GENERATION AND UTILIZATION OF KNOCKOUT MICE TO ELUCIDATE THE FUNCTIONS OF THE TGF-β PATHWAY IN MAMMALIAN ENDODERMAL SPECIFICATION AND PLACENTAL DEVELOPMENT

DISSERTATION

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By

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*****

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ABSTRACT

Ligands of the transforming growth factor beta (TGF-β) superfamily are involved in numerous developmental and disease processes. TGF-β, Activins, and Nodal ligands operate through Smad2 and Smad3 intracellular mediators. Smad2 mutants exhibit early embryonic lethality, while Smad3 mutants are viable, but show a plethora of postnatal phenotypes, including immune dysfunction and skeletal abnormalities. Previously, we have shown that the Smad2 and Smad3 genes function cooperatively during liver morphogenesis. Here we show that Smad2 and Smad3 are required at full dosage for normal embryonic development. Animals lacking one allele of each gene exhibit a variably penetrant phenotype in which structures in the anterior are reduced or lost; additionally, we demonstrate that this craniofacial defect and the previously reported hepatic phenotypes are both due to defects in the definitive endoderm. A reduction of endodermal gene expression and a failure to displace the visceral endoderm occurs despite the formation of a normal foregut pocket. This precedes any defects in anterior patterning and likely causes the abnormalities observed in craniofacial development and hepatogenesis. Furthermore, to circumvent the early lethality of Smad2 deletion and study the spatially and temporally specific functions of the gene, we generated a Smad2 conditional
allele using the Cre-\textit{loxP} system. In addition, we report that \textit{Smad2}^{\textit{loxP}}, the targeted mutation used to create the \textit{Smad2}\textit{\textit{flox}} conditional allele, is itself hypomorphic.

To elucidate the interactions of TGF-\textit{\textbeta} signaling with other pathways during murine development, targeted deletion of the \textit{Smif} gene is created. \textit{Smif} is a mammalian mRNA decapping protein which specifically interacts with Smad4. While \textit{Smif} heterozygous animals display no detectable abnormality, homozygous knockout mice are embryonic lethal between E10.5 and E11.5 due to placental failure. In addition, we are able to show that while the trophoblast cell lineages of the placenta are relatively unaffected, the vascularization of the nascent allantois is defective in the mutant embryos. Furthermore, mutant embryos exhibit a reduced number of primordial germ cells, a cell lineage which shares the same precursors with the allantois, implying the early onset of the abnormalities in their common progenitors.
DEDICATION

To my mother and father with deep respect and love
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<td>ADE</td>
<td>anterior definitive endoderm</td>
</tr>
<tr>
<td>AME</td>
<td>axial mesendoderm</td>
</tr>
<tr>
<td>A-P</td>
<td>anterior-posterior</td>
</tr>
<tr>
<td>ARE</td>
<td>adenine-uracil-rich element</td>
</tr>
<tr>
<td>AVE</td>
<td>anterior visceral endoderm</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic proteins</td>
</tr>
<tr>
<td>Cer1</td>
<td>cerberus-related 1</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>ExE</td>
<td>extra-embryonic ectoderm</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FoxA</td>
<td>forkhead box A</td>
</tr>
<tr>
<td>Gcm1</td>
<td>glial cells missing-1</td>
</tr>
<tr>
<td>ICM</td>
<td>inner cell mass</td>
</tr>
<tr>
<td>Itga4</td>
<td>integrin alpha 4</td>
</tr>
<tr>
<td>Mrg1</td>
<td>melanocyte-specific gene related gene</td>
</tr>
<tr>
<td>NMD</td>
<td>nonsense-mediated decay</td>
</tr>
<tr>
<td>P-D</td>
<td>proximal-distal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PrCP</td>
<td>prechordal plate mesoderm</td>
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<td>PS</td>
<td>primitive streak</td>
</tr>
<tr>
<td>STM</td>
<td>septum transversum mesenchyme</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Vcam1</td>
<td>vascular cell-adhesion molecule1</td>
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<tr>
<td>VE</td>
<td>visceral endoderm</td>
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CHAPTER 1

INTRODUCTION

The hope to cure the vast majority of human diseases lies in unraveling the underlying molecular and cellular mechanisms. The use of animal models provides a great tool to understand many conserved pathways shared across species. One advantage of mouse as a model system is the easy manipulation of the murine genome as compared to other small mammals; targeted mutation of the murine genome makes it feasible to dissect out the roles of single gene/signaling pathway in complicated processes such as development and cancer progression (reviewed in [1]).

Such an approach has been used to understand the roles of the TGF-β signaling pathway in murine development by introducing targeted mutations into individual components of the pathway (reviewed in [2, 3]). The purpose of this study is to investigate mouse embryos harboring mutations in both Smad2 and Smad3 (both being effectors for TGF-β signaling), in order to understand the cooperative function of the Smads in murine craniofacial and endodermal formation. In addition, a null allele of Smif, a novel Smad interacting protein, is generated and characterized and our work indicates that Smif is important for
allantoic vasculogenesis as well as placental development. Together, these analyses have helped to discover novel mechanisms involved in mammalian embryonic development.

1.1 TGF-β signaling pathway

1.1.1 Smads in TGF-β signal transduction

The TGF-β signaling is one of the major pathways essential for fundamental cell fate decisions during embryonic development and adult tissue homeostasis. Cytokines of the TGF-β superfamily comprise over 30 proteins in mouse, including TGF-βs, BMPs, Activins, Inhibins, and Nodal. These proteins are initially released into the extracellular matrix as latent pre-propeptide precursors. After activation through proteolysis, the ligands dimerize and elicit the assembly of heteromeric receptor complexes. Once activated, the receptor in turn propagates the signal through intracellular transcription factors known as Smad proteins, the primary intracellular proteins used for TGF-β signal transduction (reviewed in [3-6]; Figure 1.1).

Smads are a class of proteins which share similar structures, namely, the highly conserved amino (MH1) and carboxy-terminal (MH2) domains, as well as the less conserved linker region in the middle. Based on structure as well as function, the Smads can be divided into three major classes: R-Smads (receptor activated Smads), Co-Smads (common Smads), and I-Smads (inhibitory Smads). R-Smads function as the direct substrates for the type I receptor, and are activated through phosphorylation on a conserved C-terminal SSXS motif. The
activation of specific R-Smads depend, in part, on the active ligands; Smad2 and Smad3 transduce TGF-βs, Activin, and Nodal signals while Smad1, Smad5, and Smad8 are activated by BMPs. Once activated, the R-Smads recruit a Co-Smad (which is Smad4 in mammals) to form heterooligomers, which then translocate into the nucleus, bind to target promoters, and recruit coactivators such as p300/CBP to activate transcription (reviewed in [7-9]). Contrary to R-Smads and Co-Smads, which positively propagate TGF-β signals, Smad6 and Smad7, known as the I-Smads, antagonize TGF-β signaling. This is done by blocking R-Smad/Co-Smad4 association as a Smad4 decoy, or by masking and further targeting the receptor for degradation, respectively [10-12].

1.1.2 Smif as Smad4 interacting protein

One question concerning TGF-β signaling is how a simple pathway elicits such diverse cellular responses. The answer lies in the various nuclear proteins which function as Smad partners in transcription regulation. Therefore, research on Smad interacting proteins not only sheds light on the regulation of Smad mediated gene expression, but also helps to understand the crosstalk between TGF-β and other pathways (reviewed in [13]).

Smif was first identified as a Smad4 interacting factor by yeast two-hybrid screening [14]. The Human Smif (hSmif) gene yields a 7kb transcript which encodes a 70KD protein. Smif homologues are also present in mouse, zebrafish and Drosophila genomes. It is expressed in almost all human tissues and in mouse embryos from E7–E17. The interaction of Smif with Smad4 is highly
specific in that Smif does not bind with other Smads. Upon TGF-β or BMP stimulation, Smif associates with Smad4 and the protein complex translocates into the nucleus [14]. Consistent with the early embryonic expression in mouse, Smif has been shown to play important roles in vertebrate development. Zebrafish embryos with Smif knocked down using morpholino antisense oligos display various defects including shortened body axis and cyclopia [14], which have been observed in various mutations affecting TGF-β or BMP signaling pathways (reviewed in [15, 16]). In addition, the affected embryos display reduced expression of GATA1, a gene which has been shown to be regulated by the BMP pathway and is essential for blood cell specification [17, 18]. Together, it suggests Smif may function as a transcriptional co-activator in TGF-β/BMP signaling [14].

