Characterization of the Role of Intrinsic and Extrinsic Factors During Murine Endoderm Development

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CHARACTERIZATION OF THE ROLE OF INTRINSIC AND EXTRINSIC FACTORS DURING MURINE ENDODERM DEVELOPMENT

A Dissertation Presented

by

SIYEON RHEE

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

SEPTEMBER 2015

Animal Biotechnology and Biomedical Sciences
CHARACTERIZATION OF THE ROLE OF INTRINSIC AND EXTRINSIC FACTORS DURING MURINE ENDODERM DEVELOPMENT

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DEDICATION

To my wife Jiyeon
ACKNOWLEDGMENTS

I would first like to thank my committee chair, Dr. Tremblay for her support and mentorship over the past six years. I would also like to thank my committee members Dr. Mager and Dr. Schneyer and all of the members of Drs. Tremblay and Mager lab for support and discussion.
ABSTRACT

CHARACTERIZATION OF THE ROLE OF INTRINSIC AND EXTRINSIC FACTORS DURING MURINE ENDODERM DEVELOPMENT

SEPTEMBER 2015

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Yin Yang1 (YY1) is a ubiquitously expressed factor that plays multiple roles in early mouse development. We have found that an essential role of YY1 in the visceral endoderm (VE) of the yolk sac is the maintenance of VEGF. Furthermore, we have demonstrated that paracrine signals downstream of VEGF support the VE, assessed by maintenance of HNF4α. Because the VE is essential for yolk sac development, and thus for embryonic survival, we used an inducible-knockout strategy to demonstrate that YY1 is essential in the definitive endoderm (DE) for invasion of hepatoblasts into the surrounding mesenchyme. By E14.5 YY1-/- livers are severely hypoplastic, and although YY1-deficient DE is capable of liver bud specification, the hepatoblasts fail to initiate invasion, upregulate VEGF or maintain HNF4α. The addition of exogenous VEGF to YY1-deficient liver buds rescues HNF4α expression, while addition of a VEGFR inhibitor to wild-type embryos mimics the specific temporal and spatial phenotypes of the YY1-/- hepatoblasts. Taken together, these studies demonstrate that a conserved role of YY1 in both the VE and
liver bud is the production of VEGF. Careful analysis of early normal liver bud development reveals two temporally and spatially discreet waves of liver bud invasion. The first, initiated at E9.25, involves migration of the posterior liver bud while the second, initiated by E10.0, involves the anterior liver bud. Lineage analysis demonstrates that the anterior and posterior regions of the liver bud contribute to the caudal and rostral lobes, respectively, of the E10.25 liver. We have also previously observed differential effects upon inhibition of FGF signaling. When we impaired FGF in cultured embryos, we saw that the anterior portion of the liver bud was hypoplastic, lost molecular specification and underwent apoptosis while the posterior appeared normal. These studies, through examination of FGF and VEGF and the requirement for YY1, are the first to document that there are two spatially and temporally discreet waves of liver bud invasion that each contribute to distinct lobes.
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CHAPTER 1

INTRODUCTION

1.1 Liver development

1.1.1 Introduction

The mammalian liver is the largest internal organ; it participates in multiple functions including essential metabolic, exocrine and endocrine functions. These cover regulation of glucose level through glycogen storage, production of bile for fat emulsification and secretion of plasma proteins including Albumin and Apolipoproteins. Because the liver plays critical roles in normal physiological processes, liver dysfunction and disease results in high rates of morbidity and mortality (Si-Tayeb et al.).

Hepatocytes, the principal cell type in the liver and biliary epithelial cells, the other major cell type that contributes intrahepatic bile ducts, are derived from the embryonic endoderm. Information about the mechanisms that regulate liver development has been essential in creating efficient culture protocols for generating hepatocytes from stem cells and developing possible cell transplantation therapies. Multiple approaches over two decades have revealed important genes and signaling pathways during liver development and found the importance of a series of reciprocal interactions between the endoderm and adjacent mesoderm which enabled researchers to generate three dimensional vascularized liver from human induced pluripotent stem cells (iPSCs) (Takebe et al. 2013). These culture successes, however, do not fully recapitulate the functional adult liver. As the mechanisms
during hepatogenesis are still unclear, understanding the origins of liver cell types, proper tissue morphogenesis, growth and how liver cells communicate with other cells types will be of great importance to possible medical treatments.

1.1.2 Hepatic specification

1.1.2.1 Fate mapping of liver precursors

The liver is formed from the definitive endoderm that is one of the germ layers which gives rise to the entire gut and associated organs including thyroid, liver, lung and pancreas. Fate mapping studies in the early mouse embryo has indicated that there are two distinct populations within the epithelial sheet from embryonic day 8.25 (E8.25) (Tremblay & Zaret 2005a). One of these populations is referred as VMEL (ventral endoderm lip) as name implies, it is located in the ventral midline of the endoderm lip. The other population, the lateral liver precursors (LP) are placed in paired lateral endoderm areas of the closing ventral foregut. The two precursor populations located in the left and right lateral liver areas converge toward the ventral midline precursor population from E8.0~8.5 to form the liver bud at E9.0. Further experiments showed that the ventral midline population also contributes the other ventral foregut organs, the thyroid and ventral pancreas bud, in a temporally restricted manner (Angelo et al. 2011). Experiments with tissue explant and genetically altered animal models have shown a number of important mechanisms of liver specification including signaling from adjacent mesenchyme (Wells & Melton 2000a; Zorn & Wells 2009; Arterbery & Bogue 2013).
1.1.2.2 Inductive signals

Interactions between the endoderm and mesoderm were first shown to be essential for liver specification through the use of tissue explant experiments in the chick and mouse (Wells & Melton 2000a; Zorn & Wells 2009; Arterbery & Bogue 2013). Explant experiments show that isolated ventral foregut endoderm is specified into liver precursors only when the endoderm is in contact with both the cardiac mesoderm and STM (Fukuda-Taira 1981; Gualdi et al. 1996b). Fibroblast growth factor (FGF) signaling was the first pathway found to direct liver specification. Blocking FGF signaling in cardiac mesoderm and foregut endoderm co-culture experiments showed failure of liver induction (Jung et al. 1999a). In addition, the level of FGF is known to be important for liver induction. Higher doses induce lung instead of liver in explant experiments (Serls et al. 2005). Bone morphogenetic proteins (BMP), secreted from STM, has been shown to act coordinately as inductive signals mediating hepatic and ventral pancreatic (Chung et al. 2008). Wnt signals need to be repressed for BMP and FGF to pattern the foregut into liver (McLin et al. 2007). The difference in early patterning signals, FGF in cardiac mesoderm and BMP in STM, may imply the possibility of presence of effector for two different liver precursors.

1.1.2.3 Intrinsic factors

Even though signaling pathways affect the transcriptional regulators that induce liver specific gene expression, it is suggested that endoderm tissues must be in a competent state to respond to the inductive signals. Prior to specification,
endoderm cells convey epigenetic changes to receive further inductive signals such as FGF (Wandzioch & Zaret 2009). For example, FoxA1 and GATA4 transcription factors have the ability to bind to the enhancer region of the ALB gene in endoderm cells (Gualdi et al. 1996b). Further study shows that the role of FoxA1 is redundant with other Fox factors, FoxA1, FoxA2 and FoxA3. Loss of FoxA1 or FoxA3 does not cause an embryonic phenotype while loss of FoxA2 causes loss of foregut endoderm before liver induction stages (Ang & Rossant 1994) (Behr et al. 2007); (Kaestner et al. 1998)).

Besides FOXA and GATA, vHNF1 is also necessary for ventral foregut endoderm competence to become liver. Hepatic specification does not take place in vHNF1 null ventral endoderm, however, the mesenchymal portion of the liver forms relatively normally. Moreover, explanted vHNF1 endoderm cultured in the presence of FGF failed to express albumin suggesting an important role of vHNF1 in controlling hepatic specification (Lokmane et al. 2008a).

**1.1.3 Liver bud morphogenesis and growth**

**1.1.3.1 Liver bud emergence and invasion**

Liver bud formation begins with endodermal patterning and thickening at the 8~9 somite stage(s) while the gut is closing (Jung et al. 1999a). Liver specific genes such as alpha-fetoprotein (AFP) and Hepatocyte nuclear factor 4-α (HNF4α) are expressed in the ventral foregut endoderm at 13~15s and are the first molecular evidence of hepatic differentiation. After liver specification in the endoderm with liver bud thickening, this tissue becomes columnar in shape and
invaginates into the surrounding mesenchyme to form the liver diverticulum from E8.5–9.0 (Wells & Melton 2000a). Hepatoblasts change their shape to a pseudostratified morphology by repositioning during cell division carried out by interkinetic nuclear migration (INM) (Bort et al. 2006). The hepatoblasts then leave the endoderm epithelium, migrate through the basement membrane, and invade the STM (Tremblay 2011). Once the hepatoblasts have invaded the STM, they continue to proliferate and grow quickly. This growth is regulated by paracrine signals between hepatoblasts and adjacent mesenchyme cells as well as by genes in hepatoblasts intrinsically (Si-Tayeb et al. 2010).

1.1.3.2 Inductive signals

Induction of the liver bud appears to be completed by many factors and signaling pathways coming from the surrounding mesenchymes. The STM is a source of BMP2 and BMP4, positioning BMP signaling directly around the liver bud (Rossi et al. 2001). Hepatocyte Growth Factor (HGF) signaling, through its tyrosine kinase receptor c-Met, is necessary for hepatoblast proliferation and migration into surrounding mesenchyme in part by activating the small GTPase Arf6 (Suzuki et al. 2006). Liver bud growth requires retinoic acid signaling (Wang et al. 2006), which is controlled in part by Wnt expressed in the STM (Ijpenberg et al. 2007) suggesting that paracrine signaling from the mesenchyme regulates hepatoblast growth. A vast number of genetic studies have resulted in liver phenotypes at the inductive or slightly later stages.
1.1.3.3 Intrinsic factors

After the initial steps of induction from naive endoderm to liver diverticulum, where FOXAs, Gata4 and vHNF1 have essential roles, other factors take over the roles for further hepatoblasts’ differentiation process.

Hhex is initially expressed throughout the ventral foregut endoderm and is enriched in the lateral and midline liver progenitor regions by E8.5 and continues to be expressed in both hepatocyte and cholangiocyte lineages during development (Hunter et al. 2007). Hex mutant mice shows severe liver and gall bladder phenotypes suggesting its multiple important roles during development. It is important for the formation of pseudostratified epithelium during bud formation. Hhex-null hepatoblasts are arrested in a simple columnar state, resulting in failure to form a liver bud and invade the STM (Bort et al. 2006).

Transcription factors Prox1 and Tbx3 control migration of hepatoblasts into the STM, an essential step in hepatic development. Loss of the Prox1 gene prevents hepatoblasts from invading the STM and Tbx3 null embryos have a similar phenotype (Sosa-Pineda et al. 2000; Lüdtke et al. 2009b). These above genes represent, at least part of, a transcriptional network that coordinates the process of hepatoblast delamination and invasion. A number of genes are reported to be required to regulate proliferation and cell survival of hepatoblasts that includes c-jun (1997, 2000), Foxm1b (2004), K-ras (1997) and Seck1 (1998, 2002).
1.1.3.4 Role of Endothelial cells in liver bud development

Interaction between hepatoblasts and endothelial cells is critical for liver growth throughout budding stages. Emergence of the liver bud requires inductive signals from endothelial cells (Matsumoto et al. 2001b). Prior to hepatic blood vessel formation and liver bud expansion, endothelial cells already line the sinusoids in the STM and surround hepatoblasts beginning to interact with them physically at E9.0~9.5. In Flk1 (VEGFR2) null mice, endothelial cells do not form and hepatoblasts are induced and a liver bud is formed but fails to proliferate or invade the STM. During invasion into the STM, hepatocytes migrate along endothelial “cords” which appear to guide hepatocytes into the surrounding mesenchyme. However, the molecular mechanisms underlying these endothelial-hepatoblast interactions remain to be defined since endothelial cells are necessary for whole embryo development and a molecular marker specific to liver associated endothelial cells has not been found (Si-Tayeb et al.; Matsumoto et al. 2001b).

1.2 Yolk sac development

1.2.1 The role of visceral endoderm in yolk sac development

The yolk sac is composed of two layers of tissue; visceral endoderm derived from the primitive endoderm is placed in the outer layer and the extraembryonic mesoderm from the primitive streak during gastrulation is the inner layer. The yolk sac vasculature is composed of EC’s and smooth muscle cells that are derived from the extraembryonic mesoderm. The visceral endoderm overlaying the extraembryonic mesoderm influences the differentiation and development of
extraembryonic vasculature which includes two important developmental events: 
vasculogenesis, the de novo formation of blood vessels from endothelial progenitor 
and angiogenesis, the formation of new vessels by the sprouting of endothelial cells 
of pre-existing vessels.

The importance of the visceral endoderm in nutrient delivery has been 
demonstrated. Disruption of yolk sac endoderm function by injection of chemicals 
(e.g. trypan blue) or antibodies against yolk sac endoderm is teratogenic in rodents 
(Bielinska et al. 1999). Deletion of Apolipoprotein B, critical for nutrient transport, 
causes embryonic lethality by E10, resulting in disruption of the blood cells or 
vessel formation (Bielinska et al. 1999).

1.2.2 Inductive signals from visceral endoderm

The VE is not only necessary for the embryo structurally but for inducing 
gene expression in adjacent tissues (Bielinska et al. 1999). One of the first roles of 
the yolk sac is to develop vasculature to support the growing embryo.

The VEGF family is composed of various ligands and receptors, all play roles 
in early (vasculogenesis) and later (angiogenesis) stages of vessel formation, as well 
as cardiac and lymphatic vessel formation (Patel-Hett & D’Amore 2010). VEGFA is 
expressed in the extra-embryonic endoderm and mesoderm by E8.5 and it’s 
receptors FLT1 and FLK1 are expressed in close tissues at this time (Patan et al. 
2000). Knockout of either receptor causes embryonic lethality because of 
inappropriate angioblast migration and failure to make extra-embryonic vessels.
Signaling between VE induced IHH and endoderm-derived FGF2 are thought to promote the expression of the VEGF receptor Flk1 in adjacent mesodermal cells (Moerkamp et al. 2012). Loss of IHH results in inappropriate angioblast migration and blood island development (Dyer et al. 2001). This phenotype is mirrored by the Flk1 null phenotype. Loss of IHH by knockout or pharmacological inhibition results in defective yolk sac angiogenesis, and eventual embryonic lethality (Dryer et al. 2001; Byrd et al. 2002; Nagase et al. 2006). FGFs especially FGF2 are thought to induce angioblasts from mesoderm and stimulate angiogenesis (Flamme et al. 1997; Cox and Poole 2000). It is noted that FGFs serves in branching morphogenesis during the later process of organogenesis similar to their possible role in angiogenesis (Crivellato 2011).

1.2.3 The similarities of yolk sac and liver development

There are significant similarities between the yolk sac and liver bud development. First, many important genes are first expressed in VE and soon after in hepatoblasts such as HNF4α, AFP, TTR, HNF1α, β, Hex, Gata6, Villin, HNF3 β, GATA6, FOXA2 and Smad1,2 (Duncan 1997; Parviz 2003; Barbacci 1999; Lockmane 2008; Molkentin 1997; Bielinska 1999; Morrisey 1998; Burtscher 2009; Lee 2005; Waldrip 1998; Tremblay 2000; Sherwood 2007). Many a genetic study of liver budding has been hindered by the requirement of genes are in the VE before liver budding. As the VE feeds the embryo and is necessary for extraembryonic vasculature, impairment of VE causes embryonic lethality before or at the time of
liver budding. This observation highlights the role of the VE as a primitive gut while the DE and subsequent liver are forming.

The VE serves many of the functions that are later performed by the gut during embryo development: nutrient digestion and delivery, gas and metabolic waste removal and enzyme secretion (Bielinska et al. 1999). The YS endoderm has the ability to digest lipids, subsequently incorporating these into circulation through YS vessels, and feeds the growing embryo (Bellairs, 1964: Mobbs and McMillan, 1981, Speake et al 1998). Furthermore, it has been proposed that the VE acts as a reserve for glycogen, and vitamin A derived retinoids (Bellairs, 1964; Willier 1968; Bielinska 1999). The VE has the unique function of digesting vitamin A to synthesize components of the Retinoic Acid (RA) signaling pathway: RBP, CRABPs and CRBP (reviewed in Bielinska 1999). Even more compelling data shows that the VE produces embryonic serum proteins: a function of the liver (Young and Klein 1983; Nakazawa 2011). Recent gene expression profiling found that several lipid binding and transport proteinases well as metabolic and catabolic enzymes are enriched in VE (Nakazawa 2011).

The structure of both the VE and liver bud is indicative of their metabolic function. Both are absorptive epithelia comprised of polarized cells and their basal membranes face splanchnic mesenchyme and endothelial cells (Mobbs and McMillan 1981). These epithelial cells contain cytoplasmic organelles that facilitate their role in nutrient digestion and transportation (Nakazawa 2011). After transport from apical to basal surface, the placement of both VE and DE directly next to blood vessels allows nutrient delivery to the embryo. As this location close to blood
vessels is vital for metabolic function, it is unsurprising that DE and VE support and possibly induce vasculogenesis and angiogenesis.

Endoderm plays a supportive role in both vasculogenesis and angiogenesis, ensuring that blood vessels appropriately form close to it. Mesenchyme, in turn, signals back to endoderm to direct proper differentiation. Both VE and liver bud are in direct contact with vasculature and splanchnic mesenchyme. Consequently many vital signaling pathways are conserved between VE and the liver bud, being required first in VE and soon after in DE derivatives, especially liver. As endothelial cells coalesce into lumenized vessels, endoderm cells surround them, thus providing a direct source of growth factors and possibly structural support. Both VE and liver bud are sources of growth factors that support vasculo- and angiogenesis such as VEGF, Ang1 and RA. In the yolk sac, the process of vasculogenesis creates vessels from the blood islands between endoderm and mesoderm.

At early stages, endoderm and mesoderm are flat sheets with endothelial cells between, but as the future vasculatures form, they fold such that they surround the vessels. At this time and throughout gestation, the VE’s basal side is in direct contact with the extraembryonic vessels. Multiple knockout studies result in embryonic lethality because of failure of YS vasculature and, interestingly, many phenotypes include hemorrhage and dilated vessels between flat endoderm and mesoderm that have failed to form their characteristic folds (Kang 2013).

It is thought that organs signal vessels during the vascularization of that organ and vascular signals from endothelial cells provide patterning signals back to organs (Coultas et al. 2005; Cleaver and Melton 2003). In both the liver and the yolk
sac, endothelial cells and endoderm, or mesenchyme and endoderm participate in paracrine singling. In early liver development sinusoidal endothelial cells supply the hepatic survival factor HGF to surrounding hepatoblasts (LeCouter et al. 2003). The close interaction between the hepatoblasts and endothelial cells is essential for liver development at this early stage. Flk1 null embryos, that do not have endothelial cells, form a liver bud that does not delaminate and invade STM (Matsumoto 2001). Additionally, BMP4 and FGF8 have been implicated in endothelial to hepatocyte signaling, and loss of either signal is similar to the Flk-/- phenotype (Jung et al. 1999; Rossi et al. 2001). Rb, c-Myc, Hex, and Epo, are some of the factors expressed in endothelial cells who's loss cause liver hypoplasia, further supporting the model that these cells are vital for liver development and survival (reviewed in Arterbery and Bogue 2014).

1.3 Hypothesis

Based on the current body of evidence, our preliminary hypothesis, which we sought to investigate, was that YY1 plays an important role in murine endoderm organogenesis, more specifically, within DE derived liver development. To test this, we use a conditional knockout strategy to remove YY1 within the DE; however, because the Cre-driver first excised YY1 in the yolk sac visceral endoderm where we found it plays a critical role in yolk sac development, we were unable to examine its role in liver budding. These data, considering the role of YY1 in VEGF signaling, forced us to adjust our hypothesis as it seems that YY1 is essential for yolk sac development through a VEGF pathway. In vivo analysis of YY1 mutant embryos,
coupling exogenous VEGF rescue experiments with small molecule VEGF inhibitor mimicking experiments confirm that YY1 expression in the VE is essential for VEGF production and subsequent yolk sac angiogenesis (Chapter 2). Additionally, we wanted to investigate whether FGF is important for liver bud specification. Embryos were cultured in vivo with a small molecule FGF inhibitor yielding the novel results that while FGF is necessary for anterior liver bud specification, it is not required for posterior liver bud specification, suggesting that two distinct liver cell populations exist and are differentially affected by surrounding morphogens (Chapter 3).

Considering these findings, we refined our former models to devise two new hypotheses to serve as our current working models. **First, we assert that YY1 within the DE is essential for liver development via the VEGF pathway. Second, we hold that two distinct populations of liver cells exist and respond differentially to the loss of YY1 and the VEGF pathway.** To substantiate these claims and complete my project, we used an inducible Cre-ER to create a YY1 cKO specifically in the definitive endoderm. YY1 mutant analysis, in vivo culture, and liver bud explant culture revealed that YY1 is essential for VEGF production in the liver and subsequent hepatic angiogenesis, suggesting that the role of YY1 in the VEGF pathway is conserved within both the VE and DE (Chapter 4). Histology, Dil fate mapping, and laser ablation experiments confirmed not only the existence of two distinct liver populations with differential morphogen requirements, but also that the anterior and posterior liver buds contribute to the caudal and rostral liver lobes respectively, thus creating a new model with which to study liver organogenesis and regeneration.
CHAPTER 2

THE ROLE OF YY1 IN YOLK SAC DEVELOPMENT VISCERAL ENDODERM

EXPRESSION OF YIN-YANG (YY1) IS REQUIRED FOR VEGFA MAINTENANCE AND
YOLK SAC DEVELOPMENT

PLOS ONE 2013

2.1 Hypothesis

In demonstrating our main hypothesis that YY1 plays an important role in murine endoderm, we employed a FoxA3 Cre to generate a YY1 cKO. In attempting to knockout YY1 in the DE, we observed the loss of YY1 in both the DE and VE, causing a severe defect within the embryo yolk sac and embryo lethality forcing us to amend our preliminary hypothesis. We hypothesize that YY1 is essential for yolk sac angiogenesis via the VEGF pathway. In vivo mutant analysis coupled with both exogenous VEGF rescue experiments and VEGF pathway inhibition experiments confirmed this claim. We also pose that since the yolk sac VE and the liver bud DE share many similarities, information form the VE could be useful to study liver bud development.

2.2 Abstract

Mouse embryos lacking the polycomb group gene member Yin-Yang1 (YY1) die during the peri-implantation stage. To assess the post-gastrulation role of YY1, a conditional knock-out (cKO) strategy was used to delete YY1 from the visceral
endoderm of the yolk sac and the definitive endoderm of the embryo. cKO embryos display profound yolk sac defects at 9.5 days post coitum (dpc), including disrupted angiogenesis in mesoderm derivatives and altered epithelial characteristics in the visceral endoderm. Significant changes in both cell death and proliferation were confined to the YY1-expressing yolk sac mesoderm indicating that loss of YY1 in the visceral endoderm causes defects in the adjacent yolk sac mesoderm. Production of Vascular Endothelial Growth Factor A (VEGFA) by the visceral endoderm is essential for normal growth and development of the yolk sac vasculature. Reduced levels of VEGFA are observed in the cKO yolk sac, suggesting a cause for the angiogenesis defects. Ex vivo culture with exogenous VEGF not only rescued angiogenesis and apoptosis in the cKO yolk sac mesoderm, but also restored the epithelial defects observed in the cKO visceral endoderm. Intriguingly, blocking the activity of the mesoderm-localized VEGF receptor, FLK1, recapitulates both the mesoderm and visceral endoderm defects observed in the cKO yolk sac. Taken together, these results demonstrate that YY1 is responsible for maintaining VEGF in the developing visceral endoderm and that a VEGF-responsive paracrine signal, originating in the yolk sac mesoderm, is required to promote normal visceral endoderm development.

