular remodeling and maturation by E10.5 as previously described (Park, Lavine et al. 2006).

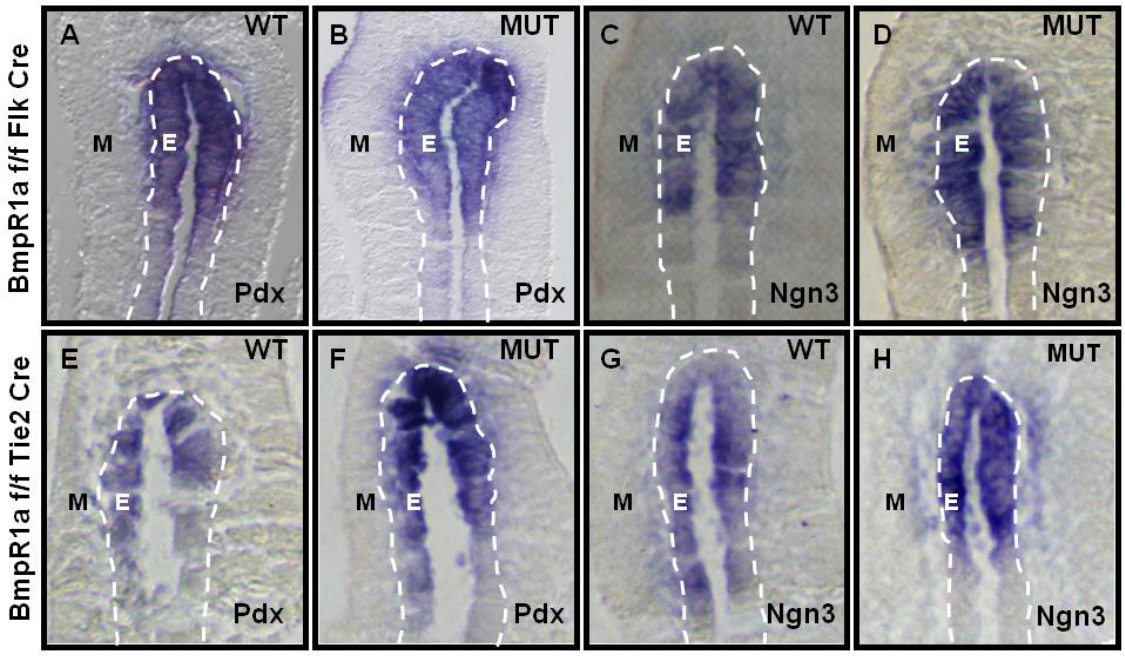

Figure 6.21. Endothelial deletion of *Bmpr1a* does not affect initial evagination or specification of the pancreatic bud. Cross sections of whole mount in situ hybridization of midgut region at E9.5. A, E) Wild-type expression of Pdx1 in the evaginating pancreatic endoderm. B.) Expression of Pdx1 and evagination of the pancreatic bud are not altered in Bmpr1a $^{f/f}$; Flk1 Cre embryos. F.) Expression of Pdx1 and evagination of the pancreatic bud are not altered in Bmpr1a $^{f/f}$; Tie2 Cre embryos. C,G) Wild-type expression of Ngn3 in the evaginating pancreatic endoderm. D.) Expression of Ngn3 and evagination of the pancreatic bud are not altered in Bmpr1a $^{f/f}$; Tie2 Cre embryos. F.) Expression of Ngn3 and evagination of the pancreatic bud are not altered in Bmpr1a $^{f/f}$; Tie2 Cre embryos.

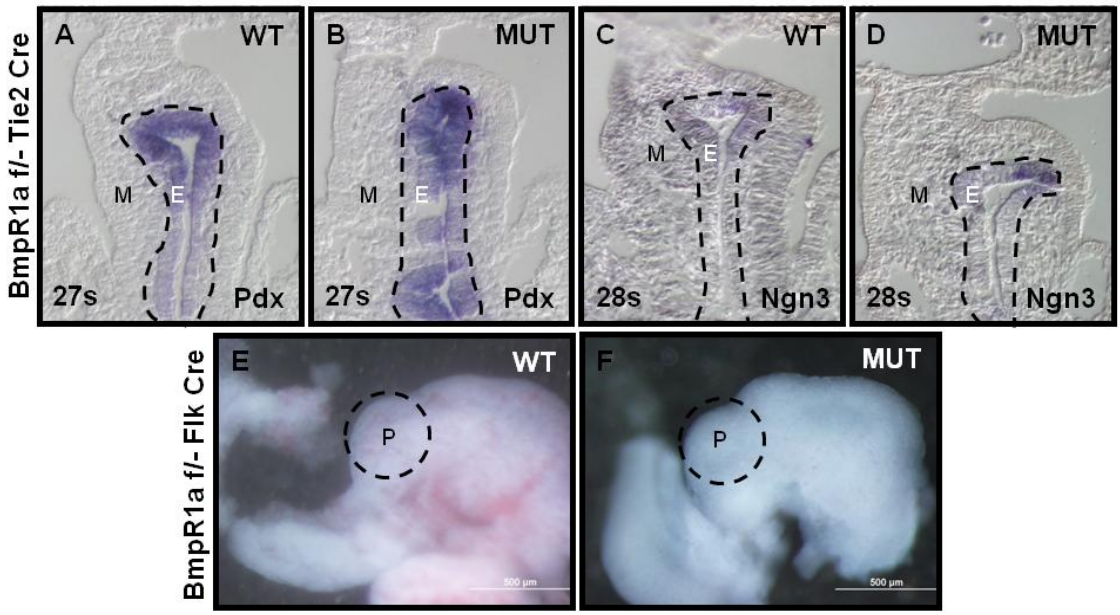

Figure 6.22. Endothelial deletion of *Bmpr1a* does not affect initial evagination or specification of the pancreatic bud. A-D.) Cross sections of whole mount in situ hybridization of midgut region at E9.5. A) Wild-type expression of Pdx1 in the evaginating pancreatic endoderm. B.) Expression of Pdx1 and evagination of the pancreatic bud are not altered in Bmpr1a $^{f/-}$; Flk1 Cre embryos. C) Wild-type expression of Ngn3 in the evaginating pancreatic endoderm. D.) Expression of Ngn3 and evagination of the pancreatic bud are not altered in Bmpr1a $^{f/-}$; Tie2 Cre embryos. E.) Dissected midgut from wild type E11.5 embryo. Pancreas is outlined. F.) Dissected midgut from Bmpr1a $^{f/-}$; Flk Cre embryo. p, pancreas.