Meanwhile, three labs independently characterized Smif as a mammalian homologue of the yeast decapping enzyme 1 (Dcp1) [19-21]. The decapping of mRNA is a key step in mRNA turnover in eukaryotes. In Saccharomyces cerevisiae, two interacting proteins are involved in mRNA decapping: Dcp1p and Dcp2p [22-24]. Dcp1p is the major decapping enzyme hydrolyzing the methylated cap on a mRNA [22], whereas Dcp2p is without intrinsic decapping activity and has been suggested to be an activator for Dcp1p in yeast [24]. Unexpectedly, it is human Dcp2 (hDcp2) but not hDcp1 that contains enzymatic activity [19-21]. Unlike hDcp2, the purified hDcp1 does not possess decapping activity in biochemical decapping assays, nor does it stimulate reactions catalyzed by hDcp2 [20]. Recently, it has been reported that hDcp1 helps to
bring other decapping activators into the protein complex protein complex [25].
Notably, there is a second homologue of yeast Dcp1p in the human genome
named hDcp1b, which, similar to hDcp1 (SMIF) shares two conserved domains
with the yeast counterpart. Despite the overall similarity to hDcp1a, there is no
evidence for the involvement of hDcp1b in mRNA decapping [19, 21].

1.2 Craniofacial development

Craniofacial abnormalities (CFA) are one of the major human congenital
defects; one in every 700 live births suffers from CFA. There are over one
hundred human genetic syndromes known to cause craniofacial abnormalities,
however the molecular basis for CFA remains largely unknown. Using mouse as
a model system helps illucidate the mechanisms for CFA (reviwed in [26, 27]).

1.2.1 The mouse AVE for specification of anterior patterning

The early post-implantation mouse embryo consists of three tissues: the
extra-embryonic ectoderm (ExE) and visceral endoderm (VE), both of which are
extra-embryonic; and the epiblast, which will give rise all the tissues of the
embryo. At E5.5, the AVE forms from a discrete population of VE cells located at
the distal tip of the conceptus, which is characterized by the expression of a
specific set of genes, such as Hex, Lim1, goosecoid, and Cer-1 (reviwed in [28,
29]). After induction, the AVE cells migrate unilaterally from the distal tip to the
prospective anterior of the embryo, thereby converting the pre-existing P-D axis
into an A-P axis (reviewed in [30, 31]). Once the AVE has reached the future
anterior, it functions to induce the underlying ectoderm to give rise to anterior
neural and non-neural ectoderm while inhibiting the expression of proximal–posterior genes in the epiblast (reviewed in [28]; Figure 1.2).

Evidence suggesting the crucial role of the AVE in mammalian anterior patterning is derived from both tissue removal/grafting experiments and mouse knockout analysis. Research has shown that forebrain markers are greatly diminished in mouse embryos that have had the AVE removed at the onset of gastrulation [32]. Conversely, when rabbit AVE was placed onto chick epiblast, anterior markers are induced ectopically [33]. In murine embryos, ablation of \textit{Lim1} causes deletion of head structures rostral to rhombomere 3 [34]. To determine the site of action for \textit{Lim1}, a chimeric analysis, in which mutant ES cells could be injected into wildtype embryos and \textit{vice versa}, was carried out. The results indicate that \textit{Lim1} functions in the VE, as embryos displayed the \textit{Lim1} null phenotype of anterior truncation when only the visceral endoderm was depleted of \textit{Lim1}. [34, 35].

1.2.2 The ADE and AME for the maintenance of anterior neural character

Shortly after the migration of the AVE, gastrulation is initiated posteriorly when the epiblast cells ingress through the PS to form the three germ layers (mesoderm, endoderm, and ectoderm). Cells located in the most anterior tip of the PS give rise to the node, ADE, and AME which includes the PrCP and notochord (reviewed in [36]). The ADE and AME move anteriorly and mediately to underlie the medial aspects of the neural plate. By E8.0 the ADE has completely displaced the AVE to the extraembryonic region and covers the entire ventral
surface of the embryo, while the PrCP lies in between the ADE and the anterior neural ectoderm (reviewed in [37]).

Multiple lines of evidence suggest that the ADE and AME are important for the maintenance and regionalization of anterior neural epithelium. Using explant -recombination assays, Ang and colleagues have shown that the ADE and AME from headfold stage embryos are able to stabilize Otx2 expression in the early streak stage ectoderm explants, while posterior mesendoderm represses Otx2 in the ectoderm explants, indicating that anterior mesendoderm functions to maintain anterior neural identity [38].

The fact that the ADE and PrCP share the same progenitors and a large profile of marker genes makes it difficult to dissect their individual roles in the maintenance of anterior identity. Indeed, it still remains controversial about when and how these two cell types start to diverge once they migrate out of the PS. Even so, numerous studies using knockout approaches have shed light on their intertwining functions. Foxa2 is initially expressed in the visceral endoderm. After the onset of gastrulation, its expression is detected at the anterior region of the PS and its derivatives, the ADE and AME [39]. When Foxa2 expression is depleted in the embryo proper but not in the extraembryonic region, the anterior neural ectoderm and anterior definitive endoderm are specified in the conditional knockout mutants, while the PrCP and notochord failed to form. However, at later stages the definitive endoderm failed to maintain its specification. Similarly, anterior neural identity failed to be established in the mutant embryos, which was illustrated by the observed anterior head truncations. These results suggest that
although PrCP is not needed for the initial specification of anterior definitive endoderm and anterior neural fold, it is required for the maintenance of both tissues [40].

*Hex* is initially expressed in the AVE and later in the ADE [41]. Murine embryos homozygous for the null allele of *Hex* display rostral truncation, as well as liver and thyroid dysplasia [42, 43]. Mutant embryos display normal expression of the forebrain markers *Hesx1* and *Six3* at late streak to early somite stages although the expression domain of both genes is significantly reduced at later stages. This indicates that it is the maintenance, but not the initial specification, of the forebrain that is affected in the *Hex* mutant. Furthermore, concepti containing predominantly Hex<sup>-/-</sup> embryonic tissue and wildtype AVE also display anterior truncation, indicating that the ADE is required in the embryo proper for normal forebrain development [43].

1.2.3 Nodal in anterior patterning

The TGF-β ligand Nodal is one of the key molecules involved in embryonic A–P patterning (reviewed in [44]). *Nodal* mRNA is first expressed throughout the primitive ectoderm and the overlying VE shortly after implantation. As development proceeds, a gradient of *Nodal* is formed within the epiblast with the highest expression in the posterior proximal quadrant. Concomitant with the formation of the PS, *Nodal* expression is gradually lost within the visceral endoderm and becomes restricted to the periphery of the nascent node [45, 46]. Murine embryos with 413.d mutation, a proviral insertion within the *Nodal* locus,
display gastrulation defects and fail to form the PS [45]. To distinguish Nodal functions in the embryonic and extraembryonic lineages, mosaic analysis was carried out, which, by using wildtype embryonic stem (ES) cells to rescue Nodal-deficient embryos, suggests that Nodal signaling in the epiblast is primarily responsible for PS formation. However, Nodal signaling has also been shown to be important in the VE for anterior patterning as chimeras with Nodal-deficient VE cells lack rostral brain structures [46]. Moreover, when Nodal was partially inactivated in the epiblast by a conditional knockout strategy using a Mox2-Cre (also known as MORE) deleter strain, reduced Nodal expression in the epiblast caused defects in ADE and axial mesendoderm specification as well as anterior neurectoderm patterning [47].

1.2.4 Smad2 and Smad3 in endodermal and anterior development

Nodal transmits its signals through Smad2 and Smad3 [48]. Smad2 transcripts are expressed ubiquitously during mammalian development [49]. Smad2 is essential for embryogenesis, as Smad2 null mutants die early during development. The severity of the Smad2 deletion phenotype varies for controversial reasons [50]. In the most severe cases, Smad2 homozygous mutant embryos do not go through gastrulation and exhibit reduction in the extraembryonic portion of the egg cylinders and lack of mesoderm, mimicking the Nodal mutation phenotype [51, 52]. A milder Smad2 knockout allele, which may encode a truncated form of Smad2 protein and thus function as a hypomorphic allele [50, 53], results in the entire epiblast to adopt an extraembryonic
mesodermal cell fate. In addition, mutant embryos failed to establish PD and AP axis; the entire epiblast becomes proximal [49]. Chimeric analysis suggested that in the extraembryonic tissue Smad2 plays essential roles in the formation of the three germ layers and the establishment of the body axis [49]. In the epiblast, Smad2 plays a crucial role in directing epiblast derivatives toward an endodermal cell fate as Smad2-deficient ES cells fail to contribute to the definitive endoderm [54], and deletion of Smad2 in an embryonic-specific fashion causes the failure of endodermal development [55].