2.3 Introduction

Yin-Yang 1 (YY1) is aptly named because of its documented roles as a transcriptional activator and repressor, binding directly to DNA via a consensus-binding site or as part of repressive complexes. In vitro analysis has revealed that YY1 is required for appropriate regulation of a variety of basic cellular processes
including proliferation, cytokinesis, epithelial-mesenchymal transition, apoptosis and DNA repair (Shi et al. 1997). Based on these diverse roles in essential cellular processes in normal cells it is not surprising that inappropriate regulation of Yy1 is believed to influence oncogenesis (Castellano et al. 2009; Atchison et al. 2011). Given the importance of YY1’s observed roles in vitro and its implication in a number of cancers, understanding the role of this gene in normal mammalian developmental processes is of great interest.

YY1 is the vertebrate homolog of the Drosophila pleiohomeotic (Pho), a member of the polycomb group (PcG) of proteins. Pho is an essential member of the multiprotein Polycomb Repressive Complex, providing DNA binding activity (Brown et al. 1998). Mammalian YY1 can substitute for Pho in wing imaginal disc development and partially rescues Pho mutant fly embryos demonstrating that these essential PcG interaction and DNA binding functions are conserved in the mammalian protein (Kwon & Chung 2003). Two high molecular weight PcG complexes, polycomb repressive complex 1 and 2 (PRC1 and 2), are conserved in vertebrates. Although YY1 has been shown to interact with vertebrate PRC2 complex members it remains unclear if YY1 targets PRC2 in mammalian cells (Satijn et al. 2001).

YY1 is expressed ubiquitously in the extraembryonic and embryonic portions of the developing mouse embryo including the germ line and all adult tissues examined (Donohoe et al. 1999; Wu et al. 2009; Griffith et al. 2011; Trask et al. 2012). Complete knockout of Yy1 results in peri-implantation lethality demonstrating a critical early role for this gene (Donohoe et al. 1999).
generation of a conditional allele has allowed for a better understanding of the tissue-specific requirements of YY1 in embryonic and adult lineages (Affar et al. 2006). YY1 has been shown to play a critical role in immunity and B-cell lineage progression (Park & Atchison 1991; Sayegh et al. 2005), where knockout in B-cells produces arrest at the pro-B cell stage (Liu et al. 2007). In the developing oocyte, loss of YY1 leads to a failure of oocyte-granulosa communication and a subsequent loss of fertility (Griffith et al. 2011). YY1 is also essential during gastrulation in the epiblast for appropriate primitive streak formation and proper regulation of the Nodal signaling pathway (Trask et al. 2012). These recent studies have identified defects in paracrine signaling in vivo upon tissue-specific deletion of Yy1.

Here we show that YY1 expression in the visceral endoderm of the yolk sac regulates VEGF in this tissue. VEGF is essential for the growth and development of both the yolk sac and embryonic vasculature. VegfA heterozygous animals have embryonic and yolk sac angiogenesis defects that are apparent by 9.5 dpc, suggesting that VEGF-A levels are critical for normal vasculature development (Carmeliet et al. 1996; Ferrara et al. 1996). Furthermore, reductions in VEGFA during early postnatal development affect embryo and organ size, revealing that the appropriate levels VEGFA signaling is also essential throughout postnatal development (Gerber et al. 1999). Finally, the reduction of VegfA in the visceral endoderm alone results in yolk sac angiogenesis defects, suggesting that the level of VEGFA produced by the visceral endoderm is responsible for angiogenesis in the underlying mesoderm (Carmeliet et al. 1996; Damert et al. 2002).
While most of the VegfA in the yolk sac is produced by the visceral endoderm, both of the cognate receptors, including Flt1 (VegfR1) and Flk1 (VegfR2/Kdr) are expressed specifically in the adjacent yolk sac mesoderm (Breier et al. 1995; Dumont et al. 1995; Miquerol et al. 1999). Consistent with these findings, knockout analysis of Flt1 and Flk1 demonstrate an essential role for these receptors in blood vessel development (Fong et al. 1995; Sato et al. 1995; Shalaby et al. 1995).

Here we use the transgenic FoxA3-Cre line (Lee et al. 2005b) to conditionally delete Yy1 in the visceral endoderm of the yolk sac and in the embryonic definitive endoderm. While the initiation of early endoderm organogenesis is only slightly delayed, yolk sac development is severely disrupted. At 9.5 dpc the mutant yolk sac is morphologically abnormal and angiogenesis, which occurs in the adjacent Yy1-expressing yolk sac mesoderm derivatives, is disrupted. A variety of visceral endoderm defects are observed at 9.0 dpc, including a loss of large apical lysosomes as well as changes in visceral endoderm-specific gene expression. Although the level of VegfA transcripts remain unchanged in the cKO yolk sac, VEGFA protein is dramatically reduced by 9.25 dpc, suggesting that YY1 regulates VEGFA translation or stabilization. Surprisingly, we show that exogenous VEGF rescues the Yy1 cKO defects in both yolk sac layers and propose that a paracrine signal produced by the VEGF-receiving YS mesoderm is required to maintain key visceral endoderm characteristics. Taken together these data support a critical role for YY1 in maintenance of VEGF levels and highlight a new role for VEGF-responsive yolk sac mesoderm-derived tissue in supporting visceral endoderm function.
2.4 Materials and Methods

2.4.1 Ethics Statement

All animal studies were approved by the Institutional Animal Use and Care Committee, University of Massachusetts, Amherst protocol #2012-043.

2.4.2 Mouse Breeding Scheme and Genotyping

Females homozygous for the Yy1 floxed allele (Yy1\(^{fl/fl}\)) (Affar et al. 2006) were mated to males heterozygous for a Yy1 null allele (Yy1\(^{+/+}\)) and for the Foxa3-Cre transgene (FoxA3-Cre) (Lee et al. 2005b) to obtain Yy1\(^{fl/+}\); FoxA3-Cre embryos. The morning of the copulation plug was defined as 0.5 dpc. After obtaining embryos at the desired stage, a portion of the yolk sac or embryo used for PCR genotyping using the primers of Yy1: 5’ACCTGGTCTATCGAAAGGAAGCAC3’, 5’GCTTCGGCTATTCCTCGCTCATAA 3’ and 5’CCAAAGTTCGAAACCTGCTTTCCT3’; Cre: 5’CATTTGGGCCAGCTAAACAT3’ and 5’ATTCTCCCACGGTTACG3’. Yy1\(^{fl/+}\); FoxA3-Cre embryos are referred to as “mutants” or “cKO” and other genotypes referred to as “wild-type” (WT) for simplicity.

2.4.3 Histology, Immunofluorescence and Immunohistochemistry

Embryos and yolk sacs were dissected and fixed in 4% paraformaldehyde (PFA)/PBS overnight at 4°C. The following day they were washed in PBS, dehydrated in an ascending methanol sequence, xylene treated, embedded in paraffin and sectioned at 7.5 μm. For histological analysis, routine hematoxylin and eosin (H&E) staining was performed on dewaxed slides.
For immunofluorescence (IF) slides were dewaxed in xylenes, rehydrated with EtOH, and subjected to antigen retrieval in Tris buffer pH 10.0 for 10 min. The slides were then washed in PBT and incubated in blocking buffer (0.5% milk powder, 99.5% PBT) for 2 hrs at room temperature and then with primary antibody in blocking buffer at 4°C overnight in a humid chamber. Slides were then washed three times with PBT, incubated for 1 hr with secondary antibody in blocking buffer at room temperature. Nuclei were counterstained with 4’, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, 1:10,000) for 3 min and then coverslipped with Prolong Gold Antifade Reagent (Invitrogen). Sections were imaged on a Nikon Eclipse TE2000-S inverted microscope with Retiga EXi Fast camera with NIS Elements imaging software. Primary antibodies used include: rabbit anti-YY1 [1:100, Santa Cruz (sc-1703)]; goat anti-HNF4α [1:100, Santa Cruz (sc6556)]; mouse anti-CDH1 [1:500, BD Bioscience (610811)], mouse anti-αSMA [1:500, Sigma (A2547)], rabbit anti-cleaved Caspase-3 [1:500, Abcam (ab13847)], rabbit anti-phosphohistone-H3 [1:500, Abcam (Ab5176)], rabbit anti-VEGFA [1:100, Santa Cruz (A120)] and guinea pig anti-PDX1 [1:1000, Abcam (ab47308)]. Secondary antibodies (Molecular Probes) were used at 1:500. IF of IgG was performed as detailed above except that a primary antibody was omitted and the secondary antibody was Donkey anti-mouse [1:500, Molecular Probe (A11036)].

Whole-mount IF of PECAM [1:50, Pharmsen (553369)] and αSMA (1:100) were performed as described (Waller-Evans et al. 2010). Following IF, yolk sacs were imaged and coverslipped on glass slides using a Nikon SMZ1500 microscope equipped with a MicroPublisher 5.0 RTV camera and Q-imaging software.
For immunohistochemistry (IHC), deparaffinized slides were subjected to 1% $\text{H}_2\text{O}_2$ for 30 min., rinsed in PBS and then blocked in PBS-0.1% Triton X-100 for 30 min. Slides were then incubated overnight at 4°C in blocking buffer as above containing primary antibodies including rabbit anti-YY1 [1:200, Santa Cruz (sc-1703)], goat anti-HNF4α [1:200, Santa Cruz (sc6556)]. After three washes in PBS, slides were incubated for 1 hr in blocking buffer containing the appropriate biotinylated secondary antibody (Vector Labs) at 1:500. The secondary was detected with Vectastain Elite ABC kit (Vector Labs) and stained using DAB (Vector Labs). For IgG localization, biotinylated anti-mouse IgG (Vector Labs) was used as outlined above except that the primary antibody was omitted. After ICH, slides were coverslipped with mounting media [Richard-Allan Scientific (8310-16)] and visualized using a Nikon Eclipse TE2000-S inverted microscope with Retiga EXi Fast camera with color filters and NIS Elements imaging software.

For LacZ staining, embryos double heterozygous for the R26R allele (Soriano 1999) and FoxA3Cre allele were stained and processed as reported (Tremblay et al. 2000).

### 2.4.4 Western Blot Analysis

5–7 yolk sacs for each sample were pooled and lysed by extraction buffer with Complete Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail (Roche). The samples were homogenized and soluble proteins obtained by centrifugation. Equal amounts of protein were loaded onto a 4%–20% gradient Tris-Glycine gel under reducing conditions. After transferring onto 0.45 μm PVDF
membranes (Millipore) following standard protocols, the membranes were blocked
(5% nonfat dry milk in TBS with 0.1% Tween20) for 1 hr and incubated overnight at
4°C with rabbit anti-VEGFA [1:1000, Santa Cruz (A120)], followed by secondary
(1:5000, Jackson Immuno Research) for 1 hr. Results were visualized using ECL
reagent (Amersham Life Sciences). Blots were stripped and reprobed with mouse
anti-GAPDH [1:5000, Millipore (MAB374)] as a loading control. Autoradiographs
were quantified using Image J software (NIH).

2.4.5 RNA Extraction, cDNA Synthesis and PCR

Whole yolk sacs or yolk sac layers (used only in Fig. S3) were dissected and
saved in RNA Later (QIAGEN) overnight at 4°C then stored at 80°C. The yolk sac
layers were separated using the trypsin/pancreatin method as described [29]. Total
RNA was extracted using the High Pure RNA Isolation kit (Roche) according to
manufacturer’s recommendation. 500 ng of RNA was reverse transcribed using the
iScript ™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturers’ instructions.
RT-PCR was performed with 36 cycles of 30 sec at 60°C, 72°C and 94°C. Hprt and β-
actin were used as internal controls. All primer sets for traditional RT-PCR used are
listed. Quantitative RT-PCR (qPCR) was performed using the following Taqman gene
expression Assays: Yy1 (MM0456392_m1), VegfA (MM00437304_m1) and Hnf4α
(MM00455964_m1). These assays were multiplexed with ActB using PerfeCTa®
quPCR SuperMix, Low ROX™ (Quanta Biosciences). Reactions were performed on a
Stratagene 3001 mx qPCR machine using Quanta’s recommend cycling conditions.
2.4.6 Apoptosis and Proliferation

3 WT and 3 cKO yolk sacs at 8.5 and 9.0 dpc were subjected to IF (as noted above) using either cleaved Caspase-3/CDH1/DAPI to assess apoptosis or PH3/CDH1/DAPI to assess proliferation. CDH1 was used to identify the visceral endoderm layer and DAPI used to distinguish individual cells. A total of 2–3000 cells were counted from 10–12 independent fields for each data point. Image-J was used to assist with counting. All statistical analysis performed on these samples and elsewhere were performed by comparing averages under the null hypothesis that there are no differences between WT and cKO samples with the student’s T-test. Statistical differences were measured by two-tailed Student’s t test analysis of variance.

2.4.7 Blood Vessel Measurements

Blood vessel size was determined by counting the same number of blood vessels on H & E stained sections from 3 mutant and 3 WT yolk sacs using the NIS Elements line measurement tool. Blood vessel density was assessed by obtaining the average of all vessels counted from the same total area from 3 WT and 3 mutant embryos.

2.4.8 LysoTracker Staining

Whole embryos with intact yolk sacs were dissected and incubated with 100 nM LysoTracker Red (Invitrogen) in PBS at 37°C for 5 min. Yolk sacs were mounted on a glass bottom culture dishes and immediately imaged.
2.4.9 Electron Microscopy

Yolk sacs (n = 2 for each age/genotype combination) were fixed with 2.5% glutaraldehyde in PBS at 4°C overnight. Samples were post-fixed in 2% osmium tetroxide in 0.1 M PBS pH 7.4, dehydrated in a graded series of acetone (10% steps) and embedded in epoxy resin. The resin was polymerized at 70°C for 12 hr. Ultrathin sections of 60 nm were cut with a Reichert Ultracut E Ultramicrotome and placed on copper grids. Sections were then counterstained with uranyl acetate and imaged with a JEOL 100 S electron microscope.

2.4.10 Whole Embryo Culture

Litters were dissected at 8.5 dpc and cultured until 9.5 dpc (26-28 hrs) as previously described (Angelo et al. 2012). VEGF [Peprotech (100-20C)] or SU1498 (Millipore) was diluted in DMSO and added directly to the culture media to obtain 200 ng/ml (VEGF) or 40 µM (SU1498), while an equal amount of DMSO-alone was added to the controls. After culture all embryos were photographed and processed for histology. Because the cKO embryos are not apparent at the onset of culture, litters were blindly divided into VEGF treatment groups and the ectoplacental cones dissected at the end of culture for genotyping.

2.5 Results

2.5.1 Yy1 is Required for Yolk Sac Development

To examine the role of Yy1 in the visceral and definitive endoderm, we used a conditional deletion strategy employing a FoxA3-Cre transgene and a conditional
Yy1 allele (Lee et al. 2005b; Affar el et al. 2006). FoxA3-Cre mediated deletion of Yy1 (referred to as “mutant”, “cKO” or “Yy1 cKO”) resulted in an embryonic delay occasionally observed by 8.5 dpc (compare inset in Fig. 2.1A to inset in G), frequently observed by 9.0 dpc and always found in cKO embryos by 9.5 dpc (compare Fig. 2.1E to K). Between 8.5-9.0 dpc the cKO yolk sac is morphologically similar to WT (compare Fig. 2.1A to G, B-C to H-I and M to O). Compared with WT, the 9.5 dpc cKO yolk sac displayed abnormal vasculature including dilated vessels and a paucity of vasculature (compare Fig. 2.1D and N to J and P). The overall numbers of vessels per area of yolk sac was significantly lower in the mutant (Fig. 2.1Q) and there were fewer vessels smaller than 100 μm and more vessels larger than 100 μm when compared with a WT size distribution (Fig. 2.1R). By 10.5 dpc, cKO embryos were considerably smaller than WT and displayed a pale thin yolk sac (Fig. 2.1F, L). Finally, because blood vessel development is dependent upon appropriate hemodynamic flow (Lucitti et al. 2007; Jones et al. 2008), it is important to note that cKO embryos do contain a beating heart through 10.5 dpc (data not shown). At 9.0 dpc, blood flow in the cKO yolk sac is indistinguishable from that of WT, although flow is impeded in the cKO yolk sacs at 9.5 dpc, coincident with the observed vascular defects (data not shown).

To gain insight into how Yy1 loss affects the visceral endoderm, we examined the ultrastructure of this tissue using electron microscopy. While the 8.5 dpc cKO visceral endoderm appears morphologically normal when compared with WT (Fig. 2.2A, E), by 9.0 dpc the large apical lysosomes observed in WT are dramatically reduced in size in cKO tissue (asterisks in Fig. 2.2B, F). IgG localization, which
accumulates in the apical regions of the visceral endoderm, and LysoTracker Red, a fluorescent cell-permeable probe that accumulates in the lysosomes of live yolk sacs, were used to further examine the alterations in lysosome size. In agreement with the TEM results, 9.0 dpc cKO embryos had reduced levels of IgG and a noticeable reduction in the size of LysoTracker filled vesicles compared with WT at 9.0 dpc (compare Fig. 2.2C to G and I to M) and both are more noticeably reduced by 9.5 dpc (compare Fig. 2.2D to H and J to N). Since the apically located vesicles are a specialized characteristic of absorptive epithelium, we also examined more general epithelial characteristics such as the cell-cell adhesion marker E-Cadherin (CDH1) and found that it too was slightly downregulated at 9.0 dpc compared with WT (Fig. 2.2K, O) and more profoundly downregulated by 9.5 dpc (Fig. 2.2L, P). Taken together these data indicate that the epithelial characteristics of the cKO visceral endoderm are disrupted by 9.0 dpc, before gross morphological defects are evident.

2.5.2 Loss of YY1 Occurs First in Visceral Endoderm and then in Definitive Endoderm

YY1 is ubiquitously expressed in embryonic and in extraembryonic tissues during all stages examined (7.5-9.5 dpc, Fig. 2.3A-C, I). To gain a better understanding of the progression of the cKO phenotype, we examined YY1 expression in cKO tissues. We find that at 7.5 dpc YY1 in the Hepatocyte Nuclear Factor 4α (HNF4α) expressing visceral endoderm is downregulated (Fig. 2.3E), and is entirely depleted in the visceral endoderm by 8.75 dpc (Fig. 2.3 F-G). We
confirmed the visceral endoderm expression of the FoxA3-Cre transgene using the R26R allele (Soriano 1999) and found that the Cre-driven LacZ expression mimicked the loss of YY1 (Fig. 2.9).

Loss of YY1 in the definitive endoderm is initiated by 8.5 dpc and is widespread between 8.5 dpc-9.5 dpc (Fig. 2.3F, L and Fig. 2.9B-C, E-F). To determine if differentiation of the definitive endoderm was impaired in cKO embryos we examined liver and pancreas development. Although Yy1 cKO embryos were often delayed, when the cKO embryos were compared to somite-matched embryos, Yy1 cKO endoderm displayed appropriate early markers such as PROX1 in the liver bud (compare Fig. 2.3J to M) and PDX1 in the ventral and dorsal pancreas buds (SFig. 2.2). As observed in the visceral endoderm (Fig. 2.3H), HNF4α expression is downregulated at 9.5 dpc in the cKO liver bud (compare Fig. 2.3K to N).

2.5.3 Angiogenesis Defects in Yolk Sac Mesoderm and Loss of VEGF

It is well documented that inductive signals between the visceral endoderm and mesoderm derivatives of the yolk sac are required to coordinate development and growth of this vital extraembryonic tissue (Dyer et al. 2001; Byrd et al. 2002; Damert et al. 2002; Bohnsack et al. 2004). To further investigate the defects observed in the yolk sac mesoderm, we examined expression of two markers associated with vascular development. The endothelial cell marker, PECAM, delineates the large primary vessels as well as the smaller secondary vessels in the yolk sac of WT 9.5 dpc (Fig. 2.4A). PECAM expression in cKO yolk sacs demonstrates that although endothelial cells have formed vessels, they are not well organized (Fig.
2.4D). α-Smooth muscle actin (αSMA), which is expressed in the smooth muscle that surrounds mature vessels, is highly expressed in large mature vessels in WT yolk sac (Fig. 2.4B-C). In the 9.5 dpc cKO yolk sac, we observe no αSMA, suggesting a defect in the later stages of blood vessel remodeling (Fig. 2.4E-F).

Based on its essential role in vascular development, we next examined VEGFA protein levels and distribution in cKO and WT yolk sacs. Immunofluorescence reveals that VEGFA is localized to the visceral endoderm and, at lower levels, in the underlying mesodermal tissue from 9.0-9.5 dpc (Fig. 2.4G-I). In cKO yolk sacs, VEGFA levels in the visceral endoderm appear slightly reduced at 9.0 dpc, more discernibly reduced at 9.25 dpc and is dramatically reduced by 9.5 dpc (Fig. 2.4J-L). Western blot analysis of pooled wild-type and mutant yolks reveals that absolute VEGFA levels are unchanged between samples at 9.0 dpc, reduced to one-third wild-type levels in mutants by 9.25 dpc and almost completely lost in the mutant by 9.5 dpc (Fig. 2.4M).

2.5.4 Analysis of Cell Death and Proliferation in the cKO Yolk Sac

Because YY1 has been implicated in cell cycle regulation (Affar et al. 2006), we next examined the role YY1 has on YS proliferation and cell death. Phosphohistone-H3 (PH3) and cleaved Caspase-3 were used as markers of proliferation and apoptosis, respectively. Analysis of sectioned WT and mutant yolk sacs revealed no differences in proliferation (data not shown) or apoptosis at 8.5 dpc (Fig. 2.4N). At 9.0 dpc, loss of YY1 in the visceral endoderm resulted in an increased percentage of cleaved Caspase-3 positive cells and a decrease in the
percentage of proliferating cells in the YY1-positive yolk sac mesoderm (Fig. 2.4N-O). Combined these data demonstrate that cKO of Yy1 in the visceral endoderm results in proliferation and apoptosis defects in the adjacent yolk sac mesoderm.

2.5.5 Examination of cKO Yolk Sac Gene-Expression

Appropriate VEGF signaling is critical for many aspects of vascular development in both the embryo and extraembryonic tissues including vascular remodeling and for eliciting an anti-apoptotic response (Fong et al. 1995; Sato et al. 1995; Shalaby et al. 1995; Carmeliet et al. 1996; Ferrara et al. 1996). At 9.5 dpc Vegfa is mainly expressed in the visceral endoderm while the cognate VEGFA receptors, Flk-1 and Flt-1, are expressed exclusively in the underlying mesoderm (SFig. 3 and ([Breier et al. 1995; Dumont et al. 1995; Miquerol et al. 1999])). RT-PCR and qPCR were used to examine Vegfa expression in whole yolk sacs at 9.0-9.5 dpc. Surprisingly, we observed no change in Vegfa mRNA at either 9.0 or 9.5 dpc (Fig. 2.5B, E), despite the reduction in protein. Hif1α, a transcriptional activator of the Vegfa locus (Forsythe et al. 1996) is also maintained at normal levels in the cKO yolk sac (Fig. 2.5B).

Other essential VE-specific markers such as Hnf4α, Ihh, vHnf1, Gata4, Gata6, Ttr and qK were examined (Fig. 2.5C) and only vHnf1 and Hnf4α, two transcription factors required for normal visceral endoderm differentiation (Chen et al. 1994; Duncan et al. 1997; Barbacci et al. 1999), were altered in the cKO between 9.0-9.5 dpc (Fig. 2.5C, E). Pgc1α was recently shown to be downregulated in YY1-deficient muscle (Blattler et al. 2012). We found that Pgc1α, which is expressed exclusively in
the visceral endoderm of the yolk sac (Fig. 2.11), is reduced in the cKO yolk sac at 9.0 and 9.5 dpc (Fig. 2.5C).