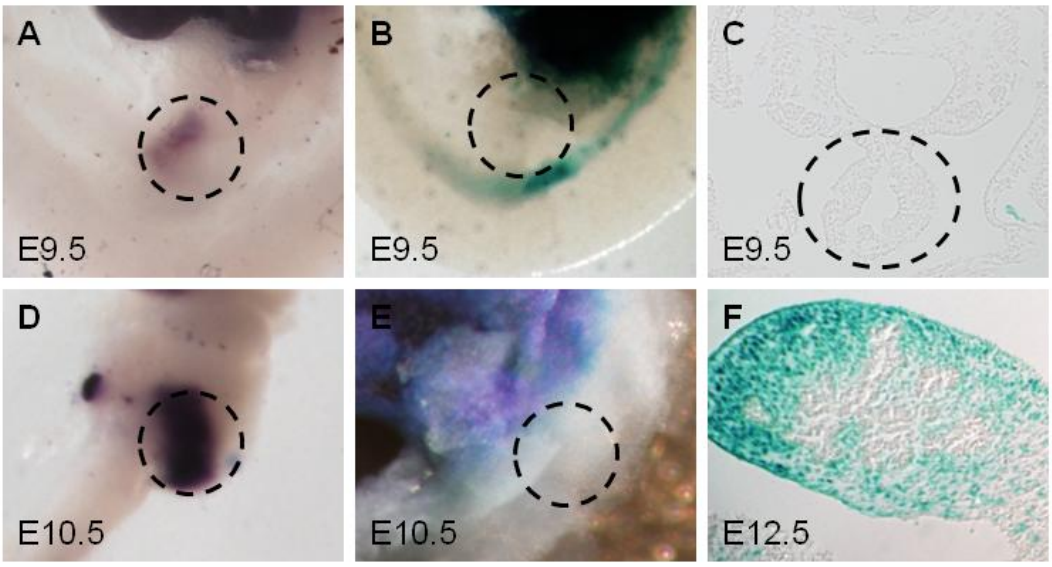

Figure 6.23. Nkx2.5 KI Cre does not show pancreatic expression until E12.5. A.) Whole mount in situ hybridization for Nkx2.5 in wild type embryo at E9.5. B.) Rosa reporter expression for *Nkx2*.5 is absent in the pancreas at E9.5. C.) Cross section of Rosa reporter expression for *Nkx2*.5 does not show expression in the pancreatic milieu at E9.5. D.) Whole mount in situ hybridization for Nkx2.5 in wild type embryo at E10.5. E.) Rosa reporter expression for *Nkx2*.5 is absent in the pancreas at E10.5. C.) Cross sections of Rosa reporter expression for *Nkx2.5* indicates expression in the pancreatic mesoderm at E12.5. Pancreas is indicated by dashed circle.

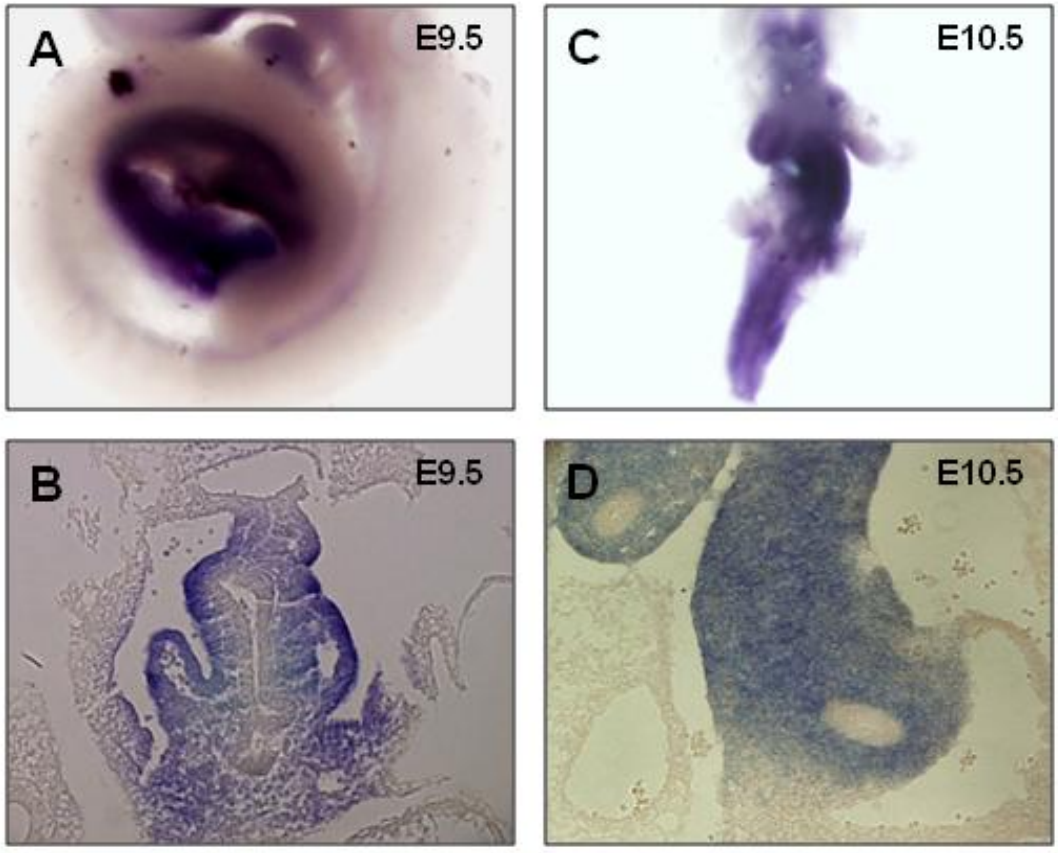

Figure 6.24. Mesoderm specific expression of *Foxf1* in gut and budding pancreas. A.) Whole mount in situ hybridization for *Foxf1* in the whole embryo at E9.5. B.) Cross section of whole mount in situ of *Foxf1* showing mesodermal specific expression in the pancreatic bud. C.) Whole mount in situ hybridization for Foxf1 on dissected gut tube from embryo at E10.5. D.) Cross section of whole mount in situ of *Foxf1* showing mesodermal specific expression in the pancreatic bud.

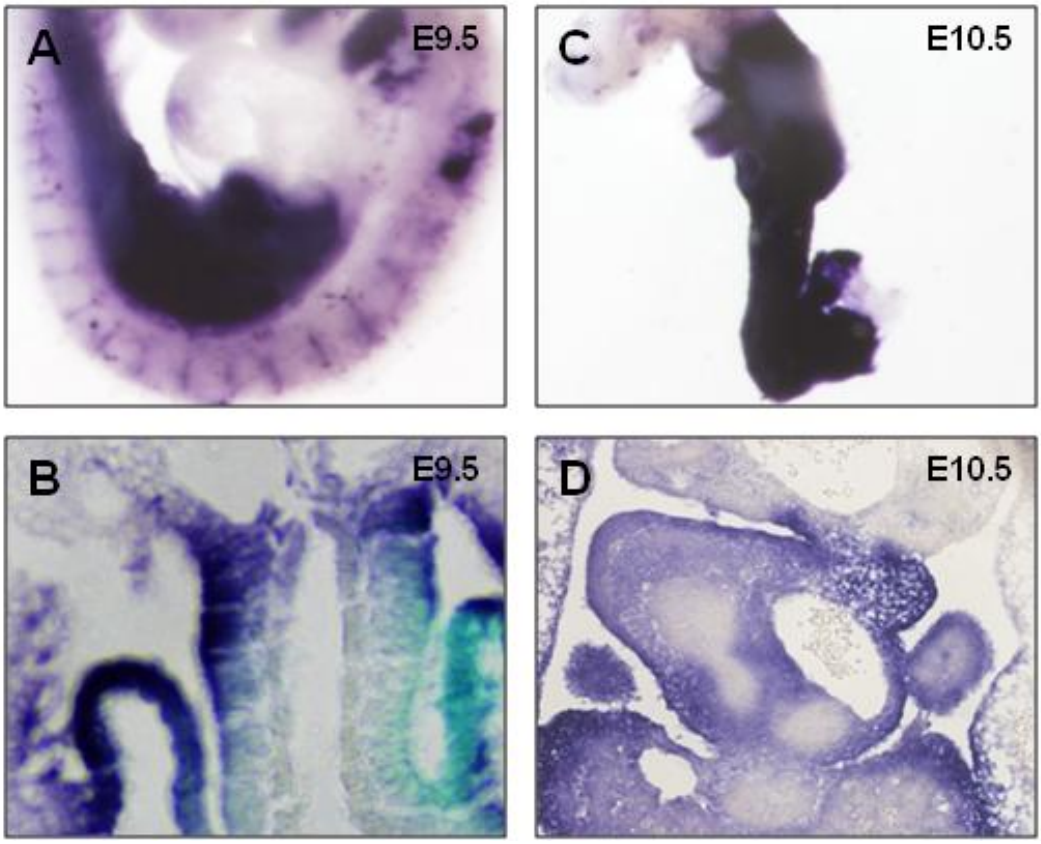

Figure 6.25. Mesoderm specific expression of *Hlx1* in gut and budding pancreas. A.) Whole mount in situ hybridization for *Hlx1* in the whole embryo at E9.5. B.) Cross section of whole mount in situ of *Hlx1* showing mesodermal specific expression in the pancreatic bud. C.) Whole mount in situ hybridization for *Hlx1* on dissected gut tube from embryo at E10.5. D.) Cross section of whole mount in situ of *Hlx1* showing mesodermal specific expression in the pancreatic bud.