Smad3, another intracellular mediator for the TGF-β signaling pathway, shares an overall 92% amino acid identity with Smad2 (Madh3 – Mouse Genome Informatics). The major structural difference between the two Smads is that the MH1 domain of Smad2 protein has a thirty amino acid insert which abolishes its DNA binding activity. Similar to Smad2, Smad3 is also expressed in early murine embryos. At pregastrulation stages, Smad3 expression is restricted to the ExE, but expands at late streak stages to compass almost in all tissues examined except the visceral endoderm, indicating Smad2 is the sole Nodal effector in the AVE [54, 56]. Despite its expression in early embryos, targeted gene disruption experiments suggest Smad3 is dispensable for murine embryonic development. Mice that lack Smad3 are viable, although exhibit several abnormal phenotypes including immune dysfunction, osteoarthritis, and accelerated wound healing [57-59]. To further understand the cooperative and/or unique roles of Smad2 and Smad3, Smad2 and Smad3 double heterozygotes were generated in our lab. Our work suggests that both Smad2 and Smad3 are required at full dosage for
normal endodermal patterning. In addition, we have shown in the absence of Smad and Smad3, the defective endoderm causes holoprosencephaly [60]. Similar conclusions are also drawn by Dunn and colleagues [56].

1.3 Liver development

The adult liver serves as the primary site for detoxification and energy metabolism. During embryonic development, the fetal liver functions as a transient site for hematopoiesis, before the hematopoietic cells finally settled in the bone marrow and spleen. Despite the complexity of its functions, the liver is a relatively simple organ, in that about 80% of the cytoplasmic mass is made of hepatocytes, a cellular derivative of the primitive gut (reviewed in [61-63]).

1.3.1 Mechanisms for endoderm competence

The ability or state which renders a cell able to progress down a given lineage in response to an inducer is called competence. Although the hepatic field is limited to anterior ventral endoderm during embryonic development, it has been shown that isolated dorsal endoderm, given proper culture conditions, can also initiate expression of hepatic specific genes [64]. This indicates that the competence for liver formation is not restricted to ventral endoderm; instead, a broader domain of endoderm acquires the potential to follow the program of hepatic specification. Both gene expression analysis and genetic evidence have indicated that one family of transcription factors are particularly important for endoderm competence the Foxa proteins.
Forka proteins are a subfamily of the winged-helix/Forkhead transcription factors. There are three mammalian Forka members identified so far: Forka1 (HNF3α), Forka2 (HNF3β), and Forka3 (HNF3γ), each of which is activated in the nascent definitive endoderm in a sequential manner [65, 66]. Of the three Forka proteins, Forka1 and Forka3 are dispensible for liver organogenesis, while Forka2 is essential for liver formation. Mice lacking Forka2 are embryonic lethal without proper formation of the gut tube [67-70]. Forka2, being one of the earliest transcription factors expressed in the future hepatic endoderm, has been shown to activate its target genes by binding to the promoter and decondensing the packed chromatin structures, thereby relieving repression of gene expression [71].

1.3.2 Specification of the hepatic lineages

The primitive gut gives rise to many internal organs along the anterior posterior axis including the thyroid, lung, liver, and pancreas. Therefore, it has been proposed that the proximal germ layers, specifically mesoderm, must help to specify different endodermal cell lineages [72].

In the case of hepatogenesis, the cardiac mesoderm has been shown to induce isolated endoderm tissue to express liver specific genes in an in vitro organ culture system. Furthermore, it has been demonstrated that it is the FGF ligands released from the cardiac tissue that function as hepatic inducers [73]. STM, another mesoderm tissue that is closely apposed to the hepatic endoderm, also contributes to hepatic induction. Specifically, BMP signaling from the STM
is required for ventral endoderm to adopt hepatic, but not pancreatic, fate ([74, 75]; Figure 1.3).

Hex is expressed in the thyroid, liver, ventral pancreas, and lung primordia as well as hematopoietic lineages during organogenesis [76]. Hex knockout mice exhibit embryonic lethality due to liver hypoplasia. At the onset of hepatogenesis, Hex mutants fail to express a set of early liver-specific genes, although the hepatic field is initially induced. Subsequently, no migration of hepatic endoderm cells is observed. Therefore, Hex is important for the specification but not the initial induction of the hepatic lineages [42, 43].

1.3.3 Liver morphogenesis

Once endodermal cells become committed to the hepatic lineages, they initiate a cascade of transcription factors beginning with Hnf4α, advancing the process of liver differentiation. Meanwhile, migrating cells emerge from the hepatic epithelium and invade into the STM, forming the primitive liver lobes. Once hepatoblasts arrive at their final site, they start to adopt the morphology of polarized epithelium, and together with supporting mesenchymal cells and endothelial cells form the intricate structures of a mature liver.

Hnf4α is a ligand-dependent nuclear hormone receptor, whose expression in mouse embryos is first detected in the VE at E4.5. Later on, it is expressed in the newly formed hepatic diverticulum, and its expression persists in hepatocytes until adulthood [77, 78]. Mouse embryos homologous for the Hnf4α null allele display gastrulation defects, due to the defective VE [79]. When supplied with
wildtype VE, however, *Hnf4α* mutant can survive till E12.0, indicating that Hnf4α is dispensable for the early specification of hepatoblasts. Nevertheless, mutant liver fails to express a panel of genes important for hepatocyte differentiation, including transcription factors HNF4α and pregnane-x-receptor (PXR)[80]. In addition, Hnf4α is also essential for the formation of liver architecture through regulation of cell adhesion molecules such as E-cadherin, ZO1, CEACAM1, and *et al* [81, 82].

1.4 **Allantoic and placental development**

The mammalian placenta is the first organ to develop during embryogenesis, and it is essential for the survival of an embryo. It functions primarily as an exchange center between fetal and maternal circulatory systems, through which the embryo gains a supply of oxygen and nutrients while carbon dioxide and waste products are removed. In addition, the placenta serves as an important endocrine organ, in that it produces and secretes a variety of hormones and growth factors throughout pregnancy which exert profound effects on both fetal development and maternal physiology. The placenta is comprised of three major cell types: the maternal components, the extraembryonic mesoderm, and trophoblast lineages [72, 83].

1.4.1 **Formation of the ectoplacental cone**

The trophoblast cells are descendent of the differentiated external epithelium of the blastocyst. In a late blastocyst, trophoblast proliferation is restricted to the polar trophectoderm, which overlies the ICM, while the mural
trophectodermal cells, which lack contact with ICM, exit the cell cycle and terminally differentiate into polyploidy trophoblast giant cells through genomic endoreduplication (reviewed in [84, 85]). After implantation, the polar trophectoderm cells proliferate and differentiate as to give rise to ectoplacenta cone and ExE, which further expand to form the chorionic ectoderm in postgastrulation embryos (reviewed in [72, 86]).

1.4.2 Allantois and its vasculogenesis

The allantois is essential for placental formation. It generates the umbilical cord which connects the developing embryo with the placenta; in addition, the allantois provides fetal vasculature to the placenta (reviewed in [87, 88]). At the neural fold stage, a nascent allantoic bud appears at the posterior end of the embryo between the yolk sac and amnion. The allantois grows into the exocoelomic cavity through combinational effects of cell proliferation, continuous addition of mesodermal cells from the posterior PS, and distal cavitation [89]. The allantoic mesodermal cells can be divided into two major cell types: the mesothelium and the inner core cells. The mesothelium, which forms the outer layer of the allantois and contains junctions between the flattened cells, are devoid of angioblasts, the precursors for endothelial cells. In contrast, the inner core cells are free of cell-cell junctions and maintain the potential to differentiate into angioblasts [90]. Vasculogenesis in the allantois takes place shortly after the emergence of the allantoic bud. Starting from the distal allantois, angioblasts amalgamate and differentiate to form nascent blood vessels, which
then spread proximally and fuse with the dorsal aorta at the base of the allantois. Unlike vasculogenesis in the yolk sac or dorsal aorta, the *de novo* formation of blood vessels in the allantois is not accompanied by simultaneous hematopoiesis [91]. Although it has been shown that the vascular labyrinthine region of mouse placenta contains hematopoietic stem cells, they are not likely precursors for the hematopoietic cells initially found in the allantois [92, 93].