Based on the lysosome defects observed in mutant yolk sacs, we examined the expression of a several genes involved in lysosome biogenesis. No changes were found in Snx2, Lamp2, Rab7, Tfeb or Lrp2 (Fig. 2.5D). However Enpp-2, which encodes for the exoenzyme Autotaxin and is required in the visceral endoderm for appropriate apical lysosome formation (Koike et al. 2009), is upregulated in cKO tissue at both 9.0 and 9.5 dpc compared with WT controls (Fig. 2.5D).

### 2.5.6 Exogenous VEGF Rescues cKO Yolk Sac

Because loss of YY1 mimics many aspects of the VEGFA heterozygous phenotype and because the cKO yolk sacs display reduced VEGFA, we next sought to determine if exogenous VEGF could rescue the cKO yolk sac phenotype. Entire litters containing cKO and WT embryos were dissected at 8.5 dpc and cultured ex vivo until they were 9.5 dpc in the absence (−VEGF, Fig. 2.6A–E, K–O) or presence of exogenous VEGF (+VEGF, Fig. 2.6F–J, P–T). Cultured WT embryos exhibit normal growth and yolk sac development (Fig. 2.6A–E), while addition of VEGF to WT embryos resulted in prominent yolk sac vascularization, an increase in apical vesicle accumulation of IgG and a slightly larger embryo (Fig. 2.6F–J).

cKO embryos cultured from 8.5 through 9.5 dpc are similar to those found in vivo (Fig. 2.6K–O). One difference is that while the in vivo developed mutants contained a few prominent dilated vessels (Fig. 2.1J, 2.4D), cKO cultured embryos contained many small non-contiguous clusters of dilated vessels. Those in the
proximal yolk sac were typically filled with nucleated blood cells (Fig. 2.6K–N).

Culture of cKO embryos with exogenous VEGF rescued several features of the mutant phenotype. VEGF treated cKO embryos produce a more normal distribution of vascular tissue and larger embryos when compared with cKO embryos cultured in media alone (compare Fig. 2.6K, K’ to P, P’), despite the absence of YY1 in the visceral endoderm (Fig. 2.6Q). Furthermore, αSMA expression is restored to normal levels in VEGF treated cKO mesoderm (compare Fig. 2.6M to R). Surprisingly we find that both HNF4α (Fig. 2.6S) and IgG levels (Fig. 2.6T) are restored in the VEGF supplemented cKO visceral endoderm. CDH1, which is reduced in mutant visceral endoderm isolated in vivo (Fig. 2.2P), is similarly reduced in cultured cKO visceral endoderm when compared with cultured WT controls (compare Fig. 2.6W to U–V). Normal levels of CDH1 are restored when cKO embryos are supplemented with VEGF (compare Fig. 2.6X to U–V). Finally, we examined apoptosis in the mesoderm of cultured embryos and found that addition of VEGF to the cKO yolk sac results in apoptosis levels that are similar to those of WT embryos (Fig. 2.6Y).

These results demonstrate that addition of VEGF to cKO embryos rescues both the vascular/mesoderm phenotypes and the visceral endoderm phenotypes. Because the visceral endoderm does not harbor either of the essential early VEGF receptors (Flt1, Flk1; Fig. 2.11) and thus cannot directly receive VEGF signals, our results suggest that the visceral endoderm receives a VEGF-responsive signal from the yolk sac mesoderm to maintain visceral endoderm characteristics. Finally, these data support the notion that the main cause of the early yolk sac failure in Yy1 cKO embryos is the reduction of VEGF.
2.5.7 Inhibition of VEGF Signaling

To confirm that many of the visceral endoderm defects observed in the cKO yolk sac is due to the loss of VEGF-signaling in the yolk sac mesoderm, we cultured WT embryos with the small-molecule SU1498, which blocks the tyrosine kinase activity of the VEGF receptor, FLK1. Addition of SU1498 to WT 8.5 dpc embryos that were then cultured for ~28 hours resulted in poor yolk sac development, delayed embryo growth and increased apoptosis in the mesoderm derivatives of the yolk sac when compared to embryos cultured in the absence of inhibitor (compare Fig. 2.7A–B to F–G and E to J). Furthermore we find that SU1498-treated embryos have reduced levels of HNF4α (Fig. 2.7H) and reduced accumulation of IgG (Fig. 2.7I) compared to untreated controls (Fig. 2.7C–D). These results demonstrate that blocking VEGF-receptor mediated signaling in the yolk sac mesoderm not only produces defective blood vessel development but also leads to a loss of visceral endoderm characteristics, supporting the hypothesis that loss of a VEGF-dependant paracrine signal from the yolk sac mesoderm is essential for maintaining the visceral endoderm.

2.6 Discussion

Here we assessed the developmental role of Yy1 in the extraembryonic and embryonic endoderm. While YY1 is not required for embryonic endoderm-derived organ specification, we find that YY1 is essential in the visceral endoderm to support angiogenesis in the adjacent mesoderm. Between 9.0–9.25 dpc mutants display a loss of VEGFA and a reduction of cell polarity markers, a lack of large apical
lysosomes and the reduced expression of several visceral endoderm-expressed
genes, including Pgc1α, vHNF1 and Hnf4α. These defects are accompanied by
decreased proliferation and increased apoptosis in the adjacent yolk sac mesoderm.
Exogenous VEGF rescued both the angiogenesis defects and many of the visceral
endoderm phenotypes including restoration of epithelial polarity, large apical
lysosomes and HNF4α expression. Inhibition of VEGF receptor signaling in the yolk
sac mesoderm produces phenotypes not only in the mesoderm but also in the
visceral endoderm. Taken together these data suggest: 1) that one role of YY1 in the
early visceral endoderm is to regulate VEGFA translation or protein stability and 2)
that a VEGFA-responsive paracrine signal generated by the yolk sac mesoderm is
important for the maintenance of visceral endoderm characteristics (Fig. 2.8).

2.6.1 A VEGF-responsive Paracrine Signal from the Mesoderm is Necessary for
Visceral Endoderm Function

It is clear that VEGF produced in the visceral endoderm is required for
angiogenesis in the yolk sac mesoderm (Damert et al. 2002). We were thus not
surprised that exogenous VEGF restored the angiogenesis defects observed in our
mutant yolk sacs. We were intrigued to find that many of the visceral endoderm
phenotypes observed in our cKO embryos are also restored by the addition of
exogenous VEGF, suggesting that the visceral endoderm phenotype is not directly
due to the loss of YY1 in this tissues. While it is possible that the visceral endoderm
defects are caused by a cell autonomous role of VEGF in the visceral endoderm, the
exclusive expression of the critical early embryonic VEGF receptors, \textit{flk1} and \textit{flt1}, specifically in the yolk sac mesoderm (Breier \textit{et al.} 1995; Fong \textit{et al.} 1995; Shalaby \textit{et al.} 1995) argues against this possibility. Instead we suggest that the defects observed in the visceral endoderm are due to a loss of a paracrine signal produced by the VEGF-responsive yolk sac mesoderm. For example, \textit{Hnf4}\textalpha{} plays an essential role in the visceral endoderm for normal yolk sac development (Chen \textit{et al.} 1994; Duncan \textit{et al.} 1997; Parviz \textit{et al.} 2003; Hayhurst \textit{et al.} 2008; DeLaForest \textit{et al.} 2011; Santangelo \textit{et al.} 2011). Exogenous VEGF restores HNF4\textalpha{} in cKO embryos, suggesting that YY1 is not required for its expression. Furthermore we demonstrate that inhibition of the FLK1 in WT embryos causes visceral endoderm phenotypes similar to YY1 cKO (Fig. 2.7). Finally, it is interesting to note that exogenous VEGF causes an apparent increase in apical IgG localization and CDH1 even in WT embryos (compare Fig. 2.6E to J, and U to V), supporting the hypothesis that VEGF signals received by the yolk sac mesoderm influences visceral endoderm phenotypes independent of YY1.

A survey of the mutations that reduce VEGF expression in the yolk sac and result in yolk sac angiogenesis defects, including \textit{VegfA} heterozygotes, \textit{VegfA} hypomorphic and \textit{Arnt} homozygous null embryos, reveals visceral endoderm phenotypes similar to that of \textit{Yy1} cKO (Carmeliet \textit{et al.} 1996; Maltepe \textit{et al.} 1997; Damert \textit{et al.} 2002). Similarly a survey of the mutations that alter the ability of the yolk sac mesoderm to respond to VEGF signaling produce a similar visceral endoderm phenotypes (Shalaby \textit{et al.} 1995; He \textit{et al.} 2010). Taken together, these
observations further support our hypothesis that a VEGF-responsive paracrine signal is essential to maintain visceral endoderm integrity.

2.6.2 YY1 Modulation of VEGF

Recent studies have found that YY1 directly binds and activates human Vegfa, B & C promoters (de Nigris et al. 2010). Contrary to this data we find that Vegfa mRNA is not depleted in the cKO yolk sac, but we do see a gradual reduction in VEGFA protein in the visceral endoderm between 9.0-9.5 dpc. Taken together with the VEGF rescue experiments, our data suggest that a reduction in VEGF protein levels is the cause of the cKO yolk sac phenotypes.

One intriguing question is how does YY1 modulate VEGF levels if not at the transcriptional level? Loss of YY1 in skeletal muscle results in a significant reduction in Pgc1α expression (Blattler et al. 2012). Similarly, in our cKO visceral endoderm we find a dramatic reduction of Pgc1α expression. Pgc1α-deficient mice develop normally, exhibiting a phenotype upon physiological stress, suggesting that loss of Pgc1α is not the cause of early embryonic lethality in Yy1 cKO embryos (Lin et al. 2004; Leone et al. 2005). A recent report has shown that with activity-induced stress, Pgc1α-deficient skeletal muscle exhibits a loss of VEGFA protein with no loss of Vegfa transcripts (Leick et al. 2009), suggesting that Pgc1α loss could play a role in the VEGFA reduction observed in our cKO yolk sacs.

2.6.3 Role of YY1 in Paracrine Signaling in vivo
Recent *in vivo* studies have shown an essential role for *Yy1* in the regulation of secreted growth factors during development, including *Bmp15* and *Gdf9* during normal oocyte development (Griffith *et al.* 2011) and *Lefty-2* during gastrulation (Trask *et al.* 2012). In these tissues a loss of YY1 results in defective paracrine signaling of key secreted growth factors, producing defects in adjacent tissues that normally receive the signal. The work presented here is another example demonstrating that YY1 acts as a critical regulator of paracrine signals during development and highlights the importance of *in vivo* functional studies.
CHAPTER 3

FGF SIGNALING IS REQUIRED FOR ANTERIOR BUT NOT POSTERIOR SPECIFICATION OF THE MURINE LIVER BUD

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Jikui Wang (First author: Initiating the project with conceptualizing the idea, culturing embryo culture, performing SISH, IF, IHC and generating figures), Siyeon Rhee (Second author: is responsible for performing IF, statistics, generating figures, helping conceptualization and contributing to complete the project)

3.1 Hypothesis

The literature supports an essential role for FGF in liver bud induction. Because most of the experiments defining a role for FGF in early liver bud development have been performed in the context of explant experiments in a dish. We chose to test the requirement for FGF in liver budding in the context of the whole embryo, by combining ex vivo embryo culture with small molecule inhibition of FGF. Surprisingly, we found that FGF is essential for anterior liver bud specification and not the posterior bud, suggesting our current novel hypothesis that there are two distinct liver cell populations, which are differentially affected by surrounding signaling cues. These novel results provide new insight into the different developmental, morphogenic, and functional roles of these disparate liver populations.
3.2 ABSTRACT

Background: The definitive endoderm arises as a naive epithelial sheet that produces the entire gut tube and associated organs including the liver, pancreas and lungs. Murine explant studies demonstrate that fibroblast growth factor (FGF) signaling from adjacent tissues is required to induce hepatic gene expression from isolated foregut endoderm. The requirement of FGF signaling during liver development is examined by means of small molecule inhibition during whole embryo culture. Results: Loss of FGF signaling before hepatic induction results in morphological defects and gene expression changes that are confined to the anterior liver bud. In contrast the posterior portion of the liver bud remains relatively unaffected. Because FGF is thought to act as a morphogen during endoderm organogenesis, the ventral pancreas was also examined after FGF inhibition. Although the size of the ventral pancreas is not affected, loss of FGF signaling results in a significantly higher density of ventral pancreas cells. Conclusions: The requirement for FGF-mediated induction of hepatic gene expression differs across the anterior/posterior axis of the developing liver bud. These results underscore the importance of studying tissue differentiation in the context of the whole embryo.

3.3 INTRODUCTION

The definitive endoderm is the embryonic germ layer that produces the gut tube and associated digestive and respiratory organs, including the lungs, liver and pancreas. The endoderm emerges as an epithelial sheet that lines the ventral surface of the early embryo. Fate mapping of the early somite (S) embryo has revealed that
the embryonic liver progenitors exist as two distinct populations within the 
epithelial sheet between 2-8 S (embryonic day 8.25; (Tremblay & Zaret 2005b; 
Angelo et al. 2012). One of these populations is composed of two symmetric patches 
of cells that lie on the lateral edges of the closing ventral foregut. The second is 
located in the ventral midline of the endoderm lip (VMEL). The left or right lateral 
progenitors produce the bulk of the left or right side of the liver bud (Angelo & 
Tremblay 2013), while the VMEL contributes to a midline “stripe” of cells 
throughout the foregut, including the liver (Tremblay & Zaret 2005b; Angelo et al. 
2012). At ~10 S the lateral progenitors abut the VMEL at the ventral midline and 
will coalesce in an anterior to posterior progression to produce a liver bud.

The first morphological sign of mouse liver development is a characteristic 
thickening of the lateral precursors that appears between 8-11 S. These thickened 
bilateral tissues coalesce with the VMEL precursors at ~10 S producing the hepatic 
diverticulum that represents the anterior portion of the liver bud, while between 
12-14 S the remainder of the thickened lateral endoderm has formed the hollow 
pseudostratified portion of the caudal liver bud (Bort et al. 2006). Expression of 
liver bud markers such as α-fetoprotein (Afp) and Hepatocyte nuclear factor 4-α 
(Hnf4α) is apparent and restricted to the morphologically evident hepatic tissue 
between 10-13 S. Thus the anterior to posterior progression of gut tube closure and 
liver bud formation are linked and coupled with liver-specific gene expression.

While it is clear that secreted factors from adjacent tissues are required for 
endoderm patterning and organ development, a number of signaling molecules have 
been implicated in liver development, including those of the Fibroblast Growth
Factor (FGF) family (Tremblay 2010b). Indeed multiple aspects of foregut differentiation have been attributed to FGF signaling in the mouse, including anterior/posterior (A/P) patterning of the foregut endoderm (Wells & Melton 2000b), the induction of liver-specific gene expression (Jung et al. 1999b; Calmont et al. 2006), liver bud invasion (Jung et al. 1999b) and liver bud proliferation/growth (Calmont et al. 2006; Berg et al. 2007).

The mammalian FGF family comprises 22 ligands, 18 of which signal through the 4 homologous transmembrane tyrosine kinase FGF receptors (FGFR1-FGFR4). The specificity of FGF-FGFR interaction depends both on their spatial and temporal expression patterns and on the differences in the FGFR’s ligand-binding capacity (Zhang et al. 2006; Turner & Grose 2010). FGF signaling can activate several intracellular downstream mediators including PLCγ, PI3K/AKT and RAS/ERK pathways, and it is believed that activation of an individual downstream mediator is context dependent (Carballada et al. 2001; Sivak et al. 2005).

Although an essential role for FGF signaling during foregut development in general and liver development in particular has been documented, understanding the exact role FGFs play during early liver budding is complicated by the diversity of FGF ligands expressed in adjacent mesodermal tissues. The most compelling evidence for a role of FGF in the earliest stages of mammalian endoderm organogenesis comes primarily from explant studies (Jung et al. 1999b; Deutsch et al. 2001; Calmont et al. 2006). Ventral foregut endoderm and underlying mesoderm explanted from 2-5 S embryos will acquire the ability to express liver-specific genes, such as albumin or Afp, after 48 hours. In the absence of mesoderm, explanted
ventral foregut does not initiate expression of these genes, suggesting liver-specific gene expression requires mesoderm-specific factors. The addition of either FGF1 or FGF2 to such explants promotes expression of albumin and Afp (Jung et al. 1999b; Deutsch et al. 2001). Because FGF1; FGF2 double knockout embryos are viable and fertile (Miller et al. 2000) it is clear that both ligands are sufficient but not required to initiate liver-specific expression from the foregut endoderm. In support of the hypothesis that FGF signals are important for liver induction, both the MAPK and PI3K pathways are activated in the pre-hepatic endoderm and nascent hepatic endoderm (Corson et al. 2003; Calmont et al. 2006). Inhibition of MAPK via ERK1/2 in explants and whole embryos leads to reduced Afp expression, while inhibition of the PI3K pathway does not, suggesting that in the context of the early endoderm, the FGF/ERK pathway is required for liver-specific gene induction (Calmont et al. 2006; Wandzioch & Zaret 2009). Finally, the use of an inducible dominant-negative FGFR in zebrafish demonstrates that active FGF signaling is required for hepatic specification (Shin et al. 2007) and that FGF signaling during liver budding is functionally conserved among vertebrates.

In the present study, we analyze the role FGF signaling plays during the initiation of liver budding in the context of the whole mouse embryo, examining both liver bud formation as well as the induction of liver-specific gene expression. In the presence of the FGFR inhibitor SU5402, liver bud morphogenesis, liver-specific gene expression and cell death are differentially affected along the A/P axis of the liver bud. FGFR inhibition causes profound defects in the anterior portion of the liver bud while these same features are much less or unaffected in the posterior...
liver bud. Because FGF mediated expression of liver-specific genes has been shown to be governed by MEK/ERK 1/2 (Calmont et al. 2006), the MEK 1/2 inhibitor, U0126, was used to determine if MEK/ERK 1/2 mediated these differential responses in the liver bud. Like FGFR inhibition, U0126 dramatically affects anterior but not posterior liver bud specification. Finally, because FGF dose can regulate liver versus pancreas gene expression in foregut explants (Deutsch et al. 2001; Serls et al. 2005), we examined how reduced FGF signals impacts ventral pancreas morphogenesis and specification in the context of the whole embryo. Taken together, these results shed light on the role FGF signaling plays in early liver and ventral pancreas development in the context of the intact mammalian embryo.

3.4 Materials and Methods

3.4.1 Embryo Culture and Small Molecule Inhibition

All animal studies were approved by the Institutional Animal Care and Use Committee, University of Massachusetts, Amherst. Embryos were obtained and whole embryo culture performed as described (Tremblay & Zaret 2005b; Angelo et al. 2012). Specifically, plugs were obtained from CD-1 (Charles River) matings and the morning of the copulation plug defined as E 0.5. To obtain the 2-14 S embryos used herein, pregnant females were sacrificed between 4-9 AM on E 8.0. Embryo dissection involved freeing the conceptus of the Reicharts membrane while keeping both the yolk sac and the ectoplacental cone intact. Dissection was performed on the 37°C heated stage of a dissection microscope in pre-equilibrated (>1 hour in a 5% O₂, 5% CO₂, 37°C incubator) dissection media [10% Fetal bovine serum, 90% DMEM]
(Lonza, 12-709)] within 30 minutes of embryo removal from the mother. After dissection, embryos were held in a 4-well dish in a 5% O₂, 5% CO₂, 37°C incubator containing pre-equilibrated (as above) culture media [75% rat serum (Valley Biomedical, AS3061), 25% DMEM (Lonza, 12-614) supplemented with Pen/Strep (Gibco), non-essential amino acids (Lonza) and GlutaMAX (Gibco)]. Embryos were maintained in static culture for no longer than 3 hours. For culture, embryos were grouped according to somite stage in bottles with up to 3 embryos containing a minimum of 2.0 ml of culture media. Prior to roller culture, the embryos were transferred to glass serum bottles, briefly gassed with 5% O₂, 5% CO₂, 90% N₂, the rubber stoppers replaced, sealed with parafilm and the bottles placed in roller culture as described (Tremblay & Zaret 2005b). The next morning, the bottles containing the embryos were briefly removed from culture, gassed with 20% O₂, 5% CO₂, 75% N₂ and returned to roller culture. Embryos were cultured for a total of 26-30 hours (unless otherwise noted). At the end of culture the extraembryonic tissues were removed, the embryo quality and somite number noted and the embryo photographed. All embryos were fixed in 4% paraformaldehyde followed by dehydration, xylene treatment and paraffin embedding.

Small-molecule inhibition was performed by the addition of SU5402 (40 µM unless noted; Calbiochem) or U0126 (100-200 µM; Calbiochem) each dissolved in DMSO, directly to the roller culture media. To maximally inhibit the intended target, the highest dose of small molecule that allows for relatively normal development was used for experiments. The amount of either drug used was similar to that used
by others to see an affect on similarly staged mouse embryos or explants in other systems (Corson et al. 2003; Calmont et al. 2006; Wandzioch & Zaret 2009). An equal concentration of DMSO was administered to the culture control embryos. Because small variations in culture conditions can dramatically affect embryo growth and viability, similarly staged controls were used each time an experimental treatment was performed and experimental embryos examined only if the control group developed normally. Because SU5402 and U0126 treatment were often performed on 5-6 S embryos cultured at the same time, the control embryo data presented in Fig. 20 and Fig. 41 are the same.

3.4.2 Histology, Immunohistochemistry/Immunofluorescence

Paraffin embedded embryos were serially sectioned at 7 μm and dried overnight. For histology, sections were stained with hematoxylin-eosin as reported (Tremblay et al. 2001). Immunohistochemistry of pERK [Cell Signaling, 1:200] was carried out as follows. Whole embryos were fixed for 30 min at 4°C and embedded as noted above. De-paraffinized sections were treated with 0.3% peroxide for 10 min at room temperature, incubated with primary antibody overnight at 4°C and then incubated with biotinylated goat anti-rabbit secondary antibody (Vector Labs, 1:2000) for one hour at room temperature. The secondary antibody was detected with Vectastain Elite ABC kit (Vector Labs) using 3’3’ diaminobenzidine (DAB, Thermo Scientific). Immunofluorescence was performed on de-paraffinized serial sections that were subjected to antigen retrieval (boiled for 3 min in 10mM citrate buffer pH 6.0) using the following primary antibodies: FoxA1 (1:1000; Seven Hills,
WMAB-2F83); FOXA2 (1:250; Santa Cruz, sc6554); HNF4α (1:200; Santa Cruz, sc6556); PROX1 (1:200; Covance, PRB-238C); cleaved-Caspase3 (1:250; Abcam, ab13847) and PDX1 (1:2000; Abcam). Secondary antibodies (1:1000, Molecular Probes) were incubated for 1 hour at room temperature. Nuclei were counterstained with 4’, 6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:10,000, Molecular Probes) for 3 min and mounted with Prolong Gold Antifade Reagent (Invitrogen). Because early liver bud development is dynamic and there is a large amount of growth during these early stages, it is important to note that all control and treated embryos were compared at similar somite stages (within 2 S of each other).

For all images and analysis divided into anterior, middle and posterior, entire transversely sectioned liver buds were divided into three equal domains from anterior to posterior (each domain contained ~4-6 sections) and representative sections from each domain displayed. To calculate the percentage of apoptosis in each liver bud domain, the total cell number (DAPI positive) and cleaved-Caspase3 positive cell number were counted from 2 representative anterior/middle and posterior sections collected from a minimum of 4 treated or control embryos.