Asymmetric expression and activity of BMPs in the developing midgut

Bmp4 expression and BMP signaling occur in a similar asymmetric pattern in the pancreatic milieu.

I have previously shown that *Bmp4* is asymmetrically expressed in the developing gut tube (Danesh, Villasenor et al. 2009). I followed *Bmp4* expression in the pancreatic milieu during budding and lateral growth between E9.0 to E10.5 (17-36 somites) (Fig6.26A-F). *Bmp4* is initially expressed symmetrically throughout the pancreatic lateral plate mesoderm at E9.0 (Fig6.26A). Expression then becomes restricted to the dorsal portion of this mesoderm, which becomes the mesogastrium between the aorta and the pancreatic epithelium by E9.75 (26-27 somites) (Fig.6.26C). Interestingly, *Bmp4* expression is similar to *Fgf9* expression at this point. Expression continues in the constricting mesogastrium as the pancreas begins to grow laterally to the left (E10.0-E10.5) (Fig.6.26D, E). Dorsal view of dissected gut tubes reveal *Bmp4* asymmetrically expressed on the right side of the gut tube at E10.5 (Fig.6.26F).

To determine whether BMP signaling is also occurring in an asymmetrical pattern, I utilized BMP response element (BRE) LacZ mice obtained from Leif Oxburgh (Blank, Seto et al. 2008). BRE LacZ mice can be used to determine target tissues of BMP activity during development. I analyzed BRE LacZ staining in the pancreatic milieu from E9.0-E10.5 (17-36 somites) to determine whether there is asymmetrical BMP activity in the gut tube that could complement the observed asymmetrical *Bmp4* expression. I found that BMP activity is present in the pancreatic endoderm and the dorsal aorta as the pancreas begins to bud at E9.0 (17-19 somites) (Fig.6.26G).

BMP2 is likely acting in an autocrine fashion to drive BMP activity in the aorta since it is expressed in the dorsal aorta at this stage (Fig.5.2C). *Bmp4*, which is expressed in the pancreatic mesoderm may signal to the pancreatic endoderm at this stage. However, I have also shown that *Bmp7* is expressed in the pancreatic endoderm at this stage, suggesting it may signal in autocrine fashion to drive BMP signal transduction in the pancreatic endoderm (Fig.5.2D). Endodermal expression continues in the dorsal pancreas, duodenum, and ventral pancreas as the pancreatic bud matures and begins lateral growth, though expression in the dorsal bud does recede at E10.5 (33-36 somites) (Fig.6.26K).

Interestingly, BMP signaling also occurs in blood vessels that span between the dorsal aorta and the pancreatic endoderm and the pancreatic bud begins to grow laterally away from the aorta at E10.0-10.75 (28-38 somites) (Fig.6.26J-K and Fig.6.27 arrows). BMP signaling can be seen in an asymmetrical pattern in these blood vessels along the dissected gut tube in a similar fashion to *Bmp4* expression pattern at E10.75 (Figs.6.26F and Fig.6.26L). These results show that BMPs may signal and function in an asymmetrical pattern to blood vessels around the developing gut tube on the right side of the developing pancreas. Additionally, it is unlikely that lateral growth of the pancreas requires BMP downstream signaling to the pancreatic endoderm since it has been shown in Fgf10$^{-/-}$ embryos, in which pancreatic endoderm bud fails to grow, that the SMP develops normally and the pancreas does not exhibit lateral growth defects.

. *BMP signaling is required for blood vessel recruitment to the pancreatic epithelium but not blood vessel specification*

To determine whether BMP signaling affects blood vessel recruitment to the growing pancreatic bud I employed in situ hybridization for *Flk1*, an endothelial specific marker, on dissected gut tubes and analyzed *Flk1* staining in the dorsal pancreas. Blood vessels appeared to be present in wild-type as well as Pdx-Noggin, Pdx-Bmp2 and Bmpr1a $^{flox / flox}$; Cagg ER Cre pancreata (Fig.6.28). However, *Bmpr1a* has been shown to be required for remodeling and maturation of blood vessels. And Flk staining in *Bmpr1a* mutant pancreata is indicative of blood vessel specification, not maturation. Therefore, misregulation of BMP signaling does not appear to affect blood vessel specification, but does block blood vessel remodeling and vascularization of the dorsal pancreas during lateral growth of the pancreas. Collectively, these data suggest that BMP signaling through BMPR1A is required for vascularization, expression of *Bapx1* and lateral growth of the pancreas. Additionally, this process is required for expression of *Fgf9* and regulation of *Fgf10* and *Barx1* gene expression. It is still unknown how Bapx1 functions to affect lateral growth of the pancreas; however, BMP function in blood vessels located on the right side of the embryo may play a role during lateral growth of the pancreas.

In the next chapter, I will address all of the data that I have presented in this dissertation to point out the significance of each experiment and also explain how individual finding relate to each other, as well as suggest future experiments.

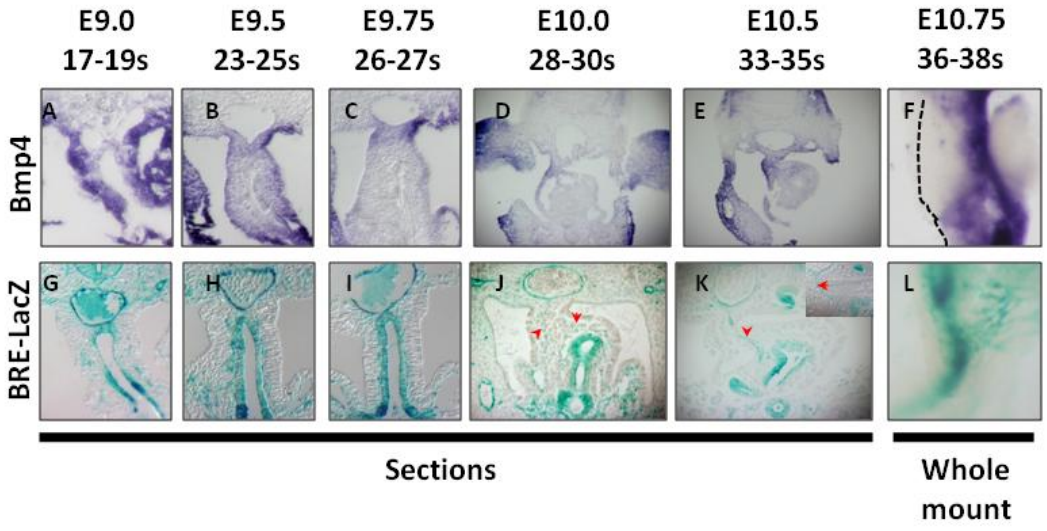

Figure 6.26. Asymmetric expression and target of BMP signaling in the developing pancreas. Top panels: Sections of whole mount in situ hybridization for *Bmp4* for the following developmental stages: A.) E9.0 (17-19 somites) B.) E9.5 (23-25 somites) C.) E9.75 (26-27 somites) D.) E10.0 (33-35 somites) E.) E10.5 (33-35 somites) F.) Whole mount in situ hybridization for Bmp4 on dissected gut tube showing lateral expression at E10.75 (36-38 somites) Dorsal view, anterior is up. Bottom panels: Sections of whole mount BRE LacZ expression for the following developmental stages: G.) E9.0 (17-19 somites) H.) E9.5 (23-25 somites) I.) E9.75 (26-27 somites) J.) E10.0 (33-35 somites) K.) E10.5 (33-35 somites) L.) Whole mount BRE LacZ staining on dissected gut tube showing lateral expression in blood vessels at E10.75 (36-38 somites) Dorsal view, anterior is up (Blank, Seto et al. 2008).