### 1.4.3 Chorioallantoic fusion

Chorioallantoic fusion refers to the process during which the allantois achieves contact with the chorionic plate and, through a series of molecular and cellular events, the allantoic mesodermal cells form cell-cell contacts with the chorionic mesothelium lining (reviewed in [83, 94]; Figure 1.4). Insights into fusion mechanisms are largely obtained from analyzing mouse mutants displaying fusion defects, such as *Vcam1* and *Itga4* mutations. Interestingly, Vcam1, a cell adhesion molecule, is expressed only at the distal region of the allantois prior to fusion. In contrast, the expression of Itga4, which encodes for Integrin α4, a possible partner of Vcam1, is restricted to the basal surface of the chorion [95-97]. The similarity between the phenotypes resulted from targeted mutation of *Vcam1* and *Itga4*, as well as the complimentary expression patterns of the two genes, strongly suggests that the Vcam1- Itga4 interaction is important for normal fusion of allantois with chorion [83]. In addition, targeted mutation of genes essential for embryonic patterning can also result in fusion defects, such as *Brachyury (T)* knockout [98]. The BMP pathway, a subset of the TGF-β family,
has also been shown to be very important for chorioallantoic fusion since embryos harboring targeted mutation alleles of *Bmp4*, *Bmp5*, *Bmp7*, and *Smad1* exhibit fusion defects [99-102]. It is likely that BMP signaling functions upstream of Vcam1, as allantoic expression of Vcam1 is absent in *Bmp4* and *Smad1* mutants [100, 102].

### 1.4.4 Formation of the labyrinth

Shortly after the allantoic attachment, the initially flat sheet of chorionic trophoblast cells folds internally. These folds are separated evenly and indicate the sites into which the fetal blood vessels will grow into, where the stromal cells and blood vessels from the allantois interdigitate with the chorionic trophoblast cells to form placental villi. The villi then go through extensive elongation and branching to create the densely packed labyrinthine structure [94]. Murine placental villus is composed of a fetal blood vessel enveloped by three layers of cells: two layers of syncytiotrophoblast cells in direct opposition to the endothelial cells, as well as a third layer of mononuclear cells of unknown origin. The fetal villi are bathed directly in spaces filled with maternal blood to facilitate efficient material exchange between the two systems, while avoiding an unwanted mixture of blood cells [83].

Labyrinth/villi formation is important for the proper functioning of the placenta, therefore, genetic mutations affecting labyrinth development often cause mid-gestation lethality [83, 103]. A good example is *Gcm1*, which encodes a transcription factor and is expressed in a subgroup of trophoblast cells at
villous branchpoints [104]. *Gcm1* null placentae display no chorionic invagination as well as failure in labyrinth formation [105, 106].

### 1.4.5 Differentiation of the trophoblast lineages

The placenta is composed of multiple specialized subtypes of trophoblast cells which not only provide structural components but also release a variety of hormones and cytokines important for placental development. As diverse as the trophoblast subtypes are, they are all derived from trophoblast stem cells which reside in the chorionic plate and follow distinct molecular pathways to form various differentiated trophoblast cells upon induction by neighboring cell types (reviewed in [86, 94, 107]). It has been shown that bHLH transcription factors Hand1 and Stra13 promote giant cell differentiation [108-110], while Mash2 is autonomously required for the formation of spongiotrophoblast, the middle layer of trophoblasts in between the outer giant cells and the inner labyrinth [111]. As for the syncytiotrophoblast, both targeted gene mutation analysis and *in vitro* differentiation of trophoblast stem cells suggest Gcm1 is essential for syncytiotrophoblast differentiation [105, 110].
Figure 1.1 TGF-β and BMP family ligands signal through distinct Smads. (Adapted from [112]).
Figure 1.2. The conversion of P-D axis into A-P axis in mouse embryo. Graphical representation of cell movement and tissue formation during the process. (Adapted from [28]).
Figure 1.3. The specification of hepatic endoderm. (A) Graphical view of a parasagittal section of a mouse embryo at E8.25. (B) Representation of magnified region in solid box in A. The hepatic field is specified by the cardiac mesoderm and the STM. (Adapted from [62]).
Figure 1.4. The mouse placenta. (A) The placenta is formed by the fusion of allantois with chorionic plate. (B) Graphical representation of murine placenta structure. The inset shows the fetal-maternal blood interface within the labyrinthine layer. (Adapted from [83])
CHAPTER 2

SMAD2 AND SMAD3 COORDINATELY REGULATE CRANIOFACIAL AND ENDODERMAL DEVELOPMENT

2.1 Introduction

The endoderm is one of the primary germ layers formed during gastrulation and as such lines the gut and associated organs. Its development is of pivotal importance during organogenesis, as thousands of children are born every year with gastrointestinal malformations, and tissues of endodermal origin are the most frequent sites of neoplasias. Even so, the mechanisms underlying endodermal determination and differentiation are still quite enigmatic.

Murine embryos are ensconced within the visceral endoderm, which is an extraembryonic cell type that does not give rise to any embryonic structures, but plays an important part in patterning the embryo. In mice, the definitive endoderm is formed during gastrulation around day 6 of development, and by day 7 comprises a layer of cells lying outside the embryo. Morphogenetic movements between day 7 and day 8 bring it within the embryo, and by day 9 of development the endoderm forms a closed tube. During organogenesis, the endoderm is regionalized under instructive cues from other embryonic structures
to form the alimentary canal and its associated organs, including the thyroid, liver, lungs, and pancreas [113].

Nodal, a ligand of the transforming growth factor-beta (TGF-β) superfamily, appears to play a crucial role during endodermal development. Nodal is expressed at the node, the murine organizing center, and embryos lacking Nodal fail to gastrulate, differentiate mesoderm, or form definitive endoderm [114]. A hypomorphic allele of Nodal has extended these findings to show that decreased Nodal signaling leads to defects in endodermal formation, and endodermal development is more sensitive to attenuation in Nodal signaling than mesodermal differentiation [115]. In addition, mutants lacking Cripto, an essential Nodal associated factor, also lack definitive endoderm [116].

Studies in other vertebrate model systems have also underlined the importance of Nodal signaling to endodermal development. Zebrafish mutants lacking the Nodal homologs Cyclops and Squint fail to differentiate endoderm [117], as do embryos lacking the Cripto homolog one-eyed pinhead [118, 119]. Indeed, enforced TGF-β signaling in zebrafish embryos, accomplished through expression of a constitutively active TGF-β receptor, is sufficient to drive embryonic cells toward an endodermal fate [120].

Ligands of the TGF-β superfamily transmit their signals through a conserved set of intermediaries, the Smad proteins. The TGF-β receptors form a heterodimeric complex that phosphorylates the receptor-activated Smads (e.g. Smad2 and Smad3) at the conserved SSXS motif in the C-terminal MH2 domain, which then associate with Smad4 and translocate to the nucleus. These Smad
proteins can then activate or repress transcription of target genes, depending on the available collection of transcriptional cofactors, such as the forkhead transcriptional activator FoxH1 [112]. Nodal transmits its signals through Smad2 and Smad3 [48]. Smad2 is essential for mammalian development, as Smad2 mutants die early during development and fail to form endoderm [49, 51, 52, 121, 122]. Hypomorphic Smad2 mutants exhibit defects in formation of the foregut [123], Smad2-deficient ES cells fail to contribute to the hindgut in a chimeric analysis [54], and deletion of Smad2 in an embryonic-specific fashion causes the failure of endodermal development [55]. Mice that lack the highly homologous Smad3 are viable, but exhibit several phenotypes including immune dysfunction, osteoarthritis, and accelerated wound healing [57-59]. Smad2 and Smad3 function cooperatively during liver development as demonstrated by the fact that double heterozygotes exhibit abnormal hepatogenesis due to a failure of hepatocytic cell adhesion [124].

Here we demonstrate that both Smad2 and Smad3 are required at full dosage for normal embryonic patterning. Animals lacking one allele of each gene exhibit a variably penetrant and expressive phenotype in which they display craniofacial and midline defects ranging from mild holoprosencephaly (HPE) to severe midline defects that compromise cardiogenesis and lead to early embryonic lethality. Other embryos exhibit a defect in which the endoderm fails to populate the liver bud, leading to hepatogenic failure. We demonstrate here that these phenotypes are due to defects in the definitive endoderm. We have uncovered a deficit in endodermal gene expression as well as a failure of the
definitive endoderm to displace the visceral endoderm. This endodermal failure, which occurs despite the formation of a normal foregut pocket, precedes and likely causes the abnormalities seen in craniofacial and midline patterning, as well as the hepatogenic defects.