### 3.4.3 In Situ Hybridization

Section in situ hybridization was performed as described (Moorman et al., 2001; Nagy et al., 2003) using Fgf2 (Hebert et al., 1990), Fgf8 (Crossley and Martin, 1995), Fgfr1 (Dudley et al., 1999) and Fgfr2 (Orr-Urtreger et al., 1991). The Fgf10, Fgfr4, Afp, Hnf4α and Prox1 probes were made using the primers listed below on E
8.5 embryonic cDNA. RT-PCR was performed and the amplified PCR fragments were then subcloned into pBS-KS or pCRII vectors. The following primers were used on isolated cDNA to create in situ probes and are each listed 5’ to 3’. Fgf10 forward (fwd), GACCGACACCACCATCCTAC, reverse (rev), CAAGCTGATTCCAGGCTATGTCTTT; Fgfr4 fwd, CTCAGCAGCTCCTCGAGTGGTC, rev, TCCTTGTCGGAGGCCATTGTCTTTC; Afp fwd, CCTTCATGTATGCCCCAGCCATTCT, rev, AAAAGGCCGAGAAATCTGCAGTGA; Hnf4α fwd, CTTCGGCATGGCCAAGATTGACAAC, rev, TATAGCCTAGTGCTGGAGGAGGAG; Prox1 fwd, AGATGACGAGAACCAACAGCTGAC, rev, AGATGAGCAGGAAACCAACAGCTGAC.

3.4.4 Data Analysis

All statistical analysis was performed on samples by comparing under the null hypothesis that there are no differences between the control and drug-treated samples with student's t-test. Values are depicted as mean ± standard error. Statistical differences were analyzed by two-tailed student's t-test.

3.5 Results

3.5.1 Examination of Endogenous FGF Signaling Components Before and at the Onset of Liver Specification

As described above, despite the fact that FGF signals are essential for early liver development, it is unclear which of the ligands or receptors are responsible for mediating this activity. If a paracrine FGF signal from an adjacent tissue is involved
in liver bud induction (an event believed to be initiated by 8 S) then the preinduced hepatic endoderm should express an FGFR before and during induction while the candidate FGF should be expressed in the mesenchyme during the same time frame. FGF2, 8, and 10 as well as FGFR1, 2, and 4 have been implicated in liver induction in the mouse (Jung et al., 1999; Kelly et al., 2001; Cai et al., 2003; von Both et al., 2004; Sirbu et al., 2008). We, therefore, carefully examined the expression pattern of candidate FGF ligands and receptors by section in situ hybridization on embryos from 3–13 S. These stages were chosen to represent specific events associated with liver budding. The 3–4 S and 5–6 S embryos represent stages before liver bud induction, the 7–8 S embryos have initiated liver bud induction and the hepatic endoderm has thickened, the 9–11 S embryos have formed the anterior liver bud and those at 12–13 S have also formed the posterior aspects of the nascent liver bud (Fig. 3.1).

Section in situ hybridization demonstrates that Fgf8 mRNA is expressed in both the prospective hepatic endoderm (arrow, Fig. 3.1A) and in the adjacent splanchnic mesoderm between 3 and 4 S (arrowhead, Fig. 3.1A). Between 5 and 13 S, the Fgf8 expression in hepatic endoderm and liver bud is lost, while its expression in the adjacent mesoderm is reduced (arrowhead, Fig. 3.1B–D). No Fgf8 expression is detected in nearby mesoderm at 12–13 S (Fig. 3.1E). Between 3 and 8 S, Fgf10 mRNA is expressed in the mesoderm proximal to the hepatic endoderm (arrowhead, Fig. 3.1F–H). Fgf10 expression is low in the septum transversum mesenchyme (STM) surrounding the liver bud between 9 and 11 S, but is upregulated in this tissue by 12–13 S (Fig. 3.1I–J). No Fgf2 was detected in any tissues surrounding the
ventral foregut (data not shown).

We next examined the expression of the FGF receptors, and found that \textit{Fgfr1} is expressed in the prehepatic endoderm and in the liver bud at all stages examined (3–13 S; Fig. 3.1K–O). \textit{Fgfr1} is also expressed in the mesoderm adjacent to the prehepatic domain from 3–8 S (arrowheads, Fig. 3.1K–M) and increases between 9 and 13 S (arrowheads, Fig. 3.1N,O). The expression of \textit{Fgfr2} in prehepatic endoderm and adjacent mesoderm is barely detectable at 3–4 S (Fig. 3.1P) and increases slightly in the prehepatic/hepatic endoderm between 5 and 13 S (Fig. 3.1Q–T) becoming detectable in the STM between 9 and 13 S (Fig. 3.1S,T). No or weak expression of \textit{Fgfr4} mRNA is observed in a subset of prehepatic/hepatic endoderm cells between 3 and 8 S (Fig. 3.1U–W) and this expression is more robust but patchy between 9 and 13 S (Fig. 3.1X,Y).

To explore the relationship between FGF signaling and hepatic specification, activation of the FGF signaling pathway was examined by investigating the expression of phosphorylated ERK1/2 (pERK) and the onset of the hepatic specification markers \textit{Afp}, \textit{Hnf4α} and \textit{Prospero homeobox 1} (\textit{Prox1}). MEK1 and MEK2 are the protein kinases that govern the intracellular branch of the FGFR signaling pathway responsible for inducing hepatic gene expression (Calmont et al., 2006). MEK1/2 specifically phosphorylates and activates ERK1/2 (Plotnikov et al., 2011) and pERK is active in the prehepatic endoderm (Calmont et al., 2006). Before liver bud induction, pERK is found mainly in the mesoderm adjacent to the prospective hepatic endoderm (arrowhead, Fig. 3.1Z). By 8 S, coincident with the onset of liver induction, pERK is readily detected throughout the prospective hepatic endoderm.
and adjacent mesoderm (Fig. 3.1AA, and Calmont, et al., 2006).

Consistent with other reports, we find that \textit{Afp} is first detected in the hepatic endoderm by 11 S (Fig. 3.1BB), while \textit{Hnf4α} and \textit{Prox1} mRNAs are initially detected in the hepatic endoderm between 12 and 13 S (Fig. 3.1CC–DD and Shiojiri, 1981; Duncan et al., 1994; Sosa-Pineda et al., 2000; Burke and Oliver, 2002). These results demonstrate that the expression of hepatic specification markers is preceded by the expression of both \textit{Fgf8} and 10 in the mesenchyme adjacent to the prehepatic endoderm and by pERK and \textit{Fgfr1} and 2 in the prehepatic and early hepatic endoderm. Taken together these results support a role for FGF signaling in hepatic induction and specification in vivo.

3.5.2 FGF Inhibition Leads to Severe Defects in the Anterior Liver Bud

While it is clear that FGF signals can induce liver-specific gene expression in murine foregut endoderm explants (Jung et al., 1999; Deutsch et al., 2001), we sought to understand the requirements of FGF signaling during liver budding in the context of the whole embryo using the FGFR-specific tyrosine kinase small molecule inhibitor, SU5402 (Mohammadi et al., 1997; Poss et al., 2000; Hoffman et al., 2002; Dell’Era et al., 2003; Izikki et al., 2009; Wandzioch and Zaret, 2009). While others have shown that SU5402 abrogates pERK expression in a quick and stable manner, within minutes after application in the whole embryo (Corson et al., 2003; Calmont et al., 2006) and at least 48 hr after application in explants (Calmont et al., 2006), we sought to confirm its inhibitory response in our culture setting. To confirm that SU5402 abolishes pERK during liver budding, 5–6 S embryos were cultured for 10
hr (to ~11 S) in the presence or absence of the inhibitor. While pERK is equally enriched in the nascent anterior liver bud and the posterior thickened hepatic endoderm in the control DMSO-added cultured embryos (Fig. 3.2A–C), SU5402 treatment abolished nearly all detectable pERK in the liver bud and hepatic endoderm (Fig. 3.2D–F) and throughout the embryo (data not shown).

To examine how loss of FGF activity affected liver bud morphogenesis, embryos were dissected before (3–8 S), during (9–12 S) or after (13–15 S) the onset of liver-specific gene expression and cultured through the liver budding stage (≥19 S) in the presence of SU5402. At the end of culture, section analysis of control cultured and SU5402 treated embryos was used to assess liver bud morphogenesis. Section analysis of control embryos cultured from 5–6 S reveals that under normal culture conditions embryonic development in general and liver bud development in particular is similar to in vivo derived embryos (Fig. 3.2G). Transverse sections through the anterior end of the thickened bud reveal a solid structure that represents the anterior-most aspect of the evaginated liver bud (Fig. 3.2H). Sections through the middle and posterior of the liver bud reveal a hollow bud-shaped structure that is easily identified based on its characteristic shape (Fig. 3.2I,J). Addition of SU5402 to 5–6 S embryos results in embryos that are smaller and sometimes delayed but appear grossly normal (Fig. 3.2K), albeit the presence of anterior head and tailbud defects. Because a high level of FGF4/FGF8 signaling is required for tailbud formation (Hoch and Soriano, 2006; Boulet and Capecchi, 2012) and telencephatic development is reliant on signaling through the FGFRs (Paek et al., 2009), these defects further prove that FGF signaling is reduced after SU5402
Detailed histological analysis of the liver buds produced by the culture of 5–6 S embryos with the FGF inhibitor reveals a consistent phenotype that is present in 90% (67/74) of these embryos (Fig. 3.2L–N). The anterior portion of liver buds treated at this stage are markedly thin and lack the pronounced anterior protrusion that would normally result in a solid anterior evagination from the gut (compare Fig. 3.2H–L). Of interest, despite the profound alterations apparent in the rostral portion of the liver bud, the posterior liver bud is morphologically normal. To gain insight into the temporal requirement for FGF in the anterior liver bud, we performed inhibition experiments on slightly younger as well as increasingly older embryos (Fig. 3.2O). Because addition of 40 µM SU5402 (the concentration used at all other stages) to the culture media of 2–4 S embryos produced extremely delayed and poor overall development, 2–4 S embryos were cultured with 30 µM inhibitor. Even at 30 µM, a significant percentage of the treated embryos were grossly abnormal with only ~20% exhibiting appropriate turning and grossly normal development. Of those with more typical development we found that 100% (9/9) had abnormal anterior liver bud morphology compared with 18% (8/47) of controls. The characteristic loss of appropriate anterior liver bud morphology was found in 94% (32/34) of SU5402 treated embryos at 7–8 S, was less prevalent when administered at 9–10 S (69%; 24/35) and further reduced when the drug was administered at 11–12 S (33%; 8/24). Abrogation of FGF signaling by means of SU5402 no longer elicits a noticeable liver bud phenotype when administered after 13 S. It should be noted that this phenotype could also be found in control embryos
younger than 11 S (2–4 S 11%; 5–6 S, 7%; 7–8 S, 6%; 9–10 S, 2%; Fig. 20). Others have found that ex vivo culture alone can affect the degree of pERK staining compared with freshly dissected embryos (Corson et al., 2003), suggesting that even brief culture can slightly abrogate FGF signaling and provides further support for the hypothesis that a reduction in FGF signaling is responsible for the observed alterations in the anterior liver bud.

3.5.3 FGF Signals are Critical for Anterior Hepatic Specification

The morphological defects observed in SU5402 treated embryos suggest that the anterior portion of the liver bud is more severely affected than the posterior liver bud by an overall reduction in FGF signaling. We next sought to determine whether these morphologically identifiable anterior defects were accompanied by molecular specification defects.

To test the molecular specification of treated embryos, we examined the expression of hepatic genes using both in situ hybridization and immunofluorescence. Afp is one of the earliest and most robust liver markers. In control embryos, serial section analysis reveals that Afp mRNA is strongly expressed throughout the liver bud (Fig. 3.3A–C). In contrast, Afp is expressed in an apparent gradient in liver buds from SU5402 treated embryos. No/weak expression is observed in the anterior while more typical expression levels are observed in the posterior (Fig. 3.3D–F). HNF4α, a transcription factor involved in hepatocyte development (Hayhurst et al., 2001; Parviz et al., 2003) and PROX1, a protein essential for hepatocyte migration (Sosa-Pineda et al., 2000) were examined in
SU5402 treated and control embryos. In control embryos, HNF4α and PROX1 are expressed in the entire liver bud at similar levels (Fig. 3.3G–I,M–O). In SU5402 treated embryos, their expression was graded, with very weak expression in the anterior bud and relatively normal expression in the posterior (Fig. 3.3J–L,P–R). In situ hybridization with probes recognizing Hnf4α and Prox1 are consistent with their immunofluorescence pattern (data not shown), indicating a transcriptional response to SU5402 treatment at these loci.

To further highlight the morphological and molecular defects associated with FGF loss, control (Fig. 3.3S) and SU5402 treated (Fig. 3.3T) embryos were sectioned sagittally. Such analysis clearly shows that while the posterior portion of the treated liver bud has thickened and maintains the normal uniform expression of HNF4α (Fig. 3.3S–S’), the anterior portion of the treated bud is thin and lacks HNF4α expression (Fig. 3.3T–T’). Taken together this data indicates that a global reduction of FGF signaling results in a marked loss/reduction of differentiation markers, specifically in the anterior liver bud.

Considerable evidence suggests that FGF signaling is responsible for cell survival (Miho et al., 1999; Montero et al., 2001; Abu-Issa et al., 2002; Li et al., 2005). The loss of anterior liver bud morphology suggested that the hepatic endoderm had undergone apoptosis. To test this idea, the percentage of apoptotic liver bud cells in SU5402 treated and control embryos was assessed in sections collected from the anterior, middle and posterior of control and treated liver buds using cleaved Caspase-3 as a marker for apoptosis (Fig. 3.3U–Z). While control embryos display a uniformly low level of apoptosis throughout the liver bud, SU5402 treated embryos
display significantly elevated levels in the anterior and middle liver bud (Fig. 3.3AA). Furthermore, apoptosis levels within the SU5402 treatment group are significantly higher in anterior sections compared with posterior sections, highlighting the fact that SU5402 treatment results in an A/P gradient of cell death in the liver bud (Fig. 3.3AA).

3.5.4 MEK1/2 Inhibition Produces Hepatic Defects Similar to FGFR Inhibition

U0126 is a highly selective MEK1/2 small molecule inhibitor that prevents liver-specific gene expression from foregut derived endoderm/mesoderm explants (Calmont et al., 2006; Wandzioch and Zaret, 2009). We cultured 5–6 S embryos with U0126 through ~22 S. Similar to SU5402 treated embryos, U0126 treated embryos are smaller than controls (Fig. 3.4A,E) and section analysis of control and U0126 treated embryos (Fig. 3.4B–D,F–H) reveals that 79% (53/67) of the treated embryos failed to form normal anterior liver bud structures (Fig. 3.4I).

To study whether MEK1/2 is also required to initiate specification markers in the liver bud, we examined the expression of these markers along the A/P axis of U0126 treated embryos. Similar to that observed after SU5402 treatment, the expression of Afp in the liver bud of U0126 treated embryos is low or absent in the anterior region but much higher in the more morphologically normal posterior liver bud (Fig. 3.5A–F). HNF4α and PROX1 expression is similarly restricted to the posterior most sections of treated liver buds but is found throughout the liver buds of controls (Fig. 3.5G–R). In situ hybridization with Hnf4α and Prox1 probes confirm the same pattern (data not shown). To highlight the posterior distribution of the
liver bud markers, U0126 treated and control cultured embryos were sectioned sagittally and expression of HNF4α and the endodermal marker FOXA1 examined (Fig. 3.5S,T). HNF4α is normally expressed throughout the morphologically identifiable liver bud (Fig. 5S″), but is excluded from the adjacent FOXA1-positive gut tube (Fig. 3.5S′). With U0126 treatment, HNF4α is confined to the posterior most region of the liver bud evagination and is absent from the anterior portion of the liver bud (Fig. 3.5T–T″).

To gain a better understanding of how loss of MEK/ERK activity affects cell viability, apoptosis was assessed in the U0126 treated and control cultured embryos using cleaved Caspase-3 as a marker of cell death (Fig. 3.5U–Z). Similar to SU5402 treatment, apoptosis was significantly increased throughout the liver bud of U0126 treated embryos compared with controls (Fig. 3.5AA).

3.5.5 Ventral Pancreas Induction After FGF Inhibition

Murine explant studies have suggested that FGF signals pattern the adjacent ventral foregut endoderm, in a concentration-dependent manner, into lung, liver, and pancreas (Deutsch et al., 2001; Serls et al., 2005). Because lung buds are not yet evident in the majority of our cultured embryos, we focused our studies on how a dramatic reduction in FGF signaling affected ventral pancreas development. Pancreatic and duodenal homeobox 1 (Pdx1), an early essential ventral pancreas marker, is not expressed when ventral foregut explants are provided with FGF, but is detected when ventral foregut explants are cultured in the absence of FGF (Deutsch et al., 2001). To determine how loss of FGF signaling affects pancreas
development in the context of the whole embryo, we examined the morphometric and molecular characteristics of the ventral pancreas in SU5402 treated and control embryos. Using PDX1 as a marker of induced ventral pancreas, no obvious change in size or position of the ventral pancreas bud was observed when comparing control to treated embryos, however a consistent increase in the intensity of PDX1 expression is apparent (compare Fig. 3.6A to D and Fig. 3S′ to T′). Despite the fact that the treated embryos are smaller, as demonstrated herein by the shortened length of the FOXA1-positive gut tube, the number of PDX1-positive ventral pancreas cells as a percentage of all foregut endoderm cells is significantly increased in treated embryos (Fig. 3.6G).

Because alterations in pathways controlling liver specification in zebrafish often affect pancreas size (Chung et al., 2008; Goessling et al., 2008; Poulain and Ober, 2011; Nissim et al., 2014), we next determined if the relative size of the pancreas bud was also altered after abrogating FGF signals. The ratio of PDX1-positive ventral pancreas sections to the number of liver and ventral pancreas sections does not differ significantly between control and treated embryos, indicating that the pancreas bud occupies the same relative amount of foregut in both treated and control embryos (Fig. 3.6H). These results demonstrate that the loss of FGF signaling results in a ventral pancreas with a higher density of PDX1-positive cells.

3.6 Discussion

The newly emergent endoderm is an epithelial sheet that has not yet been
specified into organ domains. As development progresses, domains of overlapping transcription factors are believed to initiate formation of the foregut, midgut and hindgut along the A/P axis (Sherwood et al., 2009; Zorn and Wells, 2009). Within the context of this regionalization, instructive and permissive signals from adjacent mesoderm is thought to pattern endoderm and specify the foregut endoderm toward thyroid, lung, liver, and pancreas fates. Consistent with this hypothesis, isolated foregut endoderm will not initiate liver-specific gene expression, however liver-specific gene expression can be induced by the co-culture of foregut endoderm with FGF (Jung et al., 1999; Deutsch et al., 2001; Serls et al., 2005). Herein we show that abrogation of FGF signaling in the context of the whole embryo leads to alterations in both liver bud morphogenesis and gene expression. In agreement with the described role for FGF signaling in the induction of liver gene expression, our results demonstrate that a reduction of FGF signaling has severe molecular and morphological consequences on the anterior-most portion of the liver bud. However, in contrast to the prevailing hypothesis, we find that abrogation of FGF signaling has little impact on morphogenesis and specification of the posterior liver bud. Taken together, our results are the first to demonstrate that the requirement for FGF signaling in liver bud induction varies along its A/P axis.

Although it is clear that FGF is important in many aspects of liver development, the exact ligand and receptor combination used for liver bud induction in the mouse during normal development is still unknown. To assess which FGF signaling components are expressed in the prehepatic endoderm and early liver bud domain during embryogenesis, and thus which are candidates for
generating the signals used in vivo, we examined all FGFs or FGFRs that have been implicated in liver development. Unlike FGF1 or 2, which are not required in the liver in vivo (Miller et al., 2000), FGF8 and 10 are two ligands that display knockout phenotypes and expression patterns consistent with their involvement in liver induction and morphogenesis.

Consistent with previous description, we find that Fgf10 is expressed in the cardiac mesoderm adjacent to the prospective hepatic endoderm domain during all stages examined (Fig. 3.1F–H and Kelly et al., 2001; Cai et al., 2003; von Both et al., 2004). While Fgf10 null animals form a liver, they exhibit a four-fold increase in hepatic apoptosis (Berg et al., 2007). Fgf8 is strongly expressed in the cardiac mesoderm adjacent to the hepatic endoderm domain (Fig. 3.1A–D and Crossley and Martin, 1995; Jung et al., 1999; Ilagan et al., 2006) and although a crucial role for Fgf8 during earlier development precludes analysis of its role in the liver, Fgf8 is involved in cell migration and survival in other systems (Sun et al., 1999; Ilagan et al., 2006). An examination of Fgfr expression reveals that Fgfr1, 2, and 4 are each present in both the prehepatic and hepatic endoderm, consistent with a potential role during the earliest phases of liver development (Fig. 3.1K–Y).

Genetic manipulation of a variety of signaling pathways in zebrafish highlights a reciprocal linking of foregut endoderm fate: an increase in liver size is accompanied by a decrease in pancreas size and vice versa (Chung et al., 2008; Goessling et al., 2008; Poulain and Ober, 2011; Naye et al., 2012; Nissim et al., 2014). Because FGF initiates reciprocal fates in murine foregut endoderm explants, promoting liver and excluding pancreas gene expression when added while
excluding liver and promoting pancreas gene expression when absent (Deutsch et al., 2001; Serls et al., 2005), the zebrafish and mouse data support the hypothesis that loss of FGF in the mouse embryo would cause the ventral foregut endoderm to induce a larger ventral pancreas at the expense of the liver bud. Indeed, when examined at the level of expression, loss of FGF signaling reduces the number of cells that express liver-specific genes and increases the number of PDX1 expressing cells.

However, the simple model of expanding one organ progenitor domain at the expense of the other due to a fate switch does not appear to be the case. Instead our findings demonstrate that loss of endogenous FGF signaling in the whole embryo results in the emergence of a normal sized liver bud with appropriate molecular specification restricted to the posterior end, while the normal sized ventral pancreas contains a higher density of PDX1-positive cells than control embryos. Instead of a fate switch, we propose two alternative, nonmutually exclusive hypotheses that can explain how loss of FGF affects the murine ventral pancreas. First, it is possible that loss of FGF leads to an increase in proliferation of PDX1-positive cells and, second, it is possible that FGF abrogation leads to a premature FGF dependent terminal differentiation. Both hypotheses warrant further investigation into the role that FGF plays in pancreas progenitor expansion/differentiation in the context of the embryo.

A novel finding in this report is that FGF signaling is differentially required along the A/P axis of the liver bud. Reduced FGF signaling diminishes the ability of the anterior liver bud to undergo appropriate morphogenesis and differentiation.
but does not similarly affect posterior bud formation. An intriguing question that remains unanswered is: how is this differential requirement generated? The FOXA family of winged helix transcription factors are co-expressed throughout the foregut endoderm and are essential for liver bud emergence in particular (Ang et al., 1993; Lee et al., 2005) by binding to critical liver-specific genes and acting at the chromatin level to prime the foregut endoderm for later inductive events (Zaret and Carroll, 2011). Neither FOXA1 nor FOXA2 levels are altered by FGF inhibition (Fig. 3.5T and data not shown), indicating that FGF signals are not required for appropriate pioneer factor expression in the liver bud.