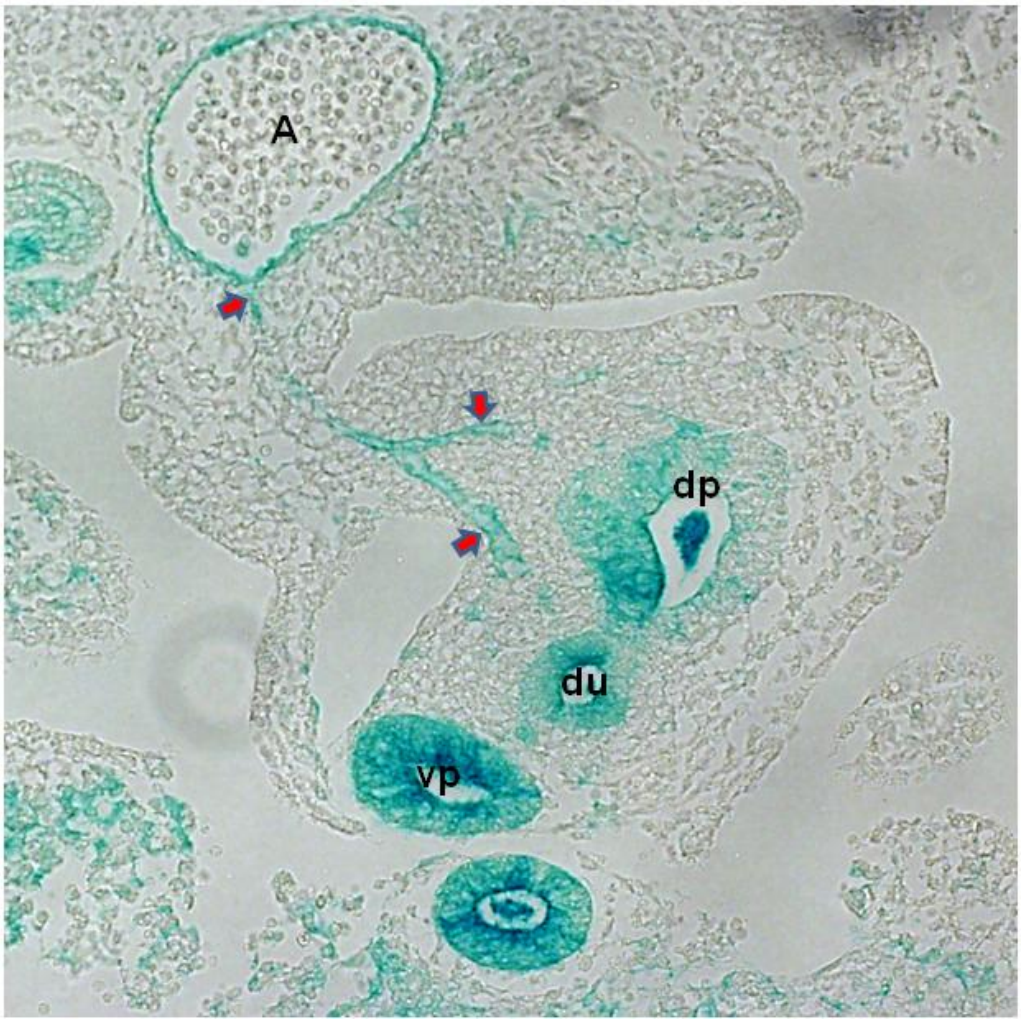

Figure 6.27. Asymmetric BMP signaling in the pancreatic milieu. Sections of whole mount BRE LacZ expression at E10.75 (36-38 somites). BRE LacZ staining is observed in the dorsal aorta, the dorsal pancreas, the ventral pancreas, the duodenum, and notably in blood vessels that sprout from the dorsal aorta and (asymmetrically on the right side) extend towards the dorsal pancreas. Arrows blood vessels that span between dorsal aorta and pancreas. Dorsal is up, left is right.

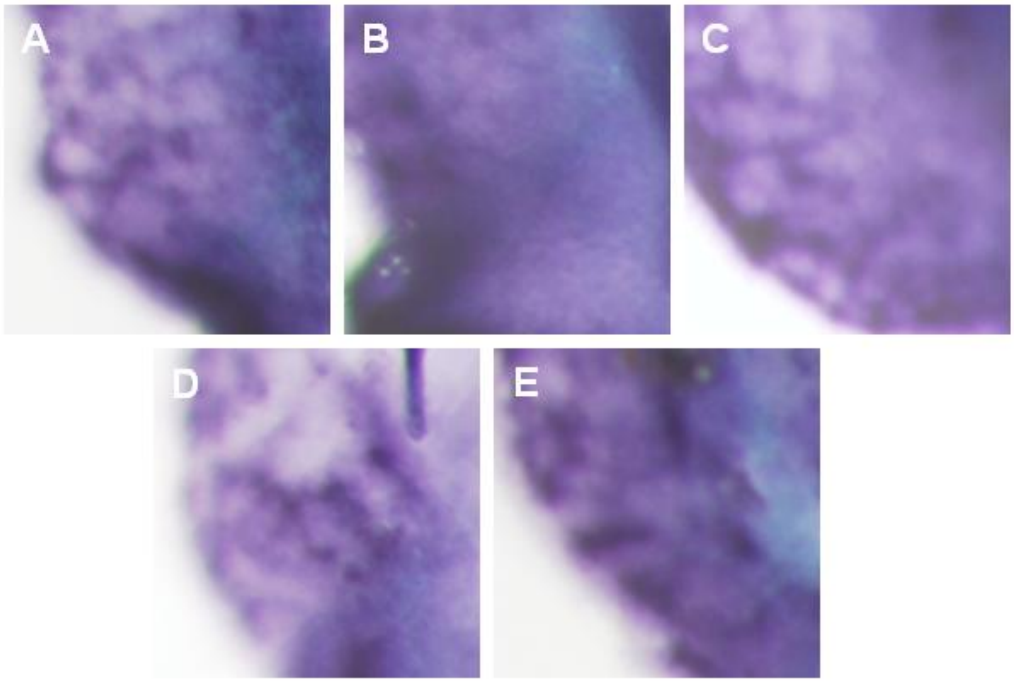

Figure 6.28. BMP signaling is not required for early blood vessel specification to the dorsal pancreatic bud. Whole mount in situ hybridization of dorsal pancreatic bud on dissected gut tubes from A.) E10.5 (35 somite) wild type. B.) E10.5 (35 somite) Pdx-Noggin-ires-eGFP. C.) E10.5 (35 somite) Pdx-Bmp2-ires-eGFP. D.) E10.75 (37 somite) wild type. E.) E10.75 (37 somite) Bmpr1a $^{flox / flox}$; Cagg ER Cre. Image shows close-up of the right side of the dorsal pancreatic bud.