2.2 Results

2.2.1 The Smad2ΔC allele results in a lack of Smad2 protein

The Smad2ΔC allele was designed to use a neomycin cassette to replace exon 10 and part of exon 11, which together encode for 87 amino acids of the Smad2 MH2 domain [52]. To examine the effects of Smad2ΔC allele on Smad2 protein expression, Smad2ΔC/ΔC ES cells were generated. Smad2+/ΔC ES cells were electroporated with a targeting vector in which the Smad2ΔC mutation was introduced with a hygromycin cassette (Fig. 2.1A). ES cells that were resistant to hygromycin and gancyclovir were isolated, and Southern analysis using the flanking probe (Fig. 2.1A) revealed two clones that had undergone homologous recombination at the remaining wild-type Smad2 allele (Fig. 2.1B). Western blots showed that Smad2ΔC/ΔChyg cells had no detectable level of Smad2 protein or any truncated product (which, if existing, would be around 47kd, Fig. 2.1C), suggesting that the Smad2ΔC mutation results in a null allele. Other groups have shown that disruption of Smad2 results in a haploinsufficiency phenotype in which some embryos exhibited gastrulation defects, while others showed craniofacial abnormalities [121, 125]. Our recent analysis showed that in crosses involving Smad2+/ΔC mice, the Smad2+/ΔC mutation resulted in embryonic
abnormalities in 10 out of the 208 Smad2^{+/ΔC} embryos examined (Fig. 2.1D). To further characterize the external embryonic tissues seen in these abnormal Smad2^{+/ΔC} embryos, marker analysis has been performed with Otx2. The presence of Otx2 signal in the anterior of these embryos, albeit weak (Fig. 2.1E), suggested that in these embryos the A-P axis is specified. Similarly, analysis of Sonic hedgehog (Shh) expression suggested the presence of axial midline tissue in the mutants (Fig. 2.1F). A similar phenotype has been seen in mutants with decreased Nodal expression [126], and has been attributed to gastrulation defects.

2.2.2 Smad2^{+/ΔC};Smad3^{+/−} embryos exhibit craniofacial defects

We have previously reported the lethality of Smad2^{+/ΔC};Smad3^{+/−} embryos due to hepatic dysplasia by E14.5 [124]. Detailed examination of embryos from Smad2^{+/ΔC} and Smad3^{+/−} crosses revealed that Smad2^{+/ΔC};Smad3^{+/−} embryos can be recovered at a normal Mendelian ratio through E10.5 (Table 2.1). However, 54 out of the 106 Smad2^{+/ΔC};Smad3^{+/−} embryos examined at E9.5-E10.5 displayed patterning abnormalities of varying severity (Table 2.1). Notably, in Smad2^{+/ΔC} and Smad3^{+/−} crosses, Smad2^{+/ΔC};Smad3^{+/−} embryos which appear with gastrulation defects were recovered at a ratio comparable to that of the Smad2^{+/ΔC}, in that out of the 25 such embryos examined at E8.5-E10.5, 15 were Smad2^{+/ΔC};Smad3^{+/−} and the other 10 were Smad2^{+/ΔC}. This suggested that the decreased expression of Smad3 did not have a major effect on the frequency of Smad2-dependent gastrulation failure.
Some of the \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} embryos examined at E9.5 and E10.5 (N=28) exhibited midline defects (Fig. 2.2A) and bore a close resemblance to the most mildly affected class of \textit{FoxH1} mutant embryos [127]. In addition, abnormal heart looping and an enlarged pericardiac cavity were often observed in this group of embryos, which was likely the cause of early embryonic lethality. The less affected mutants exhibited defects ranging from holoprosencephaly (HPE) and cyclopia, such as the E12.5 embryo shown in Figure 2.2B, to even milder phenotypes, in which the eyes were moved ventrally and the first branchial arch derivatives were reduced, but not deleted altogether, an example of which is shown at E14.5 in figure 2.2C. None of these abnormalities were observed in \textit{Smad2}^{+/\Delta C} or \textit{Smad3}^{+/-} embryos (Table 2.1), indicating the craniofacial and midline defects were the result of combined \textit{Smad2} and \textit{Smad3} deficiency.

To determine the nature of the observed patterning perturbations in the \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} mutant embryos, marker analysis was carried out at E9.5, the earliest stage at which obvious abnormalities can be observed. \textit{Fgf8} expression in \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} embryos without patterning defects was indistinguishable from that in \textit{Smad3}^{+/-}, \textit{Smad2}^{+/-\Delta C}, or wild-type siblings, while all of the phenotypically abnormal \textit{Smad2}^{+/-\Delta C};\textit{Smad3}^{+/-} embryos displayed perturbations in \textit{Fgf8} staining. In the mildly affected mutants the expression of \textit{Fgf8} appeared normal in the isthmus organizer, the pharyngeal region as well as other domains in the posterior regions of the mutant embryos, but its expression is completely lost in the telecephalic commissural plate as well as the surface ectoderm covering the nasal processes (Fig. 2.3A-C, and data not shown).
Subsequent sectioning of the embryos showed that in contrast to the wild-type embryos, where the rostral end of the neural plate directly contacted the surface ectoderm, the mutant neural plate was separated from the surface ectoderm by cells that appear mesenchymal in morphology (Fig. 2.3B and C, arrow in C). These data support the notion that the rostral end of the brain is the region that is most susceptible to patterning defects in the Smad2+/ΔC;Smad3+/- animals.

Examination of Fgf8 expression at E8.5 showed normal levels of Fgf8 in the Smad2+/ΔC;Smad3+/- mutants, suggesting that its loss was of secondary consequence to other embryonic defects.

Next, Otx2 expression was examined to determine whether there was loss of anterior cell fates in the severely affected mutant brains. While the level of Otx2 expression appeared normal in both forebrain and midbrain of the mutants, the domain of expression was much reduced (Fig. 2.3D). Notably, in contrast to the wild-type embryos, which had contiguous Otx2 expression throughout its domain, there appeared to be a break in the staining at the junction between the prosencephalon and metencephalon as well as unstained tissue at the rostral end of the brain. This is presumably due to ectopic mechenchymal cells such as those seen in figure 2.3C. Like Fgf8, Otx2 expression in E8.5 Smad2+/ΔC;Smad3+/- mutants was normal, even in those with craniofacial abnormalities.

The morphologically abnormal midline in some embryos prompted us to examine midline cell fates. Both Foxa2 (Hnf3β) and Shh are expressed in the gut and embryonic midline, and mutations of Shh result in HPE in humans [128,
Those Smad2+/\Delta C;Smad3+/\epsilon embryos that exhibited a mild craniofacial phenotype showed a loss of Shh expression in its most anterior expression domain (arrow in Fig. 2.3E), suggesting defects in the prechordal plate underlying the diencephalon. Mutants displaying more severe midline defects exhibited a more marked loss of Shh rostrally, together with overall reduction in staining intensity throughout the Shh expression domain (Fig. 2.3F). Smad2+/\Delta C;Smad3+/\epsilon mutants that were phenotypically normal also exhibited normal labeling of Shh. It is unlikely that reduction in Shh expression is the primary cause of the observed defects, as there was no observable perturbation of Shh expression in the E8.5 embryos examined (data not shown). Mutants with midline defects also exhibited deficits in the expression of Foxa2, which was also absent rostrally and reduced elsewhere compared to wild-type siblings (Fig. 2.3G). These results confirmed that the Smad2+/\Delta C;Smad3+/\epsilon embryos displaying anterior defects also exhibit deficiencies in the embryonic midline, the degree of which corresponding to the severity of the defects in the mutants.

2.2.3 Compound haploinsufficiency of Smad2 and Smad3 results in defects of the definitive endoderm.

Notochord and endoderm are both anterior primitive streak derivatives, and endodermal defects have been correlated with anterior and midline defects in multiple model systems. We therefore examined the endoderm in the Smad2+/\Delta C;Smad3+/\epsilon embryos. Foxa2 is necessary for endodermal specification [130], and it has been suggested to be a downstream target of Nodal signaling in
murine visceral endoderm [131]. Therefore the expression of Foxa2 was examined in Smad2+/ΔC;Smad3+/-, wild-type, Smad2+/ΔC, or Smad3+/- embryos at E8.5, (just before the onset of the majority of the patterning defects). Although the chordamesodermal and prechordal plate staining appeared normal in the Smad2+/ΔC;Smad3+/- embryos, endodermal staining was reduced in all of them (Fig. 2.4A). Histological sections of whole-mount embryos further illustrated this diminution of endodermal Foxa2 expression. Wild-type embryos exhibited labeling of most foregut cells (Fig. 2.4B), while the Smad2+/ΔC; Smad3+/- embryos showed significantly reduced expression in the foregut, despite normal expression in the notochord and floor plate of the neural tube (Fig. 2.4C).