Because the cardiac mesoderm has been proposed to be the main source for FGF signals and because the anterior liver bud is closest to the developing heart, one might postulate that the anterior liver bud receives the highest endogenous levels of FGF. Despite our efforts, we have not been able to discern differences in the expression of individual FGF signaling components along the A/P of the prehepatic endoderm or nascent liver bud (data not shown). Furthermore, all investigation of endogenous pERK demonstrates that FGF signaling is uniformly distributed throughout the prehepatic and hepatic endoderm during normal liver development (Figs. 1AA, 2A–C; Corson et al., 2003; Calmont et al., 2006) and uniformly lost after SU5402 or U0126 inhibition (Fig. 3.2D–F and Corson et al., 2003; Calmont et al., 2006). Thus it appears that while the anterior liver bud is much more affected by FGF loss than the posterior liver bud, this differential response cannot be explained by the distribution of the candidate FGF ligands, receptors or the downstream mediator pERK.
Another explanation for the requirement of FGF in the anterior but not posterior liver bud is that the liver progenitors within the endodermal sheet have acquired an A/P pattern before liver bud induction, that promote a differential response to external cues along this axis. The entire early organ forming endoderm has been examined in the context of the embryo to reveal the existence of distinct transcription factor domains that may provide a unique transcriptional signature to specific regions along the A/P axis of the foregut endoderm (Sherwood et al., 2009). Because these transcription factor domains exceed the number of endoderm derived organs (Sherwood et al., 2009), one possibility is that the liver precursors acquired a pattern that promotes a differential transcriptional response to FGF signals. In support of such a hypothesis, we have shown that the liver bud is produced by both the VMEL, located in the ventral midline of the foregut endoderm, and by two flanking lateral domains of endoderm in the open foregut (Tremblay and Zaret, 2005; Angelo et al., 2012). The lateral domains flank the VMEL in the closing foregut just before the onset of liver-specific gene expression. The VMEL produces a large proportion of the anterior-most liver bud, while its contribution to the remainder of the liver bud is restricted to a narrow stripe of cells within the ventral midline (Tremblay and Zaret, 2005; Angelo et al., 2012). In contrast, the lateral domains give rise to the bulk of the posterior portions of the liver bud (Angelo and Tremblay, 2013). An interesting hypothesis is that the VMEL/anterior liver bud requires FGF signaling for its induction, while lateral/posterior liver bud induction requires a separate signal. An obvious candidate for this separate signal includes the BMP pathway and studies investigating this hypothesis are ongoing. Because the
mature liver is capable of regeneration in response to multiple cues (Mao et al., 2014), it is intriguing to hypothesize that some of this plasticity is acquired during the earliest stages of liver development in the form of multiple inductive cues that are each capable of producing fully functional yet differentially responsive progenitors.

Finally, herein we find that FGF signaling is required for both normal morphogenesis and induction of gene expression in the murine liver bud in a spatially restricted manner. We and others have shown that endoderm morphogenesis and transcriptional differentiation are coupled (Loebel et al., 2011), underscoring the importance of studying complex morphogenetic processes such as liver and pancreas budding within the context the developing embryo.
CHAPTER 4

Loss of YY1 from the murine endoderm reveals a VEGF-dependent paracrine signaling mechanism required to maintain the liver bud in a temporally and spatially distinct manner.

4.1 Hypothesis

Given the previous findings: that YY1 is essential for yolk sac angiogenesis via the VEGF pathway, and that two distinct liver cell populations exist and are differentially affected by surrounding signals, we have amended our former hypotheses and propose two new ones as our working models. First, we assert that YY1 within the DE is essential for liver development via the VEGF pathway. Second, we hold that two distinct populations of liver cells exist and respond differentially to the loss of YY1 and the VEGF pathway. To validate these claims and complete my project, we used an inducible Cre-ER to create a YY1 cKO specifically in the definitive endoderm. YY1 mutant analysis, whole embryo culture, and liver bud explant culture revealed that YY1 is essential for VEGF production in the liver and subsequent hepatic angiogenesis, suggesting that the role of YY1 in the VEGF pathway is conserved within both the VE and DE. Histology, Dil fate mapping, and laser ablation experiments confirmed not only the existence of two distinct liver populations with differential morphogen requirements, but also that the anterior and posterior liver buds contribute to the caudal and rostral liver lobes respectively,
thus creating a new model with which to study liver organogenesis and regeneration.

4.2 Abstract

Yin Yang1 (YY1) is a ubiquitously expressed factor that plays multiple roles in early mouse development. We previously demonstrated that an essential role of YY1 in the visceral endoderm (VE) of the yolk sac is the maintenance of VEGF. Furthermore we found that paracrine signals downstream of VEGF support the VE, including expression of HNF4α. We use an inducible-knockout strategy to demonstrate that YY1 is essential in the definitive endoderm (DE) for invasion of hepatoblasts into the surrounding mesenchyme. By E14.5 YY1-/- livers are severely hypoplastic. Careful analysis of early normal liver bud development reveals two temporally and spatially discreet waves of liver bud invasion. The first, initiated at E9.25, involves migration of the posterior liver bud while the second, initiated by E10.0, involves the anterior liver bud. Lineage analysis demonstrates that the anterior and posterior regions of the liver bud contribute to caudal and rostral lobes, respectively, of the E10.5 liver. Although YY1-deficient DE is capable of liver bud specification, the hepatoblasts of the posterior liver bud and then those descended from the anterior liver bud fail to initiate each invasion event, upregulate VEGF or maintain HNF4α. The addition of exogenous VEGF to YY1-deficient liver buds rescues HNF4α expression, while addition of a VEGFR inhibitor to wild-type embryos mimics the specific temporal and spatial phenotypes of the YY1-/-
hepatoblasts. Taken together, these studies demonstrate that a conserved role of
YY1 in both the VE and liver bud is the production of VEGF. Active VEGF signaling
promotes a mesoderm-derived paracrine signal that is required to maintain HNF4α
expression in both the VE of the yolk sac and in the hepatoblasts of the liver bud.
Finally, these studies are the first to document that there are two spatially and
temporally discreet waves of liver bud invasion that each contribute to distinct
lobes.

4.3 Introduction

The definitive endoderm (DE) is the embryonic germ layer that gives rise to
the entire gut tube including the liver, lungs and pancreas. The DE arises at
embryonic day (E) 7.75 as a relatively unpatterned epithelial sheet that covers the
ventral aspect of the developing conceptus. Fate mapping strategies have been used
to identify the liver precursors in the endodermal sheet. By E8.25, an embryo with
2-8 somites, the liver precursors exist as two spatially distinct populations: a
bilateral precursor population that is present in the right and left lateral endoderm
and a second small precursor population that is present in the ventral midline of the
endoderm lip (VMEL). By E8.5 the two precursor populations converge at the
ventral midline of the closing foregut and initiate the morphogenetic and molecular
processes that herald the initiation of liver development- liver bud formation.

Numerous studies in a variety of organisms have demonstrated that secreted
factors from surrounding mesoderm derivatives are essential for the early stages of
liver development including its induction from the endoderm, invasion of the liver
bud into the surrounding mesenchyme and its subsequent growth (Wells & Melton 2000a; Zorn & Wells 2009; Arterbery & Bogue 2013). Cardiac mesoderm, septum transversum mesenchyme (STM) and endothelial cells are the 3 mesoderm-derived tissues that have been implicated in the earliest stages of liver development. Cardiac tissue is believed to be essential for liver bud induction, the STM has been implicated in liver bud growth and the loss of endothelial cells results in a failure of the liver bud to invade the surrounding STM. While both BMP and FGF signaling has been implicated as the secreted factors that govern these early stages, the exact nature and the exact source of the endogenous ligands has yet to be identified in mammals.

YY1 is a ubiquitously expressed Kruppel-like zinc finger protein that acts as a context dependent transcriptional activator or repressor (Thomas & Seto 1999). YY1 knockout embryos are perimplantation lethal and warranting the use of a conditional allele to study the role of YY1 during postimplantation development (Mary et al. 2012). A conserved role for YY1 during early development is in the generation of essential paracrine signals. For example YY1 in the developing oocyte is required to generate the secreted signals necessary for development of the surrounding follicle cells, in the epiblast YY1 regulates Nodal and epithelial-to-mesenchymal transition (EMT) in the primitive streak and YY1 is required to maintain VEGF levels in the visceral endoderm of the yolk sac that induce angiogenesis in the adjacent yolk sac mesenchyme. Under stress, such as exercise induced skeletal muscle injury, a role for YY1 in mitochondrial biogenesis has been established. One way that YY1 may accomplish such a diversity of roles is through
its role as a DNA binding protein in a larger chromatin-modifying complex. Indeed the DNA binding activity of the Drosophila YY1 homologue is a critical component of the drosophila polycomb repressive complex (PRC) and the mammalian YY1 can rescue drosophila (Basu et al. 2014).

Recently, interesting roles for YY1 in the liver and its precursors have been forged. YY1 is essential in the definitive endoderm for its initial migration into the ventral surface of the developing embryo and but is not required in the endoderm for normal liver bud induction. In zebrafish and high-fat diet fed mice, overexpression of YY1 in the liver promotes hepatic steotosis while downregulation of YY1 in hepatocytes of obese mice ameliorated triglyceride accumulation (Lu et al. 2014). Similarly, YY1 overexpression in hepatocytes promotes gluconeogenesis during fasting and while downregulation in the liver has apparent contrasting activities. YY1 knock-down ameliorates hyperglycemia in WT and diabetic mouse models, while heterozygosity in post-natal hepatocytes leads to insulin resistance and hepatic lipid accumulation (Lu et al. 2014).

To parse out the role of YY1 on early liver development, we employed an inducible knockout (cKO) approach utilizing the FoxA2<sup>mcm</sup> line. Like that reported earlier using a different Cre line, we find that YY1 is not required for liver bud induction, specification or early bud morphogenesis. We find that YY1 is essential for migration of the liver bud into the surrounding mesenchyme and for maintaining HNF4α expression. Non-uniform loss of HNF4α in cKO liver buds facilitated the recognition of two spatially and temporally discreet waves of liver bud invasion in WT animals. The first wave involves the posterior liver bud, which invades the STM
and forms the anterior liver lobe, while the second wave involves the anterior portion of the liver bud that appears to migrate along the sinus venosus (SV) to invade the non-parenchymal portion of the posterior/dorsal lobes. Furthermore, we demonstrate that the onset of each invasion event is coincident with expression of VEGF from the hepatoblasts and maturation of the accompanying hepatic vasculature. YY1 -/- hepatoblasts fail to invade, express normal levels of VEGF or maintain HNF4α expression and these defects coincide with a reduction in hepatic vascular maturation. The addition of a VEGFR inhibitor to WT embryos mimics the YY1 cKO phenotype and exogenous VEGF stimulates HNF4α expression from YY1 -/- hepatoblasts, establishing a critical role for hepatoblast-derived VEGF in generating the YY1 cKO phenotype. Taken together we have shown that liver bud invasion occurs in two temporally and spatially distinct phases and that YY1 is required to maintained hepatoblast fate at the onset of each invasion event by promoting a VEGF-dependent paracrine signaling loop with the underlying mesenchyme.

4.4 Materials and Methods

4.4.1 Ethics Statement

All animal studies were approved by the Institutional Animal Use and Care Committee, University of Massachusetts, Amherst protocol #2012-043.

4.4.2 Mouse Breeding Scheme and Genotyping

Females homozygous for the Yy1 floxed allele (Yy1fl/fl) were mated to males heterozygous for a Yy1 null allele (Yy1+/-; ) and for the Foxa2mcm/+ allele (Park et al.
to obtain \( Yy^{1/\Delta}; FoxA2^{mcm/+} \) embryos. The morning of the copulation plug was defined as 0.5 dpc. Tamoxifen (TM) was administrated with 0.1mg/g dosage at 7.75 dpc. After obtaining embryos at the desired stage, a portion of the yolk sac or embryo was used for PCR genotyping using the primers of \( Yy1:5'ACCTGGTCTATCGAAAGGAAC3', 5'GCTTCGGCTATTCCTCGCTCA-3' \) and \( 5'CCAAAGTTCGAAACCTGCTTTC3';: FoxA2 5'CTCAAGGGAGCAGTCTCA3', 5'ATACTATCTAG-AGAATAGGAACTTCG3' \) and \( 5'GACTTTTCTGCAACAACAGCA3' \).

\( Yy1^{1/\Delta}; FoxA2^{mcm/+} \) embryos are referred to as “mutants” or “cKO” and other genotypes referred to as “wild-type” (WT) for simplicity.

### 4.4.3 Histology, Immunofluorescence and Immunohistochemistry

Embryos were dissected and fixed in 4% paraformaldehyde (PFA)/PBS overnight at 4°C. The following day they were washed in PBS-Tween (PBT), dehydrated in an ascending methanol sequence, xylene treated, embedded in paraffin and sectioned at 7 µm. For immunofluorescence (IF) slides were dewaxed in xylenes, rehydrated with EtOH, and subjected to antigen retrieval in Tris buffer pH 10.0 for 10 min. The slides were then washed in PBT and incubated in blocking buffer (0.5% milk powder, 99.5% PBT) for 2 hrs at room temperature and then with primary antibody in blocking buffer at 4°C overnight in a humid chamber. Slides were then washed three times with PBT, incubated for 1 hr with secondary antibody in blocking buffer at room temperature. Nuclei were counterstained with 4’, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, 1:10,000) for 3 min and then coverslipped with Prolong Gold Antifade Reagent (Invitrogen).
Sections were imaged on a Nikon Eclipse TE2000-S inverted microscope with Retiga EXi Fast camera with NIS Elements imaging software. Primary antibodies used include: rabbit anti-YY1 [1:100, Santa Cruz (sc-1703)]; goat anti-HNF4α [1:100, Santa Cruz (sc6556)]; mouse anti-CDH1 [1:500, BD Bioscience (610811)], rabbit anti-cleaved Caspase-3 [1:500, Abcam (ab13847)], rabbit anti-VEGFA [1:100, Santa Cruz (A120)], guinea pig anti-PDX1 [1:1000, Abcam (ab47308)], rabbit anti-PECAM [1:500, Abcam (ab28364)], goat anti-FLK1 [1:250, R&D System (AF644)], rabbit anti-PROX1 [1:1000, Angiobio (11-002P)], mouse anti-ISL1 [1:1000, Hybridoma Bank (40.2D6)], mouse anti-NGN3 [1:1000, Hybridoma Bank (F25A1B3)], rabbit anti-SOX9 [1:2000, Millipore (AB5535)], and mouse anti-FOXA1 (1:1000, Seven Hills (WMAB-2F83)]. Secondary antibodies (Molecular Probes) were used at 1:500. For LacZ staining, all embryos double heterozygous for the R26R allele and FoxA2-Cre ER allele were stained and processed as reported (Park et al. 2008). For LacZ staining of WT and cKO livers at E14.5, liver and other internal organs were dissected together from embryos and LacZ-stained following same staining protocol. Then tissues were dehydrated and paraffin embedded to generate slides. Sectioned tissues were counterstained with Eosin for 5 min before coverslip. The tissues were imaged on either a Nikon Eclipse TE2000-S inverted microscope with a Retiga EXi Fast camera or a Nikon Eclipse Ti inverted microscope with an Andor DR-228C camera. Both microscopes use NIS Elements imaging software.

### 4.4.4 Blood Vessel Measurements

Blood vessel density was assessed by obtaining the average of all vessels
counted from the same total area from 3 WT and 3 mutant embryos using the NIS Elements line measurement tool. Hepatic blood vessel types were assessed in serial sections after staining using the following criteria: Type I: with no surrounding hepatoblasts, Type II: vessel composed of ECs partially surrounded by hepatoblasts; Type III: vessels fully surrounded by hepatoblasts. IF with PROX1 and FLK1 antibody was used to access the hepatoblasts and endothelial cells. The percentage and distribution of these three categories were assessed by counting each blood vessel type.

4.4.5 Apoptosis

Endoderm derived organ buds including the anterior and posterior liver, gall bladder primordium and ventral and dorsal pancreas from 4 WT and 5 cKO from E9.75, 10.0 and 10.25 embryos were subjected to IF using either cleaved Caspase-3/HNF4α/CDH1/DAPI or Caspase-3/FOXA1/PDX1/DAPI to assess apoptosis. HNF4α and FOXA1 were used to identify the liver bud, PDX1 for ventral and dorsal pancreas and DAPI used to distinguish individual cells. A total of 2–3000 cells were counted from 10–12 independent fields for each data point. Image-J was used to assist with counting.

4.4.6 Statistical analysis

All statistical analysis performed on these samples and elsewhere were performed by comparing averages under the null hypothesis that there are no differences between WT and cKO samples with the student’s T-test. Statistical
differences were measured by two-tailed Student’s t test analysis of variance.

4.4.7 Whole Embryo Culture

Litters were dissected at E9.25 in warmed and equilibrated (one hour in a 5% O2, 5% CO2, 37°C incubator) dissection media [DM: 10% Fetal bovine serum, 90% DMEM (Lonza, 12-709)] under a Nikon SMZ1500 or Nikon SMZ1000 dissection microscope equipped with a 37°C stage, as previously described (Angelo et al. 2012 or Wang et al. 2015). SU1498 (Millipore) was diluted in DMSO and added directly to the culture media to obtain 40 µM [SU1498], while an equal amount of DMSO-alone was added to the controls. Embryos were cultured in roller bottles in 1.5 ml culture media [CM: 75% rat serum (Valley Biomedical, AS3061), 25% DMEM (Lonza, 12-614) supplemented with Pen/Strep (Gibco), non-essential amino acids (Lonza) and GlutaMAX (Gibco)]. Media was initially equilibrated to 5% O2, 5% CO2, 37°C, then re-gassed with 20% O2, 5% CO2. Embryos were cultured for ~30 hrs, unless otherwise noted and bottles were re-gassed at least once during the culture period.

4.4.8 Embryo Dil labeling

Dil labeling was performed (CM-Dil, Molecular Probes) as described previously (Angelo et al., 2012). The position of the initial label was recorded manually and documented using epifluorescence and bright field images produced using a MicroPublisher 5.0 RTV camera and QImaging software. After ~27 hours in roller culture, as outlined above, embryos were dissected from visceral tissues, developmental progress noted and epifluorescence and bright field images were
taken. All were then fixed in 4% paraformaldehyde, sectioned sagittal, counterstained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, 1:10,000) and labeled immunofluorescently with antibodies against HNF4α and ISL1 to identify hepatoblasts and the sinus venosus (which the anterior liver bud migrates toward), respectively.

4.4.9 Liver bud explant culture

WT and cKO embryos at E9.75 were dissected as described above. Because the cKO embryos are not apparent at the onset of culture, litters were blindly divided into VEGF treatment groups and the yolk sac reserved for genotyping. The liver bud region of each embryo was carefully dissected out with sharpened forceps. Tissues were transferred onto a 4-well dish (Nunc) containing 500 ul of CM. Explant cultures were established and allowed to grow for 72 hours in a 5% O2, 5% CO2, 37°C incubator, during which time they were photographed. VEGF [Peprotech (100-20A)] was diluted in nuclease-free water (IDT11-05-01-14) and added directly to the culture media to obtain 200 ng/ml (VEGF), while an equal amount of water was added to the controls. After 24 hours culture all explants were photographed and processed for histology. For IF, cultured liver explants were rinsed in PBS and then blocked in PBS-0.1% Triton X-100 for 30 min. Tissues were then incubated overnight at 4°C in blocking buffer as above containing primary antibodies. After three washes in PBS, dishes were incubated for 1 hr in blocking buffer containing primary antibodies. After IF, tissues were imaged on a Nikon Eclipse Ti inverted microscope with an Andor DR-228C camera.
4.5 Results

4.5.1 Loss of YY1 in DE has profound affects on the liver bud

Conditional deletion of the YY1 floxed allele using the FOXA3-Cre line resulted in lethality by E10.5 due to a failure in yolk sac angiogenesis (Lee et al. 2005b; Rhee et al. 2013). FOXA3-Cre drives Cre-recombinase expression in the VE of the yolk sac beginning at E7.5 and a complete loss of YY1 in the VE is found by E8.5 while a complete loss of YY1 in the DE occurs between E8.75-9.0. Initial analysis of the liver buds from these animals revealed that while the liver buds are normally induced and express the typical repertoire of early liver bud markers including HNF4α and PROX1, they fail to maintain hepatoblast fate and appropriately invade the surrounding mesenchyme [(Rhee et al. 2013) and data not shown].

To examine the affect that loss of YY1 plays in DE organogenesis without the confounding yolk sac defect, we used the FoxA2<sup>mcm</sup> allele which allows for tamoxifen (TM) inducible deletion (Park et al. 2008) of the floxed YY1 allele (Wu et al. 2009). By crossing the FoxA2<sup>mcm</sup> mice with the R26<sup>LacZ</sup> reporter animals (Soriano 1999), we confirmed that administration of TM at E7.75 maintains the DE expression of Cre while bypassing its earlier expression in the VE (Fig. 4.7 and (Park et al. 2008)). We examined FoxA2<sup>mcm</sup>/+, YY1<sup>Δ/+</sup> embryos (hereafter termed mutant or cKO) during early liver bud invasion stages. At E9.75, the WT liver bud has invaded the STM and maintains robust expression of HNF4α, while the YY1<sup>-/-</sup> liver bud fails to migrate or to maintain normal levels of HNF4α (Fig. 4.1A, C). Like the VE of the yolk sac, the DE of the liver bud is closely associated with the developing vasculature. By E10.5 the
liver bud has expanded in part due to the rapid growth of the developing hepatic vasculature (Fig. 4.1B, B’). The developing vasculature of the YY1 cKO liver buds at this stage is disrupted (Fig. 4.1D, D’). The cKO livers contain significantly fewer vessels per liver when compared with WT (Fig. 4.1E). Because we noted that the cKO hepatoblast appeared to associate differently with the forming vasculature, we sought to quantitate that association. During WT morphogenesis at E10.5 most vessels (>60%) are surrounded by hepatoblasts (Type III), while only 15% of vessels have no direct interaction with hepatoblasts (Type I) and 25% of vessels are partially surrounded by hepatoblasts (Type II). In livers containing cKO hepatoblasts, the hepatoblasts had less contact with the vasculature compared with WT, with a significant decrease in Type III vessels (12%) accompanied by a significant increase in Type II and Type I vessels (60% and 28% respectively). By E14.5 the cKO embryos have hypoplastic livers that are significantly smaller than those of WT embryos (Fig. 4.1H-L).

4.5.2 The Anterior and Posterior Liver bud Contribute to Distinct Lobes at E10.5

Recent evidence supports the hypothesis that the anterior and posterior liver bud, each produced by distinct endodermal precursors, have differential molecular requirements for liver budding (Tremblay & Zaret 2005b; Angelo et al. 2012; Angelo & Tremblay 2013; Wang et al. 2015). Differences in the YY1 mutant liver bud along the anterior/posterior axis supported this hypothesis and, because these differences distinguished the liver lobes by E10.0, suggested that the anterior and posterior
liver bud contributed to distinct lobes. We hypothesized that these regionalized differences, made apparent with genetic perturbation, could also be observed in WT embryos in the absence of perturbation.

To determine if the anterior and posterior liver bud could be distinguished in vivo and to begin assessing if the two domains contributed differently to the embryonic liver, we initiated a histological and immunofluorescence study of the early WT liver budding stages (E9.5-10.5) using sagittal section analysis, which facilitates direct comparison of the anterior and posterior liver bud by allowing simultaneous views of the both domains. In combination with supporting histological evidence (Fig. 4.8) and using SOX9 as an STM marker and ISL1 as a marker of the secondary heart field (Fig. 4.2A-B, F-G), we find that the anterior and posterior liver bud are in contact with distinct mesenchyme populations at E9.5 (Fig. 4.2A). The posterior liver bud (outlined by blue dashed line henceforth, Fig. 4.1A) is surrounded by the STM, which is labeled at the periphery with SOX9. On the other hand, the anterior liver bud (outlined by yellow dashed line henceforth, Fig. 4.2A) is bounded anteriorly by the sinus venosus (SV) and laterally by the ISL1 positive secondary heart field. By E9.75 the invasion and growth of the posterior liver bud into the surrounding STM is observed (Fig. 4.2 B), as documented throughout the literature. At this stage we find that the anterior liver bud, which continues to be associated with the SV and the ISL1-positive secondary heart field migrates toward a distinct mesenchyme. This mesenchyme, which we now term the caudal lobe mesenchyme (CLM) is easily identified by its distinctive surrounding epithelium. Between E 10.0 and 10.25 the anterior liver bud-derived hepatoblasts
infiltrate the CLM (Fig. 4.2C-D). Frontal section analysis supports the observations made above, including the obvious presence of the CLM prior to anterior liver bud invasion, and highlights the fact that the hepatoblasts derived from the posterior liver bud invade the STM that forms the central rostral liver lobe, while the hepatoblasts from the anterior liver bud infiltrate the CLM which forms the right and left caudal lobes (Fig. 4.2F-I).