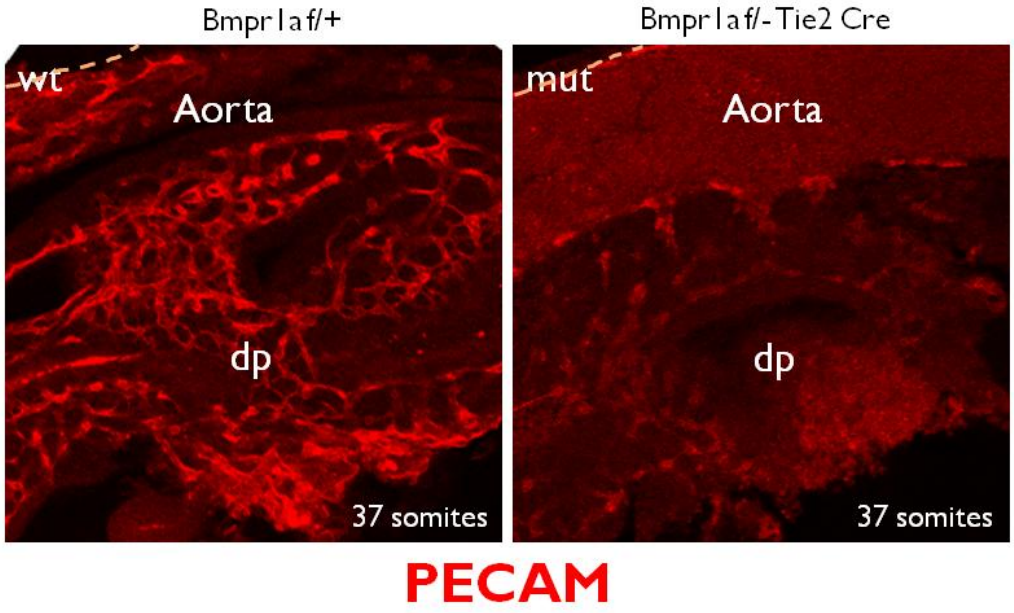

Figure 6.29. BMP-BMPR1A signaling to the pancreatic endothelium is required for vascular remodeling of the pancreatic bud. PECAM whole mount immunostaining of dorsal pancreatic bud on co-dissected dorsal aorta and gut tubes from E10.5 (37 somite) wild type and E10.5 (37 somite) Bmpr1a f/-; Tie2-Cre embryos. Dp denotes dorsal pancreas region with blood vessels spanning between the dorsal aorta and the dorsal pancreas (mesogastrium).

CHAPTER SEVEN

Conclusions and Recommendations

Conclusions

Work I have presented in this thesis includes the first comprehensive gene expression analysis for various Bmp ligands (Bmp2, 4, 7), and Bmp receptors (Bmpr1a, 1b, II) during organogenesis and murine midgut development. One of the most interesting findings resulting from my detailed Bmp expression study is asymmetric expression of *Bmp4* in the mesogastrium along the length of the developing midgut. Since very few genes are known to be asymmetrically expressed during organogenesis, this finding suggested a role for BMP signaling during LR patterning of the embryonic midgut. Accordingly, I showed that either knockdown of BMP ligands through overexpression of Noggin, or deletion of the BMP receptor 1a gene, resulted in defective lateral growth of the pancreas. Surprisingly, no other signaling molecule has been reported to be required for LR patterning of any organ during organogenesis. This makes my finding that BMP-BMPR1A signaling is required for the lateral growth of the pancreas the first of its kind during organogenesis. Additionally, I have determined that BMP-BMPR1A signaling is required for Bapx1 mediated lateral growth of the pancreas and expression of Bapx1 regulated genes (Summarized in Figure 7.1).

In this chapter, I will address each of my findings, discuss their importance, as well as suggest follow-up experiments that will add to each story.

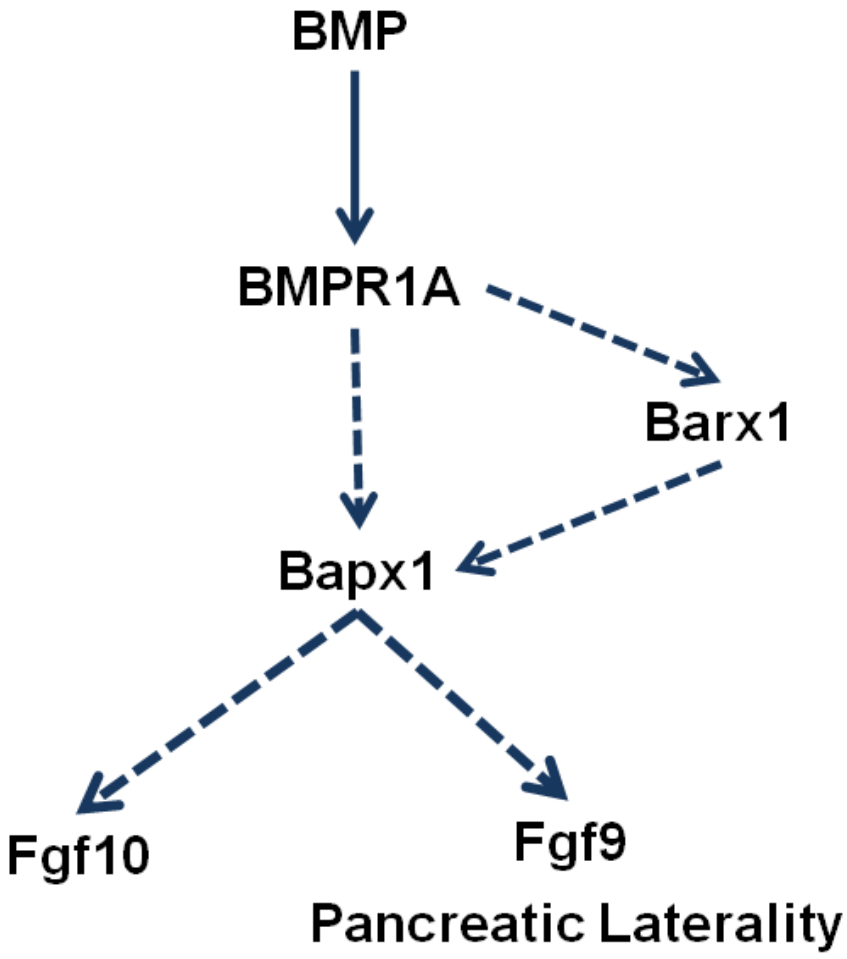

Figure 7.1. Model for role of BMP signaling through BMPR1A on *Bapx1*, *Fgf9*, *Fgf10*, *Barx1*, and pancreatic laterality (SMP formation). Solid arrow indicates direct interaction. BMP-BMPR1A signal transduction is required for regulation of *Barx1* and *Bapx1* expression in the SMP. *Barx1* is likely upstream of *Bapx1 (Kim, Miletich et al. 2007; Verzi, Stanfel et al. 2009)*, but this has yet to be established. *Bapx1* is required for lateral growth of the pancreas and regulation of *Fgf10* and *Fgf9* expression. *Fgf10* is not required for lateral growth of the pancreas (Hecksher-Sorensen, Watson et al. 2004); however, it is still undetermined whether *Fgf9* affects SMP formation and lateral growth of the pancreas.

BMP signaling is required for midgut laterality and development

Recent work in zebrafish has revealed a role for *Bmp2* during pancreatic development (Chung, Shin et al. 2008). In addition, RT-PCR analysis on murine pancreata by Dichmann and colleagues on several members of the Bmp family reveals that several different Bmps are expressed in the developing pancreas of vertebrates. The same study shows that overexpression of *Bmp6* in the developing midgut results in reduction of the pancreas and stomach, as well as asplenia. Together, these data suggest that BMP signaling may play a role during pancreatic development in the mouse (Dichmann, Miller et al. 2003). The presence of numerous BMPs in the pancreatic milieu during development and the known functional redundancy of these ligands and receptors presents a challenge for knockout studies aimed at understanding the role of BMPs during pancreatic development. As a method to bypass potential complications due to functional redundancy, I employed a BMP knockdown strategy to knock down multiple BMP ligands in the developing midgut through overexpression of a BMP antagonist, NOGGIN. A transgenic construct was created by placing *Noggin* cDNA under the control of the Pdx1 promoter and transgenic mice were produced. Broad knockdown of BMPs in the developing midgut resulted in several defects including reduced pancreas, spleen, and stomach and defects in pyloric sphincter and duodenum formation. This unequivocally demonstrates a role for BMP signaling in these tissues.