The decrease of Foxa2 expression in the Smad2+/ΔC;Smad3+/- embryos suggested that Foxa2 might be a target gene of Smad2 and Smad3 in definitive endoderm. To test this hypothesis, RNAs encoding either wild-type, or dominant negative Smad2 were injected into the 4 vegetal blastomeres of 8 cell stage Xenopus embryos, thus targeting the endoderm and most of the mesoderm. Embryos were allowed to develop into gastrulae, and Foxa2 expression was examined by real-time RT PCR. The results, shown in figure 2.4D, demonstrate that injection of wild-type Smad2 resulted in a five-fold increase in Foxa2 levels, while injection of the dominant negative Smad2 resulted in slight decreases in Foxa2. These data suggest that Foxa2 is responsive to Smad2 signaling.

To further characterize the abnormalities of the foregut endoderm, we examined Albumin, which is expressed in foregut endoderm at E8.5 [132]. However, the expression of Albumin was considerably reduced in the
Smad2^{+/ΔC};Smad3^{+/−} embryos (Fig. 4E, bottom). The fact that decreased expression of an endodermal gene occurs at a stage before the onset of any morphological defects suggests that the abnormalities in the foregut endoderm specification preceded, and were possibly the cause of the later craniofacial and midline phenotypes observed in Smad2^{+/ΔC};Smad3^{+/−} embryos.

2.2.4 Smad2^{+/ΔC};Smad3^{+/−} embryos display multiple defects in the specification of hepatogenic endoderm

The Smad2^{+/ΔC};Smad3^{+/−} embryos exhibited liver hypoplasia, if they did not arrest at earlier stages. The mutant liver suffered from abnormal cellular adhesion which could be corrected by in vitro HGF administration [124]. Since the liver is derived from the endoderm, and abnormal endoderm specification was observed in all of the Smad2^{+/ΔC};Smad3^{+/−} embryos, we examined the hepatogenic endoderm in the mutant embryos. Hex is among the earliest genes that are expressed in that part of the endoderm that will later migrate into septum transversum mesenchyme (STM) and give rise to hepatocytes, a major cell type in the liver [76]. Mice lacking Hex exhibit abnormalities of both the head and the liver [42, 43]. E8.5 Smad2^{+/ΔC};Smad3^{+/−} embryos and their littermates were labeled for Hex, which was expressed in the hepatogenic endoderm of the wild-type embryos (Fig 2.5A). We found that Hex expression was dramatically reduced in the Smad2/3 double heterozygotes (Fig. 2.5B).

The foregut pocket appeared grossly normal in the Smad2^{+/ΔC};Smad3^{+/−} embryos, although the endoderm was clearly abnormally specified. We
considered the possibility that the foregut was lined with visceral endoderm and therefore probed E8.5 Smad2^{+/ΔC};Smad3^{+/-} embryos and their siblings for alpha-fetoprotein (Afp), which stains the visceral endoderm and the parietal endoderm of the yolk sac in normal embryos (Fig. 2.5C). Ectopic Afp positive cells were observed at the anterior intestinal portal in the Smad2^{+/ΔC};Smad3^{+/-} embryos, occupying the space adjacent to the heart where the endoderm will give rise to the hepatogenic lineage in response to cardiac signals (Fig. 2.5D). This result was seen in all of the Smad2^{+/ΔC};Smad3^{+/-} embryos examined, and suggested abnormal persistence and location of the visceral endoderm. Although only some of the Smad2^{+/ΔC};Smad3^{+/-} embryos exhibited craniofacial or midline defects, all of the Smad2^{+/ΔC};Smad3^{+/-} embryos examined with endodermal markers (N=21) showed abnormal gene expression.

2.2.5 Endodermal defects in Smad2^{+/ΔC};Smad3^{+/-} embryos lead to hepatogenic failure

The presence of visceral endoderm adjacent to the heart and the lack of Hex expression suggested the existence of profound abnormalities in the hepatogenic endoderm. Although the lack of Hex in the Smad2^{+/ΔC};Smad3^{+/-} embryos may not have been cell autonomous, we felt that there still might be a role for Smad2 and Smad3 in the regulation of the Hex gene. Previously, Smad1 has been shown to bind to the Hex promoter, but not Smad2 or Smad3 [133]. However, Hex was expressed at comparable levels in E9.5 wild-type and Smad1 homozygous liver buds (Fig. 2.6A, B), suggesting that Smad1 alone does not
transactivate the \textit{Hex} promoter. However, \textit{Hex} expression was sharply reduced in E9.5 \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} embryos, suggesting a defect in the hepatogenic endoderm, rather than a delay in its development (Fig. 2.6C, D). The \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} embryos were sectioned along with their normal counterparts to closely examine sites of \textit{Hex} expression. It was clear in the wild-type embryos that the hepatogenic endoderm had already dilated, and that \textit{Hex} positive hepatocytic precursor cells were migrating into the STM (Fig 2.6E). However, \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} mutants appeared to suffer from a delay in hepatogenesis, as the endoderm was not dilated. Although a few \textit{Hex} expressing cells were evident, these did not appear to be undergoing migration or hepatocytic development (Fig. 2.6F). The lack of \textit{Hex} expression in the \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} embryos may not have been entirely cell autonomous.

Hepatogenesis is initiated under the control of cardiac mesoderm and STM, which secrete FGF and BMP signals. These ligands program the endoderm to assume a hepatogenic fate (reviewed in [62]). It is unlikely that the ectopic visceral endoderm could respond to these hepatogenic signals or contribute to the hepatocytic lineage.

\section*{2.2.6 \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} embryos develop liver despite large reductions in the hepatocytic lineage}

The observation of early hepatogenic defects in the \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} embryos is somewhat paradoxical, as the \textit{Smad2}^{+/\Delta C};\textit{Smad3}^{+/-} embryos develop livers, albeit abnormal ones. In order to further characterize this early defect, we
examined the expression of *Hnf4*, which is required for differentiation of the hepatocytic lineage, is expressed during early stages of hepatogenesis [80], and for which the protein product physically interacts with Smad3 and Smad4 [134]. *Hnf4* expression was evident in the liver bud in E9.5 embryos (Fig. 2.7A), and like *Hex*, was expressed in cells migrating out of the endoderm into the STM (Fig. 2.7C). *Hnf4* was expressed in the endoderm of the *Smad2^+/ΔC;Smad3^+/−* embryos at reduced levels, and *Hnf4*-positive cells were not seen migrating into the STM (Fig. 2.7B, D).

The lack of endodermal migration and hepatocytic differentiation could have been indicative of a delay or an outright block in hepatogenesis. To differentiate these two possibilities, E10.5 *Smad2^+/ΔC;Smad3^+/−* and sibling embryos were examined for the expression of *Afp*, an early marker of the hepatocytic lineage [135]. Chords of hepatocytes were observed to express *Afp* and populate the liver bud in the sibling control embryos (Fig. 2.7E, G). These cells were not absent in the *Smad2^+/ΔC;Smad3^+/−* embryos, but their number was sharply reduced (Fig. 2.7F, H). These results were confirmed by an examination of *Hex* expression in E10.5 embryos. Cells expressing *Hex* could be seen throughout the liver bud in normal embryos (Fig. 2.8A), and these cells were seen to populate the liver parenchyma in histological sections (Fig. 2.8C). *Smad2^+/ΔC;Smad3^+/−* embryos exhibited a reduced number of *Hex* positive cells (Fig. 2.8B) despite the fact that the lobes of the liver are clearly discernable in histological sections (Fig. 2.8D). Although some of these *Hex*-expressing cells had migrated out of the endoderm, they did not appear to be as numerous within
the liver parenchyma as in the normal embryo (Fig. 2.8D). From these data we concluded that the hepatocytic differentiation program was both delayed and attenuated in the $Smad2^{+/\Delta C};Smad3^{+/\cdot}$ mutants. It is interesting that $Hex$ expression in the thyroid was also reduced in the $Smad2^{+/\Delta C};Smad3^{+/\cdot}$ mutants compared to wt (Fig. 2.8E, F), further suggesting control of $Hex$ expression by Smad2 and Smad3. In addition, the $Smad2^{+/\Delta C};Smad3^{+/\cdot}$ mutant exhibited ectopic $Hex^+$ cells near the ventral floor of the oropharynx (Fig. 2.8F, arrowhead). These may be thyroid precursors that have failed to complete their migration to the thyroid. A similar phenotype has been seen in mutants lacking Titf2 [136].