Section analysis of a mid-sagittal and left-sagittal view highlights two cogent points (Fig. 4.8)- including that the mid-sagittal views hide the liver bud non-parenchymal cells that form the caudal/dorsal lobes are present prior to hepatoblast invasion. Frontal sections produced at these same stages reveals similar details and highlight the fact that between E9.75 and E10.0 the rostral liver parenchyma is organized in one large lobe, while the caudal lobes are found on the left and right sides and demonstrates some asymmetry with the right side occupying more space than the left side (Fig. 4.8), presumably due to the presence of the stomach on the left side.

To directly test the hypothesis that the posterior liver bud gives rise to the rostral liver lobe, and that the anterior liver bud gives rise to the caudal/dorsal lobes, we performed two complimentary experiments. The first approach includes DiI labeling followed by embryo culture (Tremblay & Zaret 2005b; Angelo et al. 2012; Angelo & Tremblay 2013) and takes advantage of the fact that the two liver precursor populations give rise to the anterior or the posterior of the liver bud. In this approach, either the lateral progenitors, which primarily produces descendants in the middle and posterior portion of the liver bud (Tremblay & Zaret 2005b;
Angelo & Tremblay 2013), are Dil (red) labeled or the VMEL progenitors, which shown primarily give rise to a large proportion of hepatoblasts in the anterior liver bud (Tremblay & Zaret 2005b; Angelo et al. 2012), are Dil labeled. We labeled 8-10S embryos in the posterior liver bud precursors and cultured them for 24-36 hours. All 5 embryos that were labeled in the posterior liver bud produced descendants that were found exclusively in the rostral lobe (Fig. 4.2. O). As previously reported, embryos labeled in the VMEL position do not develop as well as embryos labeled in any other region of the endoderm (Tremblay & Zaret 2005b) however no embryos labeled in the VMEL contributed to the upper lobe, rather they all remained either in the midline or moved to the lower lobe position (Fig. 4.2). To further test the hypothesis that the anterior and posterior liver bud give rise to distinct lobes, we used laser ablation to destroy the posterior liver bud precursors on either the right or the left side (Fig. 4.8). All ablated embryos (n=3) displayed smaller rostral liver buds on the ablated side compared with the intact side. Unlike the lateral liver bud, VMEL ablations, which are much more discreet, result in no observable changes in the liver bud (data not shown), suggesting that these ablations result in quick repair. Taken together, these observations in WT embryos support the novel hypothesis that the anterior and posterior portions of the liver bud give rise to distinct liver lobes.

4.5.3 YY1 is required in the liver bud for HNF4α maintenance

Because the early YY1cKO liver phenotypes, including loss of both vascular integrity and HNF4α, were strikingly similar to those observed upon loss of YY1
from the VE of the yolk sac, we next sought to determine if other aspects of the yolk sac phenotype were similarly regulated during early liver bud morphogenesis. By E9.5, HNF4α is normally expressed throughout the liver bud including both the posterior (blue dashed line, Fig. 4.3A-D) and the anterior liver bud (yellow dashed line, Fig. 4.3A-D). At E9.5 the YY1cKO liver buds are often delayed, resulting in lower than normal HNF4α expression in the anterior liver bud, but typical levels in the posterior liver bud. By E 9.75 the cKO posterior liver bud is often smaller than WT, the posterior liver bud hepatoblasts have reduced HNF4α expression that often maintain the epithelial marker CDH1 (compare Fig. 4.3F to B), while anterior liver bud has achieved typical levels of HNF4α. By E10.0 the defects observed at E9.75 are more pronounced and the posterior liver bud has mainly lost HNF4α and has failed to invade the surrounding STM, while the anterior liver bud is relatively normal. By E10.25 HNF4α expression in the posterior liver bud, which has produced the rostral liver lobe in WT embryos, has failed, while the caudal lobe is relatively normal. Furthermore, we find that despite the fact that YY1 is uniformly lost throughout the liver bud, HNF4α is maintained in the anterior liver bud through E10.25 but is lost in the posterior liver between E9.5-9.75.

4.5.4 Loss of YY1 in the liver bud is associated with reduced VEGFA and increased apoptosis.

We recently demonstrated that loss of YY1 in the VE resulted in both loss of HNF4α and a significant reduction in VEGF. We hypothesized that loss of YY1
resulted in a similar loss of VEGF in the nascent liver bud and proceeded to examine VEGF expression in WT and YY1 cKO liver buds. In WT embryos we find that VEGF is regulated in a temporally and spatially distinct manner in the nascent liver buds. Between E9.75-10.25 the posterior liver bud and the surrounding mesenchyme express VEGF (Fig. 4.4A-C). When compared with the posterior liver bud, VEGF expression in the anterior liver bud is significantly lower from E9.75- E10.0. By E10.25, VEGF expression from the anterior and posterior is statistically indistinguishable (Fig. 4.4A-C, G). In YY1-/ liver buds VEGF expression is low however, normal levels of VEGF are observed in the surrounding mesenchyme (Fig. 4.4D-E).

We next wanted to determine if apoptosis contributed to the reduction in liver bud mass observed in the mutant. Compared with WT, we find that between E9.75-10.0, apoptosis is significantly upregulated in the posterior but not in the anterior liver bud of mutants Fig. 4.4. A-B, D-E, G). By E10.25, apoptosis is significantly upregulated in both the anterior and posterior liver bud (Fig. 4.4. C, F, G). We next examined apoptosis in proximal endodermal organ buds, including the gall bladder, ventral pancreas and dorsal pancreas buds (data not shown). Loss of YY1 in the gall bladder primordium or ventral pancreas bud does not cause gross phenotypic differences or increases in apoptosis when compared with WT, demonstrating that the defects observed in the liver are specific. Interestingly, we found that loss of YY1 caused an as pronounced increase in cell death that was restricted to the dorsal pancreas bud.
Because VEGF is lost in our mutants and we believe that this loss at least partially underlies the phenotype observed, we also examined cell death in the EC’s adjacent to the liver bud as well as those within the adjacent mesenchyme. Intriguingly we find that cell death is significantly upregulated in the EC’s adjacent to the hepatoblasts. At E9.75 ECs, identified by Flk1 co-labeling, is significantly upregulated in the ECs adjacent to the cKO hepatoblasts in the posterior liver bud, but not those within the STM or adjacent to the anterior liver bud (Fig. 4.4. K-M, O). By E10.25 we find that that EC death is significantly upregulated in the EC’s adjacent to both the anterior and posterior liver bud (Fig. 4.4. N).

4.5.5 Loss of VEGF is the main effector of the YY1 phenotype

Based on the recent discovery that loss HNF4α in YY1-/- VE is due to loss of a VEGF dependent reciprocal paracrine signaling loop between the VEGF expressing VE and the VEGFR containing yolk sac mesoderm, we hypothesized that a similar paracrine signaling loop was also controlling early liver development. To determine if hepatoblast-derived VEGF is required to elicit a mesenchyme-derived signal that maintains HNF4α expression and invasion in the liver bud, we performed two sets of experiments. If VEGF is required as stated, then addition of a VEGFR inhibitor should mimic the YY1 -/- phenotype in the liver bud, while VEGF supplementation of cKODE liver buds should rescue HNF4α expression.

To perform the inhibition experiments, WT embryos are dissected prior to or at the onset of liver bud invasion (E9.25) and cultured for 24 hours in the
presence or absence of the VEGFR tyrosine kinase inhibitor, SU1498. After one day in culture both control and treated embryos appeared relatively normal (Fig. 4.5A, E), highlighting the fact that the requirement for VEGF in the yolk sac is confined to slightly earlier stages (Rhee et al. 2013). In the liver bud of VEGFR-inhibited embryos many the YY1 cKO<DE phenotypes were recapitulated. The posterior liver bud had dramatically reduced HNF4α and had high levels of Cleaved-Caspase activity (Fig. 4.5F, F’), while the anterior liver bud was less impacted Fig. 4.5F, F”).

Taken together these results provide further support for the hypothesis that a developmentally regulated requirement for VEGF, and not the loss of YY1, is responsible for maintaining HNF4α during the early stages of hepatic development.

To further test the hypothesis that VEGF is the main mediator of the phenotypes observed in the YY1-/- embryos, we next wanted to discern if ectopic VEGF could rescue HNF4α expression in the YY1-/- liver bud. Because VEGF is a large growth factor that, unlike small molecules, does not freely diffuse through the intact embryo, we could not use whole embryo culture to test this hypothesis. As an alternative, we devised an explant study in which the posterior liver bud along with the surrounding STM are dissected at E9.75 and allowed to grow on tissue culture plastic. Under these conditions, we typically find that the explants grow along the surface of the dish. The fibroblasts and EC’s extend the furthest while the hepatoblasts, identified by HNF4α staining after culture and by their characteristic opacity during culture, are restricted to a more central domain. Entire litters are dissected and individually recovered posterior liver bud/STM explants are bisected
and one half cultured without VEGF and the other half cultured with ectopic VEGF. The size of the original explant is recorded and the growth of each half is monitored for 24 hours. Assessment of WT cultures, which are cut to approximately the same size as the mutant explants, clearly demonstrate that such cultures contain both Flk1-positive ECs and HNF4α-positive endoderm (data not shown). Close examination of WT cultures 24 hours after explant shows that the HNF4α-positive hepatoblasts are usually positioned on top of Flk1-positive tube forming EC-derived tissue (data not shown). The addition of VEGF did not affect WT explants (Fig. 4.5J’, J”), however, the addition of VEGF to cKO liver bud significantly increased the area of HNF4α positive cells (Fig. 4.5K’, K”). These results highlight the role of VEGF as the effector molecule in YY1/-/- embryos and furthermore suggest that signaling through the VEGFR on ECs is required to maintain hepatoblast fate.

4.6 Discussion

Herein we demonstrate that the main molecular defect associated with the loss of YY1 in the liver bud is the failure to upregulate VEGF, which as expected has a profound affect on the associated EC’s. Direct manipulation of VEGF signaling demonstrates that hepatoblast-derived VEGF is required to trigger a VEGFR-dependent paracrine signal from the surrounding mesenchyme that in turn maintains HNF4α expression in the liver bud. Furthermore, careful examination of the earliest events of normal liver bud invasion reveals two temporally and spatially discrete invasion events, each coinciding with an accumulation of VEGF. The first,
occurring at E9.5 involves the posterior liver bud and the second, occurring at E10.25 involves the anterior liver bud. Although HNF4α is normally maintained throughout the liver bud in WT embryos, YY1-/- buds loose HNF4α in the posterior by E9.75 but maintain HNF4α in the anterior. HNF4α expression is lost in the cKO anterior liver bud derivatives coincident with its burst of VEGF expression and invasion of the caudal liver lobes at E10.25. Because no spatial or temporal differences in YY1 loss have been noted, the sequential loss of HNF4α expression highlights the fact that after invasion, HNF4α is maintained by a non-cell autonomous signal and is not directly controlled by YY1 itself. Signals from endothelial cells are important for apical/basal polarity with the developing hepatocyte in zebrafish (Sakaguchi et al. 2008). It is intriguing to note that HNF4α has been implicated in polarity in the murine hepatocyte and thus it is interesting to hypothesize that a conserved role for endothelial cells in liver development is to maintain the differentiation state of the hepatoblast via cell polarity.

### 4.6.1 A new model for early liver morphogenesis

Detailed examination of WT development revealed several interesting observations that aid in explaining the phenotypes observed with loss of YY1. The first is that the liver bud is composed of two domains that undergo temporally staggered development. These two domains include the anterior liver bud, which we define as that which is proximal to the sinus venosus (as highlighted by Isl1) and the posterior liver bud, which we define as that which is in direct contact with the STM.
Secondly, we find that VEGF expression commences in the posterior liver bud coincident with invasion of the STM at E9.25. The anterior liver bud begins its caudal/dorsal migration toward the non-parenchymal tissue of the dorsal lobes between E9.5-9.75. While the lower lobe can be identified by E9.5 and the anteriorly derived hepatoblast begin migrating by this time, most of the lower lobe is comprised of non-parenchymal tissue. VEGFA expression initiates in the anterior liver bud derivatives in the caudal lobe commensurate with the hepatoblasts apparent invasion of the lower lobe at E10.25.

To support the observation that the different regions of the liver bud contribute to distinct lobes by E10.0, we performed lineage tracing and ablation experiments in the context of embryo culture. We previously demonstrated that the liver bud is produced by 2 discreet precursors that preferentially contribute to distinct portions of the liver bud. The lateral progenitors, which give rise to the bulk of the liver, including the posterior liver bud, arise on the right and left lateral edges of the endodermal sheet and converge at the ventral midline of the closing gut tube. The VMEL progenitors contribute to the midline of the ventral foregut gut including a large portion of the anterior-most aspect of the liver bud. Fate mapping experiments performed by labeling one of the liver progenitors demonstrate that the anterior and posterior descendants contribute to the dorsal/caudal lobes and rostral lobes respectively. Ablation experiments, performed solely for technical reasons with the lateral progenitors, confirm that the posterior bud produces the rostral lobes. Taken together these data suggests that the lateral progenitors that
produce the posterior liver bud ultimately produce the parenchyma of the rostral lobes, while the VMEL progenitors contribute to the anterior liver bud and produce the parenchyma of the caudal lobes.

Given the compelling evidence, as outlined above, for an early segregation of the anterior and posterior liver bud to the caudal/dorsal and rostral liver lobes respectively, the remaining question is: is there a functional difference? Interestingly, recently the single early “ventral pancreas” bud, which emerges from the endoderm adjacent to the posterior liver bud, is composed of two organ progenitors, the gall bladder, which arises from the anterior portion of the ventral pancreas bud and the ventral pancreas, which arises from the posterior portion of the bud (Spence et al. 2009). While this single bud gives rise to two functionally distinct structures, another similarity exists in the formation of the adult pancreas. The adult pancreas is formed by fusion of the dorsal and ventral pancreas bud, which arise in different mesenchymal environments, have distinct molecular requirement and are believed to form distinct portions of the adult pancreas. The uncinate head is produced by the ventral bud, while the dorsal bud produces the cepharic head as well as the body and tail (Gittes 2009). A growing literature supports the molecular and histological differences between the head and the tail of the pancreas (Malaisse-Lagae et al. 1979; Clark & Grant 1983b; Wang et al. 2013b). Documented functional differences exist between the head and the tail, suggesting that the ventral pancreas assists in metabolism of environmental toxins while the dorsal pancreas functions to metabolize glucose (Trimble et al. 1982b; Vara et al. 1994; Standop et al. 2002b). Finally, the head and tail are differently susceptible to
chronic pancreatitis and pancreatic cancers (Rahier et al. 1983b; Lang et al. 2012). In light of the body of evidence supporting adult pancreatic differences resulting from distinct embryonic origins, if our hypothesis regarding different lobe contribution by distinct hepatoblast populations hold true, then future investigation into the histological, functional and pathological differences between the rostral and caudal liver lobes is warranted.

4.6.2 Functional Similarities between the Yolk Sac and Early Liver Bud

Given that the function of YY1 is believed to be context dependent, the conservation of the roles for YY1 in the liver bud and in the visceral endoderm of the yolk sac are remarkable. A significant hindrance in studying early hepatic development has been that many required genes are first essential in the visceral endoderm (VE) of the yolk sac (Chen et al. 1994; Duncan et al. 1997; Molkentin et al. 1997; Narita et al. 1997; Dufort et al. 1998; Morrisey et al. 1998; Waldrip et al. 1998; Barbacci et al. 1999; Tremblay et al. 2000; Parviz et al. 2003; Lee et al. 2005a; Decker et al. 2006; Ju et al. 2006; Watt et al. 2007; Lokmane et al. 2008b; Burtscher & Lickert 2009). Only a handful of early DE-specific genes have been identified, demonstrating that the transcriptional networks used by the VE and DE are mainly overlapping (Yasunaga et al. 2005; Hou et al. 2007; Sherwood et al. 2007; Tremblay 2010a). Indeed the mammalian yolk sac acts functionally as an embryonic gut, serving as the site of nutrient uptake, gas and metabolic waste exchange (Farrington et al. 1997; Bielinska et al. 1999). Furthermore, like the developing liver, growth and differentiation of the yolk sac is dependent upon reciprocal paracrine signaling
between the VE and the underlying mesoderm (Farrington et al. 1997; Byrd et al. 2002). The yolk sac and the liver bud are composed of two layers of tissue: the VEGF producing yolk sac VE or hepatic endoderm and the VEGFR containing yolk sac/hepatic mesoderm. Each of the mesoderm-derived tissues is produced by the posterior streak during gastrulation, requires VEGF signaling for their development and provides VEGFR-dependent paracrine signals necessary for the overlying “endoderm”. Both mesoderm-derived tissues harbor endothelial cells and other mesenchyme that is important for vascular development. An intriguing hypothesis is that the easily accessible VE or yolk sac mesoderm could be used as a proxy for early liver bud development.

One difference between the YY1/-/- phenotype observed herein and that observed in the VE of the yolk sac is the failure to downregulate E-cadherin. The VE of the yolk sac does not undergo an epithelial to mesenchymal transition (EMT) and remains as an epithelium throughout development. Loss of YY1 in cells of the primitive streak results in a failure to downregulate E-Cadherin, an essential step in the process of EMT (Trask et al. 2012). The invasion of the STM by hepatoblasts is considered a secondary EMT (Thiery et al. 2009) and here we also find that YY1/-/- cells similarly fail to downregulate E-Cadherin. Although a clear role for YY1 as an oncogene has yet to be established, it is interesting to note that increased levels of YY1 are associated with cancer progression and EMT during oncogenesis in many endoderm-derived tissues (Atchison et al. 2011) including hepatoblastoma (Shin et
Taken together these results highlight an instrumental role for YY1 in invasion during normal and pathological conditions.

An intriguing question that is not described herein includes understanding how loss of YY1 controls VEGF protein. Others have also found that YY1 directly binds and activates the VegfA promoter in human cell lines (de Nigris et al. 2010) and unpublished work from our laboratory has confirmed that putative YY1 binding sites are found in a similar region in the murine Vegf promoter and that YY1 associates with that promoter/chromatin complex at least in WT VE cells (M. Trask, J.Mager, S. Rhee, K. Tremblay unpublished). However, qPCR analysis from dissected liver buds indicates that as in the yolk sac, Vegf transcripts are not altered. A literature search reveals that no known protein modifications or direct regulators of VEGF protein exist except post-transcriptional regulation, primarily by miRNAs (Hua et al. 2006; Jafarifar et al. 2011; Sun et al. 2013). Others have found that YY1 controls a subset of miRNAs involved in muscle development (Lu et al. 2012) and it is thus possible that YY1 indirectly controls VEGF by regulating miRNAs that target VEGF. The question of how YY1 controls VEGF will be an interesting avenue of future experimentation.

4.6.3 A new look at hepatic endoderm and mesenchyme interactions

Sinus Venosus

The intimate interaction between the sinus venosus, a portion of the secondary heart field, and the migrating hepatoblasts suggest that they are required
for either the development or the migration of the liver. While a role for beating cardiac tissue in liver gene expression has long been documented in explant and transplant studies (Le Douarin 1975; Gualdi et al. 1996a)) the identification of the sinus venosus as the subset of cardiac tissue that guides and potentially supports the formation of the caudal murine liver lobes is unexplored. It is interesting to note that similar to the role we propose for the sinus venosus in mouse, others have documented that in the chick, “liver bud morphogenesis requires interaction of the hepatoblasts with the ductus venosus – a remnant of the vitelline veins- which ends at the sinus venosus. After invagination, the chick hepatoblasts initially elongate along this tissue and then proliferate to form the hepatocellular cords.

4.6.4 Coordinated development of liver bud and endothelial lineages

Normal liver development, homeostasis and regeneration require the coordinated development of hepatocytes and the surrounding vascular tissue (Matsumoto et al. 2001b; LeCouter et al. 2003; Ding et al. 2010b; Hu et al. 2014). As early as E9.25 of liver bud development, Flk-1 positive ECs surround the evaginated bud and loss of the Flk-1 positive cells results in a failure of the specified liver bud to invade the surrounding mesenchyme. These results demonstrate that ECs are required for hepatoblast invasion, but do not establish a direct link between ECs and hepatoblasts. Intriguingly, like that observed with EC loss, we find that loss of YY1 in the liver bud does not impact liver development until the onset of invasion. Herein we establish that loss of hepatoblast VEGF results in the loss of proper hepatic vascularization.
Although we have uncovered a vital VEGF-dependent reciprocal paracrine signaling pathway that underlies the maintenance of hepatoblast differentiation. There are still several questions that remain unanswered. Which hepatic mesenchyme is responsible for HNF4α maintenance? While our evidence suggests that the VEGFR lies on the endothelial cells it is possible that the signal required for hepatoblast maintenance comes from an endothelial-mesenchymal relay such as that described in the ventral pancreas bud (Jacquemin et al. 2006).
CHAPTER 5

FUTURE DIRECTION

5.1 Use the new liver morphogenesis model to fully Understand Liver Development

One of the biggest findings in this body of work is the proposed new liver developmental model. I have shown that there are two liver precursors developing in a unique spatiotemporal manner. The posterior liver bud invades the STM for upper lobe formation while anterior liver bud migrates dorsolaterally into caudal lobe mesenchyme (CLM). It appears that these two populations of hepatoblasts are directed by two different sets of signaling cues. Posterior liver bud hepatoblasts migrate toward ECs in the STM while the sinus venosus appears to provide the attractant cues for the anterior liver bud. YY1 and VEGFA paracrine signaling pathways distinctly affect posterior liver bud invasion; this strongly supports the two liver population story. This may create a new model, but how can this model be used? What could be the impact of this model? Hypothesis for developing new liver model would be that two liver bud model can be used for reinterpreting previous results and refining better analyzing for liver phenotype. Under the current liver development model (Si-Tayeb et al. 2010), researchers could easily misinterpret their research results causing them to lose their chance to reveal true effectors. If the liver bud is treated as a homogenous population, the lack of a phenotype in the anterior or posterior liver bud would be interpreted as no phenotype. If the field treats the liver bud as two populations that respond differentially to signals, then
we can fully understand the impact of signaling factors in the liver. This new model can have an enormous impact on the field, reinterpreting previous results which are already published by other researchers, understanding why previous experiments have had partial early phenotypes, and give a better understanding of the way that the liver develops into the functional adult organ.

There are several examples throughout the literature of phenotypes that may have been misinterpreted through the assumption that the liver is a homogenous population. The Takahashi research group in Japan published two papers describing the affects of STM signals in 2011 (Saito et al. 2011) and 2013 (Saito et al. 2013). The first paper described the role of Mab21l2 in heart and liver development and the second one focused on its role on gall bladder development during mouse embryogenesis. In both papers, Mab21l2 null embryos failed to form STM (Saito et al. 2011) (Saito et al. 2013). They showed liver defects in early (E9.5-10.5) and later stages (E11.5). However, a two liver population model could better explain their interpretation of these defects. Their mutant embryos show different phenotypes in the anterior and posterior. Because the anterior bud is able to maintain expression of Alb, HHex and Hnf4α RNA, they conclude that there is no change in gene expression in the liver. They do not show the posterior liver bud, and there is the possibility that it does not form in these embryos because of loss of STM. Their second paper states that there is ectopic liver dorsolaterally that expresses Alb RNA. This, however, is the normal location of the caudal lobe hepatoblasts migrating from anterior liver bud to CLM. The anterior liver bud does not seem to be affected by STM defects and would be expected to be fine in the two liver population model.
Additionally, Gata4 loss shows severe STM defect but did not show the anterior liver bud (Zhao et al. 2005). They show posterior liver bud region in WT and anterior liver bud region in mutant embryos and concluded that there was no different gene expression change. Here again the posterior liver bud could be missing because of the lack of STM while the anterior appears normal and maintains normal gene expression. Data from our lab shows that BMP inhibition affects posterior but not anterior liver bud (data not shown) while FGF inhibition affects anterior but not posterior bud (Wang et al. 2015). This is potentially just one example of differential phenotypes within the liver and STM mutants could present many more.