A role for BMP during pyloric sphincter development has been shown in chick; however, it will be interesting to understand how BMP signaling modulates spleen and stomach development. Nothing is known about BMP signaling in the spleen and BMPs

have not been shown to be required for spleen development. A likely explanation for the reduced spleen phenotype in Pdx-Noggin transgenics is defects in SMP formation and lateral growth of the midgut, which provides the mesodermal precursors necessary for spleen development.

Additionally, we have shown that BMP signaling is required for stomach development as Pdx-Noggin transgenics exhibit reduced stomach. Regulation of stomach development by BMPs may occur via regulation of smooth muscle formation, since we see that staining of smooth muscle actin increases significantly in the transgenic stomach (data not shown from C.Soukup). Of interest would be to determine what downstream pathways are being affected by BMPs in the developing stomach. What is the mechanism by which smooth muscle actin staining goes up in the transgenic stomach? One approach could be the employment of microarray studies on wild type vs. transgenic gastric stomachs at E15.5 to identify genes that may be regulated by BMPs.

Our studies also show that BMP signaling is required for pancreatic growth since Pdx-Noggin transgenic pancreata are reduced in size. It will be interesting to determine whether this phenomenon is due to reduced proliferation or precocious differentiation of pancreatic progenitors. BMPs may function to maintain the pluripotent state of pancreatic cells and removal of BMP ligand may result in early differentiation of pancreatic progenitors. To test this possibility, we have performed in situ staining for early markers of pancreatic progenitors in Pdx-Noggin transgenics. Levels of *Ngn3* and *Sox9* appear to be reduced or absent in the transgenic pancreatic bud (data by C. Soukup, not shown), suggesting precocious differentiation of pancreatic progenitors. However, *Nkx2*.2 remains expressed in the pancreas, suggesting that the earliest endodermal cells in the gut are not

affected by knockdown of BMPs. Conversely, BMPs may be required for proliferation of pancreatic progenitors and removal of BMP ligands in the developing pancreas may result in the observed reduction in pancreatic size. It will be necessary to test this possibility by doing Ki-67, pH3, or BrdU staining of Pdx-Noggin pancreata to check for reduced levels of proliferation. Additionally, TUNEL staining will reveal whether the phenotype is due to cell death in the pancreatic anlage.

BMP overexpression prevents differentiation of the pancreatic primordium

Overexpression of *Bmp6* in the pancreas during midgut development has been shown to result in reduced pancreas, stomach and asplenia (Dichmann, Miller et al. 2003). Consistently, we have shown that overexpression of *Bmp2* in the pancreatic midgut results in reduced pancreas, stomach and asplenia. We also showed that *Bmp2* overexpression blocks differentiation of the developing pancreas. *Bmp2* transgenic mice exhibited pancreata that were unable to differentiate into insulin, glucagon, or amylase producing cells, suggesting that BMPs may play a role in maintaining the pluripotent state of pancreatic progenitor cells, preventing pancreatic differentiation, or blocking pancreatic proliferation. Interestingly, although both knockdown of BMPs and overexpression of BMPs in the developing midgut result in reduced pancreatic development. Since overexpression of *Bmp2* appears to prevent pancreatic differentiation, it would be interesting to determine what step during pancreatic development is regulated by BMPs. One way to do this would be to stain for different cell types in Pdx-Bmp2 transgenic pancreata to determine at what stage of cellular

differentiation the pancreatic progenitors stall. The observation that *Bmp2* overexpression results in reduced pancreata suggests that perhaps the role of BMPs during pancreatic development would be the maintenance and not the proliferation of multipotent pancreatic progenitors. To test this, it will be necessary to do Ki67 and TUNEL assays on sections of Pdx-Bmp2 transgenic pancreata to determine proliferation and cellular death respectively. Together, these experiments will forward understanding to the regulatory role of BMPs during pancreatic development.

Several Bmp ligands and receptors are co-expressed in the developing embryo

Bmps have been shown to be expressed in a multitude of different tissues during pancreatic development, and it is not surprising that BMP signaling is vital for the development of the tissues in which they are expressed including the heart, lung, brain, and limbs. The inherent redundant signaling pathways used by multiple BMP ligands have made them challenging to study in some tissues. Expression analysis of BMPs for the most part can be found scattered in piecemeal reports in numerous papers. Even the most detailed of Bmp expression studies are usually limited to only a couple of Bmp ligands or receptors and almost always limited to only a few embryonic developmental stages. For this reason I analyzed the expression patterns of several common Bmp ligands that have been shown to have redundant signaling pathways, (*Bmp2,4,7*), as well as their cognate receptors (*BmpR1a, Bmpr1b, BmpRII*).

Of interest is the expression patterns of other Bmps and how they are co-expressed in the embryo. This list would consist of the remaining members of the 60A

class of BMPs of which *Bmp7* is a member; these would include *Bmp5*, *Bmp6*, *Bmp8A*, and *8B*. In addition, remaining receptors that should be addressed include type 1 activin receptors Alk1and Alk2, and type 2 activin receptors IIa, IIb (Suzuki, Thies et al. 1994; Dewulf, Verschueren et al. 1995; Kawabata, Chytil et al. 1995; Liu, Ventura et al. 1995). In situ expression analysis of the remaining Bmp ligands and receptors in this manner will definitively identify embryonic tissues where Bmps are co-expressed and help future planning of conditional knockout strategies where BMP signaling in specific tissues is addressed.

Bmp ligands and receptors are co-expressed in the developing midgut

Due to the high level of redundancy between BMP ligand and their cognate receptors, I wanted to understand what BMP ligands and receptors may play a role during midgut development by analyzing the expression profiles of several Bmp ligands and receptors. The role of BMP signaling in the midgut is largely unknown; however, I have previously shown that Bmp signaling is required for midgut development including stomach, spleen and pancreatic growth. Bmp ligands and receptors have been shown to be expressed in the developing pancreas (Dichmann, Miller et al. 2003). However, this data was obtained by RT-PCR analysis and does not address spatial expression. To address spatial-temporal expression of Bmp ligands and receptors during pancreatic development, we characterized the expression of several Bmp ligands (*Bmp2,4,7*), their receptors (*BmpR1a,1b,II*), as well as the BMP antagonist, *Noggin* during pancreatic development using whole mount in situ hybridization. Stages analyzed ranged from the

onset of murine pancreatic bud development at E9.0 to later stages where the pancreas is highly differentiated and branched (E14.5).

Several Bmps are expressed during the onset of pancreatic bud development in the pancreatic anlage as well as adjacent tissues in the pancreatic milieu. *Bmp2* is expressed in the aorta flanking the pancreatic bud, *Bmp7* is expressed in the endoderm of the developing pancreas whereas *Bmp4* is asymmetrically expressed in the pancreatic mesoderm, particularly in the pancreatic mesogastrium, which serves as the connection between the pancreas and the body wall. Asymmetric expression of *Bmp4* in the developing gut tube suggests a role for BMPs in LR patterning of the midgut. Since I have shown that BMP signaling is required for lateral growth of the pancreas, it would be interesting to assess the role of *Bmp4* on LR patterning during midgut turning. This question can be addressed by obtaining floxed *Bmp4* mice from Brigid Hogan and crossing them with gut mesodermal specific Foxf1-Cre and / or Hlx1-Cre using the transgenic mice lines I have developed. This cross will delete asymmetric *Bmp4* expression in the gut tube and address its role in LR patterning during gut turning.