### 2.2.7 Variable Penetrance of Phenotypes in $Smad2^{+/\Delta C};Smad3^{+/\cdot}$ embryos

The wide variations in the phenotypes seen in the $Smad2^{+/\Delta C};Smad3^{+/\cdot}$ embryos prompted us to examine the cause. Although levels of Smad2 and Smad3 are reduced in these mutants by one half [124], it is possible that levels of phospho-Smad2 or phospho-Smad3 are altered to a greater extent. To examine this possibility, western blots of tissues derived from both normal and holoprosencephalic E9.5 $Smad2^{+/\Delta C};Smad3^{+/\cdot}$ mutants were probed with a phospho-Smad2 antibody. We were unable to detect consistent differences between those $Smad2^{+/\Delta C};Smad3^{+/\cdot}$ mutants that exhibited craniofacial defects and those that did not, suggesting that varying levels of phospho-Smad2 were not the cause the observed phenotypic variability (data not shown).

Another possibility underlying the variation in phenotypic abnormalities would be modifier genes within the strain background, which could vary between
individuals. To test this hypothesis, Smad2 and Smad3 mutant alleles were bred onto the A/J strain background. Out of the 29 E10.5 embryos examined for crosses of Smad2$^{+/\Delta C}$ and Smad3$^{+/\Delta C}$ mice on the A/J strain background, we recovered 3 Smad2$^{+/\Delta C}$;Smad3$^{+/\Delta C}$ embryos (Table 2.2), which failed to exhibit either craniofacial or liver defects, the latter judged by staining with Afp. This prompted us to examine animals at postnatal stages, as roughly half of the Smad2$^{+/\Delta C}$;Smad3$^{+/\Delta C}$ embryos died of liver defects without accompanying craniofacial abnormalities [124]. Unexpectedly, these crosses resulted in the birth of 3 Smad2$^{+/\Delta C}$;Smad3$^{+/\Delta C}$ mice (Table 2.2), which are one month of age as of this writing showing no observable abnormalities. Although we were able to recover occasional Smad2$^{+/\Delta C}$;Smad3$^{+/\Delta C}$ mice at weaning on a mixed 129Svev/NIHBlack Swiss background (Table 2.1), their occurrence was rare, none passed both Smad2 and Smad3 alleles through the germ-line, and most perished shortly after weaning ([124] and data not shown). These data suggest that modifier loci within the strain background can affect the penetrance of the Smad2 and Smad3 mutations on liver development. Further analysis will be needed to determine the effect of strain background on Smad2 and Smad3 functions in craniofacial and midline patterning.

2.3 Discussion

2.3.1 The Smad2$^{\Delta C}$ allele results in a null mutation of Smad2.

A number of groups have reported mutations at the Smad2 locus that have produced different phenotypic abnormalities in the homozygous embryos.
Defects of the extraembryonic ectoderm and endoderm, which were seen in the
$\text{Smad2}^{\Delta C/\Delta C}$ mutant embryos, have been reported by some groups [52, 121, 125],
while others observe a defect in the anterior/posterior axis resulting in a loss of
epiblast cell fates [49, 122]. In this report we have generated ES cells that are
homozygous for the $\text{Smad2}^{\Delta C}$ mutation in order to determine if protein is
produced. Western analysis suggests that Smad2 protein is absent in these cells,
including any possible truncated product. Given that the phenotype seen in the
$\text{Smad2}^{\Delta C/\Delta C}$ mutants was more severe than that seen in other studies, we were
concerned that the $\text{Smad2}^{\Delta C}$ allele could create a dominant negative mutation
that would inactivate other Smad proteins and exacerbate the phenotypic
abnormalities seen in the homozygous mutants. However, overexpression of a
similar $\text{Smad2}^{\Delta 9,10}$ mutant RNA in $\text{Xenopus laevis}$ embryos does not cause any
observable defects, despite the fact that a dominant negative $\text{Smad2}$ allele
recapitulates previously observed phenotypes in $\text{Xenopus}$ [50]. The $\text{Smad2}^{\Delta 9,10}$
allele phenocopies the $\text{Smad2}^{\Delta C}$ mutant [50], suggesting that both alleles are
indeed null.

A haploinsufficiency phenotype has been reported for some $\text{Smad2}$
mutant alleles [121, 125], and in recent crosses we are able to detect gastrulation
defects in roughly 6% of all the embryos examined with a $\text{Smad2}^{\Delta C}$ allele, and did
not appear to be dramatically exacerbated in the absence of a $\text{Smad3}$ allele. It is
possible that these were not seen in our original report due to some alteration in
the strain background. Although we have observed $\text{Smad2}^{+/\Delta C};\text{Smad3}^{+/-}$
embryos with craniofacial abnormalities, we did not detect the reported
craniofacial abnormalities seen in $Smad2^{+/\Delta}$ embryos [125]. However, most of our analysis was focused on embryonic stages in which a milder craniofacial defect could have remained undetected.

2.3.2 Anterior development in $Smad2^{+/\Delta C};Smad3^{+/\Delta}$ embryos.

Nodal signaling is required for anterior development. One way to address the requirement for graded Nodal signaling in these processes is the attenuation of Smad2 and/or Smad3, effecters of the Nodal signaling pathway. A previous report showed that deletion of $Smad2$ in epiblast tissues resulted in defects in the specification of endoderm and axial mesendoderm, which together led to craniofacial and midline defects. They showed further that these defects were intensified in the presence of $Smad3$ mutations [55]. Here we adopt a strategy to delete one allele each of $Smad2$ and $Smad3$, expecting to maintain higher Nodal activity, thus addressing which of the anterior primitive streak derivatives is more susceptible to the reduction of Nodal signaling. Our results show that the specification of anterior endoderm is affected in all of the double heterozygous embryos, suggesting that it is the specification of anterior definitive endoderm that requires the highest level of Nodal signaling. Notably, we observed a small number of $Smad2^{+/\Delta C};Smad3^{+/\Delta}$ embryos which display craniofacial or midline defects as early as E8.5, a phenotype quite similar to that upon epiblast-specific deletion of $Smad2$. However, these account for a minority of the abnormal $Smad2^{+/\Delta C};Smad3^{+/\Delta}$ embryos, suggesting that migration or specification of axial
mesendoderm is not the cause of the abnormalities seen in the majority of the 
$\text{Smad2}^{+/\Delta C};\text{Smad3}^{+/}\text{-}$ embryos.

Results from a number of studies indicate that induction of anterior cell
fates in vertebrates requires the node, an organizing center homologous in
function to the Spemann organizer of amphibian embryos. In addition, a head
induction center is required which in mammals is an extraembryonic region, the
anterior visceral endoderm (AVE, reviewed in [29, 137]. Alterations that affect
the AVE can interfere with anterior patterning, and $\text{Smad2}$ itself has been shown
to be required in the visceral endoderm for anterior specification [49]. However,
it is unlikely that the phenotype seen in the $\text{Smad2}^{+/\Delta C};\text{Smad3}^{+/}\text{-}$ embryos is a
result of abnormal AVE function because $\text{Smad3}$ is not expressed in the visceral
endoderm [54], and epiblast-specific deletion of $\text{Smad2}$ causes anterior defects
[55]. Indeed, in a majority of the $\text{Smad2}^{+/\Delta C};\text{Smad3}^{+/}\text{-}$ embryos anterior patterning
is largely normal until somitogenesis is well underway. Accordingly, E8.5
$\text{Smad2}^{+/\Delta C};\text{Smad3}^{+/}\text{-}$ embryos failed to reveal any diminution of $\text{Otx2, En1, Fgf8,}$
or $\text{Six3}$ levels, even in embryos exhibiting mild craniofacial defects (data not
shown), despite the changes in endodermal gene expression. In addition, $\text{Foxa2}$
expression in the axial mesendoderm of the $\text{Smad2}^{+/\Delta C};\text{Smad3}^{+/}\text{-}$ embryos was
normal at E8.5 while endodermal staining was demonstrably reduced.

Anterior defects have been seen in the absence of members of the TGF-β
signal transduction pathway, including mutants of $\text{Nodal}$ [115], $\text{FoxH1}$ [127], and
$\text{Smad2}$ [122]. In addition, embryos lacking one allele of $\text{Nodal}$ and one of $\text{Smad2}$
or both alleles of the $\text{Activin Receptor IIA}$ exhibit similar defects to those seen in
the Smad2$^{+/\Delta C};$Smad3$^{+/ \cdot}$ embryos [125, 138]. However, in the Smad2$^{+/\Delta C};$Smad3$^{+/ \cdot}$ mutants, the morphogenic movements that form the foregut are largely normal, whereas in other cases foregut formation is visibly perturbed [115, 127]. It is therefore likely that in a number of previous cases the craniofacial abnormalities observed may not be entirely due to defects in the definitive endoderm.