The above few examples highlight the supporting evidence in the literature that there are two different populations within the liver bud that respond to different mesenchyme cues and that the assumption that the liver bud is homogenous has led to misinterpretation of phenotypes. The many mutants that have STM, later liver, or CLM phenotypes but no interpreted early liver phenotype need to be reexamined under the new model as a possible research. This work will reveal true differences in patterning and signaling to the liver early in development. The early liver bud is possibly much more complex than the people studying it have thought.

5.2 What are the mesodermal sources guiding two different liver buds?

In this thesis, I have shown there are two distinct liver precursors developing in unique spatiotemporal way. The posterior liver bud invades the STM for upper lobe formation while anterior liver bud migrates into caudal lobe mesenchyme. It
appears that two populations of hepatoblasts are directed by two different signaling
cues. Posterior liver bud hepatoblasts migrate toward ECs in the STM while the
sinus venosus appears to provide the attractant cues for the anterior liver bud. The
difference in mesenchymal cues becomes particularly evident in YY1 mutant
embryos. While the whole liver bud eventually suffers from the loss of YY1, and
consequent loss of VEGF, the posterior bud dies almost a full day before the anterior
bud.

Loss of hepatoblast marker, HNF4 α, increase in epithelial cell junction
maker, CHD1 and loss of VEGF are observed in the posterior liver bud at E9.5, while
the anterior bud looks comparable to wild type. This posterior phenotype is
accompanied by increased apoptosis, especially in cells adjacent to the hepatoblasts
that appear to be poised for invasion but are failing to do so. These edge cells are
positioned to receive signals from the adjacent STM and endothelial cells. In wild
type embryos, these cells would be invading the STM in the direction of endothelial
cells. Interestingly, the endothelial cells of the sinus venosus and vitiline vein are
located nearby, just anterior and lateral to the bud, but the posterior YY1 null
hepatocytes does not migrate toward these cells. One hypothesis could be that the
posterior hepatoblasts could receive growth factors from all endothelial cells (Ding
et al. 2010a). If this were the case one would expect the upper portion of the
posterior hepatoblasts to have a slightly less severe phenotype than those farthest
from vasculature. This is not the case; the whole posterior bud appears to suffer cell
death and inappropriate molecular patterning.

The posterior liver bud follows the same steps of apoptosis and
inappropriate gene expression experienced by the posterior bud by E10.5 when it attempts migration into its respective mesenchyme. WT anterior hepatoblasts migrate away from the STM, along the adjacent sinus venosus into a pocket of CLM. In the YY1 null liver, the hepatoblasts on the outer edge “leading” the migration suffer apoptosis and loss of HNF4α first. This is reminiscent of the posterior bud phenotype and indicates that these cells need mesenchyme/endothelial signals, to survive their next step in development. What is the most notable is the difference in time of the same phenotype in two proximal portions of the same liver bud. This could be explained by the presence of unique mesenchyme signals guiding each population.

The process of two seemingly similar portions of organ progenitors giving rise to an organ through different mesenchyme signals is not unique to the liver; the pancreas develops as separate ventral and dorsal buds, in unique mesenchyme environments, that fuse to form the adult pancreas. The first known molecular marker of pancreas identity in the mouse is PDX1, which is expressed in both the ventral and dorsal bud by E9.0. They both then go on to express differentiation markers such as Ngn3, insulin, and glucagon and are thought to contribute both exocrine and endocrine cell types to the adult pancreas.

Although both the ventral and dorsal pancreas buds are molecularly similar throughout development and become an adult organ, each in thought to be induced by a unique set of signals. The ventral pancreas develops contiguous to the liver, from the posterior ventral foregut, in close association with the STM, while the dorsal bud develops in proximity to the notochord, dorsal aortae and somites. It is
thought that a gradient of FGF signaling, high near the heart and low near the pancreas, is thought to induce ventral pancreas fate (Rossi et al. 2001). The dorsal pancreas is thought to be induced by endothelial cells of the dorsal aortae or by suppression of Shh by the notochord (Jacquemin et al. 2006). The difference in inductive signaling is somewhat reminiscent of the liver bud’s location between two different mesenchyme and endothelial populations and its apparent ability to respond differentially to resulting cues.

The question of how two different mesenchymal sources control two different hepatoblast populations will be an important path of future experiments. The answers are not just important in vitro for stem cell differentiation protocols and cell replacement therapies, but also to uncovering a possible functional difference in portions of the adult organ. In pancreas there is evidence that the head, derived from the ventral bud, can respond to chemical stress and the body and tail, derived from the dorsal bud, responds to glucose stress (Malaisse-Lagae et al. 1979; Trimble et al. 1982a; Rahier et al. 1983a; Standop et al. 2002a). The pancreas head is small, has fewer islets and is less responsive to metabolic demands. The body and tail perform the bulk of the functions of the pancreas (Clark & Grant 1983a; Wang et al. 2013a). It would seem that the dorsal pancreas bud would be vital, however, loss of the dorsal bud results in diabetes while loss of the ventral bud is lethal in the embryo (Lång et al. 2012). This observation indicates that the pancreas head is not as important for common functions but it vital under certain conditions. It is interesting to consider that there may be a similar mechanism in the planning of the liver. The anterior bud gives rise to the small caudal lobes, while the bulk of the liver
is derived from the posterior liver bud. Maybe the smaller liver lobes are similarly acting as a “backup” for the liver system in the event of injury and could be less sensitive to certain chemical or physical stresses. The liver can regenerate in response to chemical injury, vascular starvation, ablation and ductal ligation. It makes sense that there are varied response systems for the variety of possible injury mechanisms. The discovery of differences in the embryo could lead to better knowledge of this unique regenerative adult organ.

5.3 Investigation of how YY1 regulates VEGFA in VE and liver bud

VEGFA protein level was significantly reduced in YY1 null VE and hepatoblasts while Vegfa transcripts are maintained at normal levels. It is thought that VEGF is regulated by YY1 indirectly, however, there is no evidence showing direct control of VEGF protein except post-translational regulation, especially via MicroRNAs (miRNAs). One hypothesis to answer the question “how does YY1 regulate VEGFA?” is YY1 regulates miRNAs that repress VEGF translation.

MicroRNAs (miRNAs) are non-coding single-stranded RNAs of 21 to 25 nucleotides that negatively regulate their targets at the post-transcriptional level through binding to their 3’ UTR regions leading to translational repression. Since VEGFA was first proposed as a target of miRNAs in 2004, there has been increasing evidence that miRNAs play important roles in regulating VEGFA in vascular development as well as in vascular diseases. Several studies have been performed to identify miRNAs targeting VEGFA mRNA; specifically their binding site(s) within the 3’ untranslated region (UTR) region. Among many miRNAs, I determined three
miRNAs including miRNA 20a, b and 93 as candidate miRNAs that regulated by YY1 and affect VEGF post translation. My preliminary results display that miRNA 20a, 20b and 93 are increased by loss of YY1 in our in vivo VE tissue while YY1 and HNF4α mRNA are reduced suggesting that miRNAs may be a main effector of VEGF post-translational repression in YY1 null VE and liver bud. For further experiment, studying YY1’s role in regulating miRNAs that represses VEGF post-translationally is the most suitable approach to answer the question.

5.4 Studying liver bud differentiation: hepatoblasts vs cholangiocytes

Regeneration of the liver is a vital process in response to physical or chemical injury. A hallmark of this process is the presence of OVAL cells identified by expression of SOX9 and HNF4α (Michalopoulos 2011). These cells have the capability to differentiate into cholangiocytes and hepatocytes to repopulate the liver. Upon injury, hepatocytes have the capability to regenerate the liver through proliferation. When severe injury occurs, and there are not sufficient hepatocytes to repopulate the liver, the proliferation of hepatocytes is inhibited and biliary epithelial cells proliferate to repopulate (Michalopoulos 2011). In multiple genetic knockouts detrimental to the liver, such as C/EBPα, TBX3 and PROX1, the result of lack of molecular differentiation signals is an increase of SOX9/HNF4α positive OVAL cells (Shiojiri et al. 2004; Suzuki et al. 2008; Lüdtke et al. 2009a; Seth et al. 2014). This adds to the evidence that depletion of differentiation signals results in a regenerative response.

Models of liver regeneration are produced by injury of the adult liver,
however, the source of regenerating cells is variable depending on the method of injury and therefore, the source of repopulating cells may be dependent of the method of injury. There is contradicting information that indicates several different adult cell populations at those responsive to injury and responsible for regeneration (Kelley-Loughnane et al. 2002; Zhang et al. 2008; Ding et al. 2010a; Ochoa et al. 2010; Michalopoulos 2011; Stanger 2015). What is consistent among models is that the liver requires both bile ducts and blood vessels to regenerate? Hypothesis would be YY1 plays an important role for cell fate decision between hepatocytes and cholangiocytes. Interestingly, in the YY1 DE cKO mouse embryo at E14.5 and later stages (E18.0) there are SOX9 positive cells scattered through the hypoplastic liver (data not shown, to be updated in the final thesis). At these stages, SOX9, a marker of cholangiocytes; however, the presence of both SOX9 and HNF4α positive cells is indicative of progenitor OVAL cells. Interestingly, a hallmark of loss of YY1 in the liver bud is an increase in OVAL cells in the embryonic liver, as determined by the presence of SOX9 and HNF4α. The fetal liver could represent a population of quiescent stem cells that mirror the adult regenerative population. Upon severe injury in the adult, or differentiation signal starvation in the embryo, there appears to be a down regulation of HNF4α, a marker of hepatoblasts, and upregulates SOX9 expression to form SOX9/HNF4α positive OVAL cells. Interestingly, this is a systemic response that appears to be unspecific to a particular liver bud population. This could be a model to escape the effects of chemical or physical injury because upon depletion of molecular signals in the embryo there is a systemic response that is not population dependent.
5.5 Investigation of the developmental roles of YY1 in lung morphogenesis

Like the liver, the lung develops from endoderm organ buds surrounded by mesenchyme that secretes signals to pattern the endoderm as it grows and differentiates (Morrisey & Hogan 2010). At E9.5 in mouse, endoderm buds form on either side of the gut tube and undergo branching by E11.5, forming tip and stalk regions that are identifiable morphologically, molecularly and by differences in proliferation (Morrisey & Hogan 2010). The stages of lung development are reminiscent to liver development involving movement of the endoderm bud into adjacent mesenchyme and well documented signaling from mesenchyme to endoderm (Morrisey & Hogan 2010). Similar to the liver, loss of YY1 in lung endoderm results in phenotypes in both endoderm and mesenchyme (data not shown, to be updated in the final thesis). These phenotypes are indicative of a paracrine feedback loop and suggest the leading distal cells require YY1 or YY1 supported signaling.

YY1 endoderm null lungs are small in size and contain fewer branches, indicating defects in proliferation/apoptosis and patterning at E14.5. As early as E9.75 YY1 cKO lungs show apoptosis in the endoderm and by E10.5 this phenotype is pronounced. E10.5 marks a time point when the lung buds are growing but have not yet started to branch. Interestingly, apoptosis at this time point is clustered into discrete areas of the lung buds. Preliminary data shows that Cleaved caspase 3 is expressed in a cluster, usually on one side of one lung bud in each sample. Careful analysis should be performed to identify if it is the same region of the same lung bud in all cases. This could indicate that this apoptotic area is a site of differentiation,
and possibly the cells suffering the effects of loss of YY1 are those in need of growth or transcription factors.

Like liver, it is known that BMP and FGF from mesenchyme pattern the gut tube before lung budding and pattern the lung buds during differentiation. Interestingly, BMP4 and FGF10, both expressed in the STM, are expressed in lung mesenchyme and required for proper outgrowth and branching morphogenesis (Min et al. 1998; Sekine et al. 1999; Shu et al. 2002; Rajagopal et al. 2008). Loss of FGF10 in the mesenchyme surrounding the lung buds, or loss of its receptor FGFR2 in the endoderm, inhibits lung bud extension. This is similar to the phenotype seen in YY1 KO lungs but much more severe. The difference in severity does not discount the possibility that FGF10 could be affected in our YY1 KO and could be explained by the efficiency of KO in the system used to generate our preliminary data.

Immunofluorescence analysis of YY1 cKO embryos at E9.5-9.75 shows that there are some YY1 positive remaining cells. By E14.5 around 50% of endoderm cells express YY1 in patched pattern of YY1. This could be explained by repopulation of lung endoderm by the remaining YY1 positive cells proliferating faster than YY1 null epithelium and escaping the apoptosis seen in YY1 null cells. Knockout in this system uses FOXA2 Cre-ER to drive excision of YY1 upon addition of TM. Future experiments need to be performed to find an effective method of maintaining YY1 cKO, perhaps using multiple TM doses to examine later time points in development.

Examination of YY1 cKO embryos at later time points is necessary to analyze the branching phenotype and possible perturbed patterning candidates such as FGF10, BMP4 and VEGF. The process of branching is occurs when additional buds
form on the primary buds and elongate, creating branches (Morrisey & Hogan 2010). The distal cells of these branches express FGF10, which is thought to be regulated by BMP4, which is expressed in the distal epithelium of the stalks. The balance between these two factors is thought to control branch morphogenesis, and addition of BMP4, deletion of its receptor Bmpr1, inhibits the ability to respond to FGF cues (Weaver et al. 2000). VEGF has been well studied in the formation of blood vessels in the lung but not in the development of the airways themselves (Morrisey & Hogan 2010). However, preliminary observations of WT embryos show that VEGF is more highly expressed in distal cells that stalk and that these distal cells are close to FLK1 positive ECs while the stalk is not in direct contact with ECs (data to be added to final version). Given what is known about the necessity for endothelial cells in liver outgrowth, and that genetic mutants lacking endothelial cells do not survive to lung branching stages, future studies will take advantage of the YY1 KO to study the role of VEGF in airway outgrowth (Matsumoto et al. 2001a).

Future studies are necessary to build on preliminary data from the YY1 cKO lung as it appears to be a valuable model to study paracrine signaling between mesenchyme and endoderm, and to identify novel regulators of lung morphogenesis such as VEGF. In the future this model should be characterized at pre branching time points (E9.75 and E10.5) to identify the role of YY1 in controlling the signals necessary for survival and further differentiation. The unique pattern of cell death in these embryos could give clues about which cells are responding to mesenchyme-derived signals in preparation for branching. After optimization of out FOXA2 Cre-ER system to maintain cKO of YY1, later stages should be studied (E14.5, E15.5) to
examine the affect of YY1 on branching morphogenesis, and its possible regulation of BMP and FGF signals who’s mutants somewhat phenocopy YY1 cKO. Finally, this system can be used to study VEGF and possibly identify a similar feedback loop as seen in the liver or a new attractant cue that guides or promotes branching elongation. This work will shed light on the mechanisms at play in early and late lung development and could give new insight into the origin of lung diseases.
Figure 2.1. *FoxA3-Cre* mediated *Yy1* cKO deletion results in prominent yolk sac defects at 9.5 *dpc.*

A-L) Bright field images of WT (A–F) and cKO (G–L) embryos (EM) alone or embryos within their yolk sacs (YS) at the indicated stages. A, G) 8.5 *dpc* mutant embryos (inset in G) are sometimes slightly delayed compared with WT (inset in A) but display no noticeable yolk sac defects. B–C, H–I) 9.0 *dpc* mutants display relatively normal yolk sac blood vessel development. D–E, J–K) 9.5 *dpc* mutant yolk sacs have dilated vessels (asterisk in J) and poor vessel organization (compare D to J). cKO embryos (K) are smaller than WT embryos (E) from the same litter. F, L) While prominent large blood vessels are easily detected in 10.5 *dpc* WT yolk sacs (F), the yolk sacs of mutants are uniformly pale (L). M–P) A comparison of WT and mutant H&E stained yolk sac sections demonstrates that while no differences are found at 8.5 *dpc* (M, O), the 9.5 *dpc* cKO yolk sac (P) has fewer and larger vessels compared with WT (N). Q) Investigation of the same sized area at 9.5 *dpc* revealed significantly fewer vessels in mutant compared with WT yolk sacs (** *= p<0.001; error bar = standard error). R) A size distribution chart at 9.5 *dpc* reveals that mutants contain fewer of the small vessels (<100 µm) and more of the larger vessels (>100 µm) compared with WT yolk sacs.
Figure 2.2. *Yy1* cKO visceral endoderm displays a reduction of apical lysosome size and other epithelial characteristics.

A–B, E–F) Transmission electron microscopy (TEM) of WT (A–B) and cKO (E–F) visceral endoderm (VE) sections reveals large apical lysosomes in 8.5 and 9.0 dpc WT visceral endoderm (asterisks, A–B) and in 8.5 dpc cKO visceral endoderm (asterisks, E). At 9.0 dpc the size of the apical lysosomes are greatly reduced in the cKO (compare asterisks, in F to B). C–D, G–H) IgG localization (green) at the apical surface is readily noted in 9.0–9.5 dpc WT yolk sac sections (YS, C–D) while IgG distribution is reduced in the mutant at the same stages (G–H). Inset is a higher magnification view of a portion of the visceral endoderm. I–J, M–N). Whole-mount LysoTracker Red staining reveals large filled lysosomes from 9.0–9.5 dpc (I–J) in WT tissue while the LysoTracker-filled areas are reduced in the mutant samples (M–N). K–L, O–P) Immunolocalization of E-Cadherin (CDH1; green) reveals epithelial cell-cell adhesions in the visceral endoderm of WT yolk sac sections from 9.0–9.5 dpc (K–L). CDH1 expression is slightly reduced at 9.0 dpc and more profoundly reduced at 9.5 dpc in cKO visceral endoderm (O–P). ME = mesoderm; N = nucleus.
Figure 2.3. Efficient excision of YY1 in definitive and visceral endoderm is accompanied by reduced HNF4α.

Immunofluorescence analysis of sectioned WT (A–D, I–K) and cKO tissue (E–H, L–N) at the stages indicated. A–C, I) YY1 (green) is ubiquitous in WT embryonic and extraembryonic tissues. A–B, D, K) HNF4α (red, orange when co-expressed with YY1) labels the visceral endoderm (A–B, D) and the developing liver bud (K). E–G, L) In cKO embryos, YY1 is downregulated in the extraembryonic visceral endoderm (VE) at 7.5 dpc (E) and is completely lost in the embryonic visceral endoderm by 8.75 dpc (F), when YY1 is also depleted in the definitive endoderm (DE) of the foregut. By 9.25 dpc YY1 is lost in most cells of the liver bud (L). E–F, H, N) Although HNF4α is present in the YY1-deficient visceral endoderm until 8.75 dpc (E, F) it is greatly reduced in both the visceral endoderm and in the nascent liver bud by 9.5 dpc. J, M) Despite the loss of YY1 in the nascent liver bud, the liver bud differentiation marker PROX1 is maintained in the cKO liver bud (M) at levels comparable to that observed in WT (J). The dotted line in C–D and G–H represent...
the division between the visceral endoderm and mesoderm derivative of the yolk sac, while in I–N the dashed line outlines the liver bud (LB).

Figure 2.4. cKO embryos display a variety of defects in the yolk sac mesoderm. A–B, D–E) Whole mount immunofluorescence of 9.5 dpc WT (A–B) or mutant (D–E) yolk sacs (YS) using the endothelial marker PECAM (green) and the vascular smooth muscle marker (αSMA) demonstrates that the large disorganized vessels in the cKO (D) are not surrounded by αSMA (E). C, F) Section immunofluorescence of WT (C) and cKO (F) 9.5 dpc yolk sacs demonstrates loss of αSMA in the cKO. G–L) Section immunofluorescence of VEGFA (green) demonstrates relatively uniform VEGF levels in the 9.0, 9.25 and 9.5 dpc WT yolk sac (G–I) while VEGF distribution in the visceral endoderm of the mutant is progressively diminished at each stage (J–L). M) A Western blot of whole yolk sacs at the indicated stages. The ratio of VEGFA to GAPDH signal intensities for the cKO relative to each stage-matched WT control is displayed under each band. N) Cleaved Caspase-3 staining was used to assess the percentage of cell death in the yolk sac layers of WT and cKO sections at 8.5 and 9.0 dpc. A significant increase in apoptosis was observed in the cKO mesoderm (ME) at 9.0 dpc. O) Phosphohistone-H3 (PH-3) staining was similarly used to assess proliferation and a significant decrease in proliferation was found in the cKO yolk sac mesoderm at 9.0 dpc. *** = p<0.001, ** = p<0.01; error bars = standard error; dotted line is drawn between the visceral endoderm (VE) and mesoderm derivatives (ME) on yolk sac sections.
Figure 2.5. Changes in yolk sac gene expression in cKO embryos.
A–E) RT-PCR and qPCR performed with cDNA prepared from whole 9.0 and 9.5 dpc cKO and WT yolk sacs. A) As expected, Yy1 is significantly downregulated in whole cKO yolk sacs. β-actin and Hprt expression are used as loading controls. B) No expression differences between WT and cKO samples are noted for VegfA using primers that recognize all (Exon 2–3) or the alternative VegfA isoforms (Exons 3–8) nor in the Vegf transcriptional regulator Hif1α. C) While many visceral endoderm-specific genes show no expression differences, expression of vHnf1, Hnf4α and Pgc1α were all downregulated in cKO samples when compared to WT at 9.0 and 9.5 dpc. D) An examination of genes involved in lysosome biogenesis reveals no expression differences between WT and cKO yolk sacs with the exception of Enpp-2, which is upregulated in mutant samples at both stages examined. E) qPCR reveals that Yy1 is expressed at ~30% of WT levels in whole yolk sacs, where mesoderm derivatives maintain Yy1. qPCR was used to confirm that VegfA expression is not significantly altered between cKO and WT and that expression of the visceral endoderm gene, Hnf4α is significantly downregulated in cKO yolk sacs. *** = p value<0.001; error bars = standard error.
Figure 2.6. Exogenous VEGF rescues Yy1 cKO yolk sac defects.
A–Y) WT and cKO embryos cultured from 8.5–9.5 dpc in the presence (+VEGF) or absence (−VEGF) of VEGF. A–E) WT cultured embryos display normal yolk sac vasculature (A–A’), typical embryonic size (inset in A) and the presence of YY1 (brown) in the visceral endoderm (VE) and in yolk sac mesoderm (ME; B). In WT yolk sac sections, αSMA (red) surrounds mature vessels (C), HNF4α (red) is expressed in the visceral endoderm (D) and IgG is localized to the apical visceral endoderm (E). F–J) WT embryos cultured with exogenous VEGF display robust yolk sac vasculature (F, F’), YY1 expression in both YS layers (G), normal αSMA in mature vessels (H), typical HNF4α in the visceral endoderm (I) and high levels of apical IgG (J). K–O) Cultured cKO embryos demonstrate poor vascular development, including pooled blood in the proximal yolk sac (K–K’), no YY1 in the visceral endoderm (L), reduced αSMA (M), reduced HNF4α (N) and decreased apical IgG (O) when compared with WT cultured embryos (A–E). P–T) cKO embryos cultured with exogenous VEGF display normal yolk sac vasculature (P–P’) and increased embryo size (inset in P) when compared to cKO embryos cultured without exogenous VEGF (K–K’). cKO embryos cultured with VEGF lack visceral endoderm YY1 (Q) but have increased αSMA in the yolk sac mesoderm (R) and increased levels of HNF4α (S).
and apical IgG (T) in the visceral endoderm when compared to untreated cKO embryos (M–O). U–Y) Immunofluorescence against cleaved Caspase-3 (CASP3, green) and CDH1 (red) of sectioned yolk sacs revealed that typical CDH1 expression found in WT (U) and WT cultured with VEGF (V), was downregulated in cultured cKO embryos but more normal visceral endoderm expression restored when cKO embryos were cultured with VEGF (X). Y) Quantification of cleaved Caspase-3 positive cells demonstrates that the addition of VEGF to cKO embryos restores WT levels of apoptosis. *** = p<0.001; error bars = standard error; dotted line is drawn between the visceral endoderm (VE) and mesoderm derivatives (ME) on yolk sac sections.