Interestingly, analysis of BRE LacZ mice reveals that BMP target tissues include both the pancreatic endoderm as well as an asymmetric pattern complementary to that of *Bmp4* expression. Asymmetric BMP signal transduction is occurring on the left side of the pancreas in blood vessels that span the dorsal aorta and the dorsal pancreatic bud. I also plan to assess the role of *Fgf9* in the pancreas since it is expressed in the pancreatic mesoderm at this stage and may be responsible for proper vascularization of the pancreas.

Bmp2 expression is evident in the dorsal aorta, which lies adjacent to the pancreatic bud, transiently in contact with the pancreatic endoderm. Later in pancreatic

development as the pancreas continues to proliferate, differentiate and branch to form the mature pancreas, *Bmp7* is highly expressed in the pancreatic endoderm at the tips of the pancreatic branches located closest to the duodenum.

Bmp receptors are also coexpressed in various compartments of the pancreatic milieu during bud initiation. *BmpR1a* and *BmpRII* are ubiquitously expressed in the pancreatic endoderm, the pancreatic mesenchyme as well as the dorsal aorta. However, *BmpR1b* appears to be expressed only in the pancreatic mesenchyme, and specifically, in the mesothelial 'cap' that begins to grow to the left side during midgut turning. Later in pancreatic development, *BmpR1a* and *BmpRII* continue to be ubiquitously coexpressed in developing pancreas, whereas *BmpR1b* is notably absent. The presence of various Bmp ligands and receptors in surrounding tissues and within the pancreas during development suggests a highly complex signaling and likely redundant role for BMPs during pancreatic development and therefore predicts that phenotypes from knockout studies of single BMPs or their cognate receptors during pancreatic development may be masked by compensatory BMP pathways.

My expression analysis reveals that several Bmp ligands and receptors were also expressed in the stomach and spleen as well. This finding supports our finding that knockdown or overexpression of BMPs in the midgut milieu during development affect both stomach and spleen development. Bmp expression in the stomach and spleen has not been investigated. Future studies should include expression analysis of remaining Bmp family members and how they are co-expressed in the developing midgut. This list would consist of the remaining members of the 60A class of BMPs including *Bmp7*, *Bmp5*, *Bmp6*, *Bmp8A*, and *8B*. Additionally, the remaining receptors that should be assayed

include type 1 activin receptors *Alk1and Alk2*, and type 2 activin receptors IIa, IIb (Suzuki, Thies et al. 1994; Dewulf, Verschueren et al. 1995; Kawabata, Chytil et al. 1995; Liu, Ventura et al. 1995). Understanding expression patterns from these additional Bmp ligands and receptors will definitively identify areas where Bmps are co-expressed in the various developing midgut organs and help for future planning of conditional knockout strategies in the developing midgut for BMP signals.

BMPR1A is required for pancreatic laterality

BmpR1a function in the pancreas has been shown to be required for glucose stimulated insulin secretion (GSIS) (Goulley, Dahl et al. 2007). However, a developmental role for BMP ligands and receptors in pancreatic development has not been described in mammals. I have shown that Bmp receptors 1a and II are ubiquitously expressed in the developing pancreas and therefore could serve as transducers of BMP signals in the pancreas. To address this possibility, we employed conditional knockdown strategies to globally knock down *BmpRII* or *BmpR1a* in the embryo.

To test the role of *BmpRII* during pancreatic development, we obtained E15.5 *BmpRII* from conditional embryos in which floxed *BmpRII* was deleted with the globally expressed post gastrulation Mox2-Cre line. These embryos were generated and provided by H. Beppu. My analysis of these embryos did not reveal any pancreatic phenotype with respect to histology, lateral growth, or differentiation. This suggests that the BMP type II receptor that BMPs are signaling through in the developing pancreas may be the activin type II receptors. Consistently, activin receptor IIB and to a lesser extent activin receptor

IIA mutant embryos exhibit reduced pancreas, spleen, and stomach, as well as defects in differentiation of pancreatic cells (Kim, Hebrok et al. 2000). Therefore, BMP signaling in the pancreatic milieu during development likely occurs through Activin receptor IIB. An interesting follow-up experiment would address the contribution of *BmpRII* during pancreatic development by producing Actin receptor IIA or IIB / BmpRII double mutants.

To identify the type I BMP receptor that plays a role in BMP regulation of pancreatic development, I started with global conditional knockout of *BmpR1a*, a globally expressed pancreatic gene, by crossing floxed *BmpR1a* mice with Cagg ER Cre globally expressed tamoxifen inducible Cre line. Resultant mutant pancreata were smaller in size and exhibited defects in pancreatic development. Molecular characterization revealed that levels of *Pdx*-1 (data not shown) and *BapX1* were reduced, suggesting that pancreatic differentiation and laterality may be disrupted. However, the phenotype observed here was not as severe as the Pdx-Noggin transgenic midgut, likely due to one of two reasons. One explanation is that *BmpR1a* may not be sufficiently knocked down with our inducible Cagg-Cre TM system, and that remaining *BmpR1a* is sufficient for pancreatic development. This option has been addressed by producing BmpR1a $^{f/-}$; Cagg ER Cre embryos, however, the increased penetrance is also highly lethal to the embryo and embryos die almost immediately after injection with tamoxifen, making analysis difficult. Another explanation is that a different Bmp receptor, such as BmpR1b, which I have shown to be expressed in the pancreatic mesenchyme, may be compensating for signal transduction. A logical follow-up experiment would include analysis of double mutants of *BmpR1a* and *BmpR1b*. This strategy should address redundant roles of BMP

signal transduction that may be responsible for masking a more severe phenotype seen in Pdx-Noggin transgenics.

Potential tissue targets of BMPs during pancreatic development

Global knockdown of *Bmpr1a* resulted in failure of SMP formation and defective lateral growth of the pancreas. Since *BmpR1a* is ubiquitously expressed in all tissues in and around the pancreas during bud evagination, I wanted to determine which tissue in the pancreatic milieu is being induced to drive lateral growth of the pancreas. To do this, I analyzed BMP signal transduction in the pancreas during embryogenesis and employed several conditional knockout strategies to delete *BmpR1a* in the pancreatic endoderm and the dorsal aorta.

To analyze BMP signal transduction and identify tissues in which BMP signaling occurs in the mouse, we obtained BMP reporter element (BRE) LacZ animals that are used as indicators of BMP activity (Blank, Seto et al. 2008). Staining revealed that BMP activity occurs in the pancreatic endoderm and adjacent dorsal aorta during the onset of pancreatic budding. Interestingly, at the onset of lateral growth of the pancreas, BMP activity is maintained in the dorsal pancreatic endoderm and aorta, but now also observed in blood vessels that extend from the dorsal aorta by angiogenesis, from the right side of the growing dorsal pancreas. BMP activity in the dorsal pancreatic endoderm may play a role in growth / differentiation of the pancreas since overexpression of *Bmp2* in the pancreas blocks pancreatic growth and formation of exocrine and endocrine lineages. However, endodermal BMP activity is unlikely to have an effect on lateral growth of the

pancreas since it has been shown that the pancreatic endoderm is not required for lateral growth of the pancreas in *Fgf10*$^{-/-}$ mutants. Through deduction, it is reasonable to hypothesize that BMP activity in either the dorsal aorta or the blood vessels that span between the dorsal aorta and the right side of the pancreatic bud is secondarily required for lateral growth of the pancreas. In other words, BMP signaling is required cell autonomously in aortal endothelial cells, while as yet unknown signals from the aorta derived vessels are required non-cell autonomously in adjacent SMP. It will be very exciting to determine the effect of blocking BMP activity in these various tissues to analyze the indirect effect on pancreatic growth, differentiation, and laterality.

Knockout of *BmpR1a* in the pancreatic endoderm did not have a developmental defect; however, some animals exhibited smaller body mass and defects in glucose homeostasis, as previously reported (Fig.6.15and (Goulley, Dahl et al. 2007)). Study of pancreatic histology as well as marker analysis of differentiated pancreatic cells including endocrine and exocrine cells revealed no great differences between wild type and knockout embryos examined for insulin, glucagon, muc1 and amylase.

BmpR1a was also knocked down in the dorsal aorta to address the possibility that BMP signaling in the developing midgut may be through indirect means. The hypothesis here being that BMP ligands could be signaling through BMP receptors on the dorsal aorta which can then feed back with paracrine production of a different factor that allow the pancreas to grow and differentiate. To test this hypothesis, I conditionally knocked down *BmpR1a* from the dorsal aorta by crossing floxed *BmpR1a* with two separate Cre lines, Flk1-Cre and Tie2-Cre, both of which have been shown to be expressed in the dorsal aorta. Mutant embryos did not exhibit defects in early pancreatic bud evagination

or differentiation, suggesting that BMP signaling through the BMPR1A located on the dorsal aorta is not required for early pancreatic development. Vascular specification is not blocked in these mutants; however, vascular remodeling and SMP formation are disrupted (E10.5).

As a future study, it will be interesting to assess the role of *Bmp4* and *Fgf9* in the pancreatic mesenchyme. There are no Cre lines available today that are expressed in the pancreatic mesenchyme, so one had to be created. Several genes that have been shown to be expressed in the mesenchyme in scattered reports were analyzed by in situ hybridization including *Nkx2*.5, *Capsulin, Rarb2, Foxf1* and *Hlx1*. My goal was to find genes that were expressed in the pancreatic mesenchyme, but not in most other tissues where BMP signaling has been shown to be vital for development. In situ analysis of these gene candidates revealed two candidates, *FoxF1* and *Hlx1* for their strong expression in the pancreatic mesenchyme and absence from vasculature at E9.5, before lateral growth of the pancreas initiates. The promoters from these genes were subsequently cloned (Fig.3.1 and Fig.3.2), and transgenic animals were constructed. Subsequent studies will include crosses between floxed *BmpR1a* and these mesenchymal specific Cres. Additionally, since *BmpR1b* is also shown to be expressed in the developing pancreatic mesenchyme, it would be necessary to also produce double knockouts of *BmpR1a* and *BmpR1b* to account for functional redundancy between the Bmp receptors.

An exciting experiment would be the analysis of *Fgf9* null or conditional mesodermal knockout pancreata. *Fgf9* is first expressed in the pancreatic milieu in a similar fashion to *Bmp4* at this stage in the mesogastrium at E9.5 and then becomes

restricted to the SMP by E10.5. *Bapx1* null animals show reduced *Fgf9* expression; however, it is unknown whether *Fgf9* downregulation causes *Bapx1* mediated defective lateral growth of the pancreas (Hecksher-Sorensen, Watson et al. 2004). FGF9 signaling in the lung mesenchyme has recently been shown to be required for distal capillary development in part by its regulation of *Vegfa* expression in the lung mesenchyme (White, Lavine et al. 2007). Since *Fgf9* is down-regulated in *Bmpr1a* knockdown pancreata and exhibits overlapping expression with *Bmp4* in the mesogastrium at E9.75, it is possible that FGF9 may function during the onset of lateral growth of the pancreas to promote blood vessel growth in the dorsal pancreatic bud also possibly via VEGF. *Fgf9*-/- embryos survive until P0 (Hung, Yu et al. 2007) and could be analyzed at E10.5 to determine whether deletion of *Fgf9* affects lateral growth of the pancreas. If an effect is seen on lateral growth of the pancreas in *Fgf9* null animals, it would be exciting to determine whether mesodermal expression of *Fgf9* is required for lateral growth of the pancreas. To do this experiment, an *Fgf9* floxed animal has been developed (Lin, Liu et al. 2006) and could be obtained. *Fgf9* floxed animals can be crossed with *Foxf1* or *Hlx1* mesodermal Cre lines I have developed to assess whether mesodermal expression of *Fgf9* is required for lateral growth of the pancreas and whether these animals exhibit defects in blood vessel formation between the dorsal aorta and the dorsal pancreas.

As mentioned before, it will also be interesting to determine whether asymmetric gene expression of *Bmp4* seen in the mesogastrium of the developing gut tube is required for lateral growth of the pancreas. I show that *Bmp4* is the only laterally expressed Bmp during lateral growth of the pancreas and may play a role during SMP formation and subsequent lateral growth of the pancreas. Deletion of *Bmp4* is lethal (Winnier, Blessing

et al. 1995); however, *Bmp4* floxed animals have been constructed (Kulessa and Hogan 2002) and it would be very interesting to determine whether mesodermal deletion of *Bmp4* by Foxf1 or Hlx1 mesodermal Cre results in defective laterality of the pancreas.

The work described in this thesis demonstrates that BMP signaling through BMPR1A is required for *Bapx1* mediated SMP formation and lateral growth of the pancreas (Fig.7.1.). A number of factors are also misregulated in this pathway including *Fgf10, Fgf9* and *Barx1*. Although *Fgf10* null animals exhibit defective pancreatic endoderm formation, lateral growth of the pancreas is not disrupted (Hecksher-Sorensen, Watson et al. 2004).

Interestingly, *Barx1* was not reported to be misregulated in *Bapx1* mutant pancreata; however, I see dorsalization of *Barx1* expression in the SMP in *Bmpr1a* mutant pancreata. One explanation would be that *Barx1* is either upstream of *Bapx1*, or involved in a separate signaling pathway form *Bapx1* that is downstream from *Bmpr1a*. There is some evidence to suggest that *Barx1* is upstream of *Bapx1* since *Bapx1* expression is lost in the absence of *Barx1* in the mouse stomach (Verzi, Stanfel et al. 2009). Interestingly, the *Barx1* mutant midgut exhibits failure of dorsal and ventral pancreas to fuse, the spleen was hypoplastic and embedded within the dorsal pancreas, and the stomach failed to rotate. At E11.5, the SMP fails to form in *Barx1* mutants and the spleen and pancreas failed to grow laterally (Kim, Miletich et al. 2007).

Collectively, these observations suggest that BMP signaling is upstream of BMPR1A which is required for regulating *Barx1* mediated expression of *Bapx1* (Fig.7.1). Specifically, BMP-BMPR1A signaling to the pancreatic endothelium is required for vascularization of the pancreatic bud. Furthermore, *Bapx1* is required for *Fgf10* and *Fgf9*

expression as well as formation of the SMP and subsequent lateral growth of the pancreas. Since it is known that *Fgf10* while required for proper pancreatic branching, is not required for SMP formation or lateral growth of the pancreas, *Bapx1* mediated lateral growth of the pancreas may be through expression of *Fgf9*. Future studies should address the role of *Fgf9* in SMP formation and lateral growth of the pancreas, specifically with respect to blood vessel formation between the dorsal aorta and the dorsal pancreas since *Fgf9* has been shown to play important roles in blood *Vegfa* expression and blood vessel formation and blood vessels located on the right side of the pancreas are being induced by BMP signaling.

In conclusion, results in this dissertation have identified BMP-BMPR1A signaling as the first extrinsic signal transduction pathway required during LR patterning of any organ during organogenesis. These studies demonstrate that BMP-BMPR1A signaling the the pancreatic endothelium is required for vascularization of the pancreas as well as *Bapx1* mediated SMP formation and lateral growth of the pancreas. It is my belief that the proposed future studies presented in this section will help identify both the specific BMP ligand that is required for lateral growth of the pancreas, as well as the mechanism by which deletion of *Bapx1* blocks SMP formation.

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