Figure 2.7. Inhibition of FLK1 in WT embryos results in yolk sac defects similar to Yy1 cKO.
A–J) WT 8.5 dpc embryos cultured until they reached 9.5 dpc in the absence (A–E; –SU1498) or presence of the small molecule SU1498 (F–J; +SU1498). Compared with control embryos (A–E), SU1498 treated embryos displayed clear yolk sac abnormalities, including pooled blood in the proximal yolk sac (F), a small embryo (G), reduced HNF4α (H), reduced apical IgG localization (I) and higher amounts of cleaved Caspase-3 (CASP3) staining (J). Asterisks in J indicate cleaved Caspase-3 positive cells; dotted line represents the division between the yolk sac mesoderm (ME) and visceral endoderm (VE).
Figure 2.8. Loss of YY1 leads to defects in paracrine signals necessary for angiogenesis and visceral endoderm integrity.

A, B) A summary of the signaling events downstream of YY1 in WT and mutant yolk sacs. A) In the presence of YY1, normal VEGF levels produced by the visceral endoderm (VE) allow the underlying mesoderm derivatives (ME) to undergo events associated with vascular remodeling. The underlying vascular tissue is the source of a VEGF-dependant paracrine signal(s) that is required by the visceral endoderm to maintain characteristics such as epithelial polarity, large apical lysosomes and HNF4α expression. B) In the absence of YY1 in the visceral endoderm, decreased levels of paracrine VEGF result in defective angiogenesis, increased apoptosis and decreased proliferation in the adjacent mesoderm. Because of reduced VEGF signaling, the yolk sac mesoderm does not generate the paracrine signal(s) needed to maintain epithelial characteristics in the visceral endoderm, resulting in decreased HNF4α, a loss of large lysosomes and reduced CDH1 levels.
Figure 2.9. *FoxA3-Cre* activity monitored with the *R26R* allele. A–F) *R26R;FoxA3-Cre* double heterozygotes were dissected at the stages indicated and Cre activity monitored by LacZ staining (blue). Whole mount images (A–C) and eosin counterstained transverse sections (D–F) of the same embryo at the indicated plane (green line, A–C). A, D) At 7.5 dpc, LacZ expression is confined to and mosaic within in the visceral endoderm (VE) and not yet found in the early definitive endoderm (bottom white portion in A). B, E) At 8.5 dpc LacZ activity is found throughout the visceral endoderm of the yolk sac and is mosaic within the definitive endoderm (DE). C, F) LacZ expression is found throughout the definitive endoderm including the liver bud (LB) at 9.5 dpc. ME = yolk sac mesoderm.
Figure 2.10. Pancreas specification in Yy1 cKO definitive endoderm. A–D) Immunofluorescence of transverse sections of WT (A–B) and cKO (C–D) 9.5 dpc embryos using YY1 (red), PDX1 (green) and the nuclear stain DAPI (blue). Co-expression of all 3 markers (yellow) is found in both the ventral (A) and dorsal pancreas buds (B) in WT embryos. C–D) Despite the loss of YY1 in the definitive endoderm (blue cells adjacent to asterisk) and its derivatives, both the ventral pancreas (green, C) and dorsal pancreas (green, D) express PDX1. VP = ventral pancreas bud; DP = dorsal pancreas bud.
Figure 2.11. Yolk sac separation reveals layer-specific gene expression patterns. cDNA obtained from WT 9.5 dpc yolk sacs isolated whole (YS) or separated into visceral endoderm (VE) and mesoderm (ME). RT-PCR reveals that VegfA is expressed mainly in the VE. Hnf4α and Pgc1α are expressed exclusively in the visceral endoderm while the VEGF receptors, Flt1 and Flk1, are confined to the mesoderm layer. β-actin expression was used as a loading control.
Figure 3.1. FGF signaling components are expressed in the prehepatic and early hepatic domains before the onset of hepatic markers. A–DD:
Representative transverse sections through the endoderm containing the liver progenitors or nascent liver bud (outlined by the dashed line) at the somite stages (S) indicated followed by in situ hybridization with the indicated probes or immunohistochemistry with pERK. **A–E:** *Fgf8* mRNA is expressed in the putative hepatic endoderm (arrows) and adjacent mesoderm (arrowheads) at 3–4 S (A), is restricted to the adjacent mesoderm from 5–11 S (B–D) and is barely discernible at 12–13 S (E). **F–J:** *Fgf10* is expressed in the mesoderm adjacent to the hepatic endoderm between 3 and 8 S (F–H), is absent in the hepatic region at 9–11 S (I) but is apparent in the septum transversum mesenchyme (STM, lollipop) surrounding liver bud at 12–13 S (J). **K–O:** *Fgfr1* is enriched in the prospective hepatic domain before liver bud formation and in the nascent liver bud at all stages examined. Once the liver bud has formed, *Fgfr1* is also expressed in the STM (N,O). **P–T:** *Fgfr2* is absent from the prehepatic region at 3–4 S (P) but is detected in the prehepatic and hepatic endoderm from 5–13 S (Q,R). *Fgfr2* is expressed in the STM from 9–13 S (S,T). **U–Y:** *Fgfr4* mRNA is absent from the prehepatic endoderm at 3–4 S (U) but is present in the prehepatic endoderm from 5–8 S (V,W). *Fgfr4* exhibits patchy expression in the early liver bud (X,Y). **Z–AA:** pERK is exclusive to the mesoderm before hepatic induction (4S; Z) and expands into the hepatic endoderm at the onset of hepatic induction (8S; AA). **BB–DD:** *Afp, Hnf4α,* and *Prox1* mRNA are first detected at the stages indicated. Arrowhead = mesoderm proximal to hepatic endoderm; arrow = hepatic endoderm; lollipop = STM; dashed line = liver bud. Scale bar = 100 µm.
Figure 3.2. Reduced FGF signaling before induction leads to distinct morphogenetic defects in the liver bud. A–N: Control and SU5402 treated embryos were cultured from 5–6 S and transversely sectioned through the liver domain. A–F: Embryos were cultured for 10 hr and pERK was detected by immunofluorescence. pERK is detected at uniform levels in the hepatic diverticulum.
(dotted line, A) at the anterior of the liver domain and in the thickened hepatic endoderm (arrows, B, C) in the middle and posterior sections. SU5402 treatment results in a uniform loss of pERK in the hepatic diverticulum (D) and in the thickened hepatic endoderm (E, F). G–N: After ~26 hr of culture this control embryo reached 22 S (G) while the SU5402 treated embryos (K) are often slightly delayed and noticeably smaller. Hematoxylin and eosin stained sections through the liver bud were counted and representative sections from the first third (Anterior; H, L), second third (Middle; I, M), and last third (Posterior; J, N) are shown. The dashed line outlines the liver bud. Compared with controls, the treated liver buds displays abnormal morphology in the anterior sections (L) while the remainder of the liver bud is smaller but relatively normal (M, N). O: A comparison of the percentage of control or SU5402 treated embryos with abnormal anterior liver bud development. The embryos are grouped according to the somite number at which culture was initiated. The numbers above each bar represent the number exhibiting the phenotype in transverse section/ the total number examined. Scale bar = 100 µm; dotted line = liver bud.
Figure 3.3. Reduced FGF signaling leads to marked differences in gene expression and cell-death in the anterior versus the posterior liver bud. A–R: In situ hybridization (A–F) or immunofluorescence (G–R) with liver-bud markers performed on transverse sections from control and SU5402 treated cultured embryos. Liver buds are divided into thirds and representative anterior, middle and posterior sections documented. A–F: While Afp expression is uniform throughout the control liver bud (A–C), the treated embryo displays greatly reduced Afp in the anterior and middle section (D,E) compared with the posterior section (F) or those from the same region in controls (A,B). G–R: HNF4α (G–I) and PROX1 (M–O) are uniformly expressed throughout the liver bud of control embryos. In treated liver buds, HNF4α and PROX1 are absent from the anterior (J,P), exhibit detectable levels of expression in the middle (K,Q) but express relatively normal levels in the
posterior (L,R). **S,T:** Sagittal sections through control (S) and SU5402 treated (T) embryos allows for a clear view of the anterior/posterior (top/bottom) and dorsal/ventral (right/left) axis. The magnified images (white boxes in S, T) highlight the uniform expression of HNFα throughout the control liver bud (S'), while HNF4α is restricted to the posterior liver bud in treated embryos (T'). The adjacent ventral pancreas bud expresses the early pancreas marker, PDX1, throughout the ventral pancreas bud of control (S') and treated (T') embryos. **U–Z:** Apoptosis was assessed in transverse sections of the anterior, middle and posterior of control (U–W) and SU5402 treated (X–Z) embryos using cleaved-Caspase 3 (green). **AA:** The percentage of apoptotic cells in the liver bud of control (n = 5) and SU5402 treated embryos (n = 4) was determined. **P < 0.01; *P = 0.045; dashed line = liver bud. Scale bar = 100 µm.

**Figure 3.4.** Altered anterior liver bud morphology after MEK inhibition. **A–H:** Control (A) and U0126 treated (E) embryos were cultured from 5–6 S through 20–22 S and transversely sectioned as indicated by the white line. Hematoxylin and
eosin stained sections through the liver bud (dashed line) were counted and representative sections from the first (Anterior; B, F), second (Middle; C, G) and final third (Posterior; D, H) are shown. The anterior-most liver structure in control embryos is a solid structure (B) but after treatment, the anterior-most region of the liver bud is often devoid of such a structure (F). The middle and posterior liver bud of treated embryos (G,H) are comparable to controls (C,D). I: The percentage of embryos cultured from 5–6 S through 20–22 S that display abnormal anterior morphogenesis is 7% in control and 79% when U0126 is administered. The numbers above each bar represent the number exhibiting the phenotype in transverse section/the total number examined. Dashed line = liver bud. Scale bar = 100 µm.
Figure 3.5. MEK signaling is required for anterior hepatic progenitor differentiation.

A–R: In situ hybridization (A–F) or immunofluorescence (G–R) was performed on transverse sections from control and U0126 treated embryos cultured from 5–6 S through 19–22 S. Representative anterior, middle and posterior liver bud sections are shown. A–F: While liver buds from control embryos express uniform *Afp* (A–C), the liver buds of treated embryos display greatly reduced *Afp* in the anterior sections (D) compared with those from middle or posterior sections (E,F) or from the same region in the control (A). G–R: HNF4α (G–I) and PROX1 (M–O) are expressed throughout the liver bud of control embryos. After treatment with U0126, HNF4α and PROX1 are lost in the anterior (J,P), low in the middle (K,Q) and expressed at near normal levels in the posterior (L,R) liver bud. S,T: Immunofluorescence of sagittal sections (anterior/posterior = right/left; ventral/dorsal = top/bottom) through the liver bud of control and U0126 treated embryos. The endoderm marker FOXA1 highlights the evaginated liver bud and adjacent endoderm in control and treated embryos. While HNF4α is expressed throughout the entire liver bud in control embryos (S), it is relegated to the posterior-most (left) portion of the U0126 treated liver bud. U–Z: Cleaved Caspase-3 is used as an apoptosis marker in liver buds from control (U–W) and U0126 treated (X–Z) embryos. AA: The percentage of apoptotic cells was determined in sections derived from the anterior, middle and posterior of the liver bud in U0126 treated (n = 6) embryos and somite matched controls (n = 4). ***P < 0.005; dashed line = liver bud. Scale bar = 100 µm.
Figure 3.6. Loss of FGF signaling increases the proportion of PDX1-positive cells in the foregut endoderm. A–F: Somite-matched control (A–C) and SU5402 treated (D–F) embryos are transversely sectioned and subject to immunofluorescence with the pancreas marker PDX1 (green), the endoderm marker FOXA1 (white) and counterstained with DAPI (blue). The ventral pancreas bud is outlined by a dashed line. G: The percentage of PDX1 positive ventral pancreas as a total all foregut endoderm nuclei on representative sections from control (n = 4) and SU5402-treated (n = 4) embryos was calculated. The percentage of PDX1 positive cells in the endoderm of treated embryos was significantly increased when compared with controls. H: To determine if pancreas size was altered after SU5402 treatment, the ratio of PDX1 positive ventral pancreas containing sections to the total number of morphologically identifiable liver and PDX1 positive ventral pancreas sections was determined in control (n = 4) and treated samples (n = 4). No significant differences were found in this ratio. Scale bar = 100 μm; ***p = 0.0037
Figure 4.1. Loss of YY1 in the DE results in liver hypoplasia and vascular defects.

A-D) Transverse sections of YY1cKO^{DE} (YY1 cKO) embryos reveals that YY1 (green A, C) is lost throughout much of the liver bud by E9.75. B, D) YY1cKO;R26R embryos stained for LacZ (blue) and counterstained with eosin to reveal expression of the reporter. At E10.5 it is clear that when compared to WT (B) the cKO liver buds (D)
have fewer hepatoblasts (blue), it is also clear that the associated vasculature is less regular than that in WT. White boxes in each represent the magnified view (B',D').

H) A characterization of vessel/hepatoblast interaction revealed that there are 3 types of vessels found in WT: Type I vessels have no surrounding hepatoblasts, Type II vessels have some surrounding hepatoblasts and Type III vessels are completely surrounded by hepatoblasts. G) A comparison of the distribution of WT and cKO Type I-III vessels revealed that the mutant displayed a significant increase in Type I and Type II vessels and a significant decrease in Type III vasculature. I-M) A comparison of E14.5 WT (H-I) and cKO (J-K) demonstrates that the cKO embryos are slightly smaller but have hypoplastic livers (J,K). A comparison of liver to body weight reveals that this ratio is significantly reduced in the cKO embryos (L).

Figure 4.2. The anterior and posterior liver buds contribute distinctly to the rostral and caudal liver lobes. A-D) Sagittal section analysis of WT embryos at the liver forming regions. Immunofluorescence performed with ISL1 (green, secondary heart field), SOX9 (white, STM) or HNF4α (red, hepatoblasts) as indicated. Section analysis at E9.5-9.75 (A-B) reveals that the anterior liver bud (yellow dashed line) is in contact with the sinus venosus (SV) and with the ISL1+ mesenchyme, while the posterior liver bud (blue dashed line) is in contact with the STM. (C) By E 10.0 the anterior liver bud has migrated dorsally and posteriorly into the caudal lobe mesenchyme (CLM). (D) By E10.25 the rostral and caudal liver lobes are both visible. E) A cartoon highlighting the position of the anterior liver bud (yellow) posterior liver bud (blue), and the STM, SV and CLM. F-J) Frontal section analysis of WT embryos as in A-D above highlights the fact that the ISL1+ mesenchyme separates the right and left anterior liver bud. These sections also convincingly show that the CLM has formed the right and left caudal lobes prior to hepatoblast invasion. K-P) Individual embryos labeled with DiI (red) in the VMEL (K) or Lateral liver progenitors between 4-10S (O) as indicated by the corresponding cartoon,
were cultured for 30+ hours and co-localization of the Dil labeled descendants with hepatoblasts noted in the resultant embryos. L-M) Section analysis of two VMEL labeled embryos, demonstrated the presence of Dil in the caudal liver bud (L”,M”) while the rostral liver bud did not contain Dil labeled hepatoblasts. P-Q) This embryo was Dil labeled in the posterior liver bud and labeled descendants are restricted to the rostral lobe (O’) but absent from the caudal lobe (O”).

**Figure 4.3. YY1 is essential for maintenance of HNF4α from the posterior liver bud.** A-H) Sagittal section analysis of WT (A-D) and cKO (E-H) embryos at E9.5 (A, E), 9.75 (B,F), E10.0 (C,G), E10.25 (D,H). A-D) Immunofluorescent imaging with CDH1 (epithelial marker, green), HNF4α (hepatoblast, red) and DAPI (nuclei, blue) reveals that in WT E9.5-E10.0 liver buds, the hepatoblasts in the anterior (A”, B”” C”) and posterior (A’, B’, C’) liver bud uniformly express HNF4α and that the anterior hepatoblast retain epithelial expression of CDH1 while the invaded posterior hepatoblasts have mainly lost CDH1 expression. By E10.25 the hepatoblasts in the rostral (D’) and caudal (D”) lobes express similar levels HNF4α and have mainly downregulated CDH1 in these lobes. E-H) In cKO liver buds HNF4a is maintained at E9.5 but decreases from E9.75 to E10.25 in the posterior liver bud (F’, G’, H’). By E10.0 HNF4a is downregulated and CDH1 is increased in both caudal lobe and rostral lobe (G-G’). At E10.25 both rostral and caudal lobes have lost molecular specification (H-H”). I) An illustration of the sagittal liver bud shown that the rostral lobe (blue) is first affected at E9.75 and both lobes suffer by E10.25.
Figure 4.4. The reduction of apoptosis occurs in hepatoblasts losing HNF4α and VEGFA and their adjacent endothelial cells specifically

A-G) Immunofluorescent analysis of VEGFA (white) and HNF4α (hepatoblast, red) in WT (A-C) and YY1 cKO (D-F) performed at E9.75-10.25 reveals increasing expression of VEGFA over time first in posterior liver at E9.75 (A’) and maintained at E10.0 (B’) and E10.25 (C’) and later in anterior liver starting at low levels (E9.75 A”, E10.0 B”) and reaching comparable levels by (E10.25 C”). YY1 cKO embryos show significant decrease in both posterior (D’, E’, F’) and anterior liver bud (D’, E’, F’) throughout E9.75~10.25. VEGFA intensity for all stages was quantified (G).

H-N) Immunofluorescence of NGN3 (hepatoblast, green), FLK1 (endothelial cell, red), Cleaved caspase 3 (apoptosis marker, white) and DAPI (nuclei, blue) reveals that apoptosis is rare in WT (H-J) but in YY1 cKO occurs in hepatoblasts where VEGFA is reduced and endothelial cells related to those hepatoblasts specifically first in the rostral lobe at E9.75-E10.0 (K’, L’) then in the caudal lobe (M”). Apoptotic hepatoblasts (N) and ECs (O)s were counted for each condition.
**Figure 4.5. VEGF paracrine signaling is required for liver bud maintenance.** To assess the role of VEGF in liver bud maintenance a loss of function and VEGF rescue experiment were performed. A-I) Control or SU1498 treated embryos, dissected at E9.25 and cultured through the E10.5 (~30 hours; A,F). Sagittal section analysis was performed with CDH1 (epithelial marker, green), HNF4α (hepatocyte marker, red) and Cleaved caspase 3 (apoptosis marker, white). Mid-sagittal (B,G) and left-sagittal (C,H) sections reveals that while the gall bladder primordium (gbp) is of a typical size in the treated embryos, the posterior liver bud of SU1498-treated embryos (blue dashed line and G'-H') has lost HNF4α and has increased apoptosis when compared with either WT posterior liver bud (B'-C') or with the anterior liver bud after SU1498-treatment (G''-H''). The cartoon at the end of each panel (E,I) summarized the outcome of each experiment. The gall bladder primordium is represented by the pink bud extending into the STM. J-P) To determine if VEGF can rescue YY1-deficient hepatoblasts, the posterior liver bud and surrounding STM from WT and YY1cKO embryos were dissected free of the surrounding tissue (J) and bisected (K, N). Each bisected half was cultured in the presence or absence of ectopic VEGFA and their growth monitored over the X days in culture. While widespread HNF4α expression is noted in WT liver buds in the absence (L) or presence (M) of ectopic VEGF, no HNF4α expression is found in cKO liver buds in the absence of VEGF but widespread HNF4α is found in the presence of ectopic VEGF. Due to differences in severity noted between cKO embryos, liver bud growth
and the extent of HNF4α expression was always directly compared between VEGF- and VEGF+ liver buds.

Figure 4.6. Summary of WT and YY1cKO liver bud development. Model presenting a paracrine signaling loop through endoderm VEGF to STM that results in mesenchyme signals that support the endoderm. Loss of YY1 results in loss of VEGF and consequent loss of molecular hepatic specification as assessed by HNF4α. The extent of HNF4α expression was always directly compared between VEGF- and VEGF+ liver buds.
Figure 4.7. *FoxA2-CreER* can be used to activate Cre in the DE. A) To activate the inducible-Cre and bypass the bulk of the early visceral endoderm expression, Tamoxifen (TAM) is introduced at E7.75 and the resultant FoxA2<sup>mcm</sup>/+;R26R<sup>+/-</sup> embryos dissected at the indicated stages, stained with LacZ to determine Cre activity and photographed in whole mount at E8.5, 9.0 and 9.5 (B,C,D) with the yolk sac (*) intact. Little LacZ activity is noted in the yolk sacs, while extensive staining is observed throughout the remainder of the FoxA2 expression domain including DE and liver bud, which is confirmed by transverse section followed by eosin counterstaining (B’,C’,D’).
Figure 4.8. The caudal lobe mesenchyme is present prior to anterior liver bud invasion. Mid-sagittal and left-sagittal sections of the liver forming area of E9.5, 9.75 and 10.0 embryos and immunohistochemistry performed with HNF4α (brown) to reveal the hepatoblasts and counterstained with hematoxylin. A) At E9.5 the posterior liver bud (blue dashed line) has begun invading the surrounding STM, while the anterior liver bud is in contact with the SV and is at the entrance to the CLM. Unlike the STM at these early stages, the CLM is surrounded by an epithelial layer. A’) This left-sagittal section demonstrates that the anterior liver bud derivatives abut and not yet entered into the blood filled CLM and that the posterior liver bud has not yet migrated into the lateral areas of the STM. B) This 9.75 embryo demonstrates that the posterior liver bud has migrated further into the STM. B’) Laterally the posterior liver bud has begun invading the STM while the anterior liver bud has entered the CLM. C) By E 10.0 a mid-sagittal section reveals the presence of the gall bladder primordium (pink dashed line) and the putative ventral pancreas progenitors (green dashed line). The posterior liver bud has invaded the STM and the anterior liver bud is in contact with SV. C’) A more lateral section reveals that the posterior liver bud has invaded the lateral STM while the anterior liver bud has more fully integrated into most of the CLM.
Figure 4.9. Lateral progenitor ablation disrupts posterior liver bud formation. A) One side of the laterally located liver precursors were ablated in WT embryos, allowed to culture through the liver budding stage. B-E). After sham-ablated or ablation the embryos are indistinguishable in whole mount. Sagittal section analysis reveals the presence of an HNF4α positive anterior and posterior liver bud (C). The ablated embryo displays an abnormal posterior liver bud that has not yet invaded nor turned on HNF4α, while the anterior liver bud displays HNF4α and appears relatively normal.
BIBLIOGRAPHY


Kelley-Loughnane N., Sabla G.E. & Ley-Ebert C. (2002) Independent and overlapping transcriptional activation during liver development and regeneration in mice. ... 

required to form distinctive large lysosomes in the visceral endoderm cells of the mouse yolk sac. J Biol Chem 284, 33561-70.


Suzuki A., Sekiya S., Büscher D. & Belmonte J.C.I. (2008) Tbx3 controls the fate of hepatic progenitor cells in liver development by suppressing p19ARF expression. ...  


Tremblay K.D. (2010b) Inducing the liver: Understanding the signals that promote murine liver budding. *J Cell Physiol*.


Zhao R., Watt A.J., Li J. & Luebke-Wheeler J. (2005) GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. ... *and Cellular Biology*.