On the other hand, in the Smad2$^{+/\Delta C};$Smad3$^{+/ \cdot}$ embryos the primary defect would appear to be abnormalities in the endodermal compartment, as they precede any other morphological disturbances. The endoderm is demonstrably abnormal, as it lacks a normal complement of endodermally expressed genes, and it fails to displace visceral endoderm at the most anterior region, as evidenced by the expression of visceral endoderm markers in the anterior intestinal portal. These endodermal defects likely result in an abnormal midline, and then anterior patterning later in embryogenesis. It is still possible that unseen defects exist elsewhere in the embryo, requiring the use of conditional mutagenesis to concretely demonstrate the importance of the endoderm in midline patterning.

2.3.3 TGF-β signaling is essential for endodermal specification

Nodal signaling is also essential for endodermal development. Expression of a Nodal antagonist such as Antivin results in a failure of endodermal specification in zebrafish [139]. Increased TGF-β signaling through the forced expression of an activated TGF-β receptor is sufficient to endodermalize
zebrafish embryos, further underlining the importance of TGF-β signaling to endodermal development [120]. A chimeric analysis, in which ES cells with disruptions in both Smad2 alleles were injected into wild-type blastocysts, suggested that Smad2 was indispensable for hindgut formation, as the cells did not contribute to this tissue [54]. However, foregut formation was unaffected, because Smad2 mutant ES cells could contribute to the foregut with no observable adverse consequences [54]. FoxH1 mutant embryos also exhibit defects in endodermal formation, with the hindgut apparently more sensitive to the loss of FoxH1 than the foregut [127]. Our data shows that foregut endoderm appears considerably more sensitive to the reduction of Smad2 and Smad3 than formation of the hindgut, as we are able to derive holoprosencephalic animals with normal hindgut derivatives. It is quite possible that the requirement for Smad3 is more pronounced in the foregut, and that Smad2 and Smad3 have some functions in the foregut endoderm that are independent from FoxH1, possibly due to different cofactors.

2.3.4 Smad2 and Smad3 are required for hepatogenic competence of the foregut endoderm

We have also shown in this study that Smad2+/ΔC;Smad3+/− embryos develop liver defects because of defective endodermal competence. The expression of Hex, a gene needed for hepatogenic development, is greatly reduced in all of the Smad2+/ΔC;Smad3+/− embryos, and results in a severe decrease in the hepatocytic lineage. The defects in the Smad2+/ΔC;Smad3+/−
embryos are milder than those observed in the total absence of Hex, of which liver development is completely abrogated [43]. In the Smad2^{+/\DeltaC};Smad3^{+/-} embryos, the liver bud forms even with a greatly reduced number of hepatoblasts, and the liver grows although at a slower rate [124]. Endodermal hepatogenic precursors are delayed in their exit of the gut primordium, and subsequent migration into the septum transversum. An RT-PCR analysis of E13.5 Smad2^{+/\DeltaC};Smad3^{+/-} livers failed to reveal expression of a panel of hepatocytic genes, further suggesting the absence of the hepatocytic lineage in the Smad2^{+/\DeltaC};Smad3^{+/-} mutants. It is interesting that hepatogenesis can proceed despite the severe reductions seen in the number of hepatocytic cells.

Our model for the effects of Smad2 and Smad3 on Hex expression involves both direct and indirect mechanisms as shown in figure 2.9. First, Hex expression from the hepatogenic endoderm is lost due to an indirect mechanism. The definitive endoderm fails to cover the heart in the Smad2^{+/\DeltaC};Smad3^{+/-} embryos, and is therefore unavailable to receive hepatic inductive signals. This defect appears to be due to a failure in endodermal migration, as BrdU analysis has suggested that the proliferation of the mutant foregut endoderm is normal.

Second, Hex expression from the hepatogenic endoderm is lost due to a direct mechanism. Smad2 and Smad3 likely transactivate the Hex promoter directly. Hex has been shown to be a Nodal target gene in Xenopus laevis [140], and the murine Hex promoter has Smad binding sites [133]. Further evidence for regulation of Hex by Smad2 and Smad3 comes from reduced expression in the developing thyroid. Although we cannot rule out generalized defects of the
foregut endoderm as being the cause of diminished thyroid *Hex* expression, it is noteworthy that the lungs in the *Smad2^+/ΔC;Smad3^+/−* embryos, which are not dependent on *Hex* expression, develop normally despite the fact that they are a foregut derivative.

Finally, Smad2 and Smad3 control the expression of *Foxa2*. In our model *Foxa2* then regulates *Hex* expression, which has been previously demonstrated in HepG2 cells [141]. It is likely that the reductions in *Foxa2* contribute heavily to the endodermal defects seen in the *Smad2^+/ΔC;Smad3^+/−* embryos. Disruption of *Foxa2* in mice leads to failure in endodermal specification [68], while overexpression of *Foxa2* in ES cells can drive their endodermal development [142]. Chimeric animals made from wild-type tetraploid cells and *Foxa2* mutant ES cells failed to differentiate endoderm, and exhibited ectopic visceral endoderm within the gut, similar to the *Smad2^+/ΔC;Smad3^+/−* embryos [130]. *Foxa2* is one of the earliest proteins to bind to the albumin promoter, where it interacts with histones to facilitate opening of chromatin to a more transcriptionally active state [143]. The expression of *Foxa2* in the *Smad2^+/ΔC;Smad3^+/−* mutants is variable, in that some mutants have severe reductions in its endodermal expression, while other mutants are less perturbed. Those with more severe losses of *Foxa2* expression will likely continue in development and become holoprosencephalic. Those with less severe reductions in *Foxa2* will probably not suffer craniofacial abnormalities at all. However, they will go on to exhibit liver defects, as Smad2 and Smad3 have multiple channels through which they can modulate *Hex* expression. Our results
show that injecting Smad2 RNA into Xenopus embryos is sufficient to increase expression of Foxa2, and dominant negative Smad2 genes can reduce expression of Foxa2, albeit only mildly. However, it is not clear at this time whether Smad2 and/or Smad3 can bind directly to the Foxa2 promoter, or whether their effects on Foxa2 expression are mediated indirectly. It is possible that Smad2 and Smad3 regulate the expression of Foxa2 directly, as there are numerous Smad binding elements within the Foxa2 promoter. In addition, XFKH1, a close Foxa2 homolog in Xenopus, has been shown to be directly activated by Smad2 in response to activin [144]. On the contrary, it is also possible that the increases in Foxa2 expression seen in our Xenopus experiments were due to secondary effects, such as increases in endodermal tissue. Other lines of evidence link Nodal and Foxa2, including the fact that Foxa2 functions synergistically with Nodal during murine left/right axis determination [145] and craniofacial development [44]. Furthermore, expression of Foxa2 can rescue floorplate defects in Oep mutant embryos [146], suggesting that Foxa2 is a Nodal target. However, the regulatory elements controlling Foxa2 expression within the endoderm are at present unclear.

In summary, our data suggests that Smad2 and Smad3 function in the endoderm to generate signals that pattern the mammalian anterior, and that Smad signaling is required for the hepatogenic competence of the foregut endoderm. Further work will be needed to demonstrate which molecules are required for both patterning events, and on which cells they exert their functions. Conditional knockouts of the Smad genes and the appropriate Cre driver strains

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will no doubt be invaluable in discovering how these developmental events are controlled.

2.4 Materials and Methods

2.4.1 Generation of homozygous ES cells

To create a targeting vector that disrupts the second allele of Smad2 in Smad2ΔC/+ ES cells, a derivative of pPNT [147] was created with a hygromycin resistance cassette in place of neo. The targeting vector was then assembled in the same way as the neo targeting vector [52]. Electroporation and selection were performed by standard procedures. Genomic DNA extracted from ES colonies was digested with HindIII and SalI (Roche), run overnight on 0.7% agarose gels, and transferred to nitrocellulose filters (Hybond) via vacuum transfer using a Vacugene pump vacuum apparatus (Pharmacia). Probes were labeled using a random primer labeling kit (Roche). Hybridizations were performed using standard procedures, and blots were exposed to Biomax MR film (Kodak).

2.4.2 Western blot

Wild-type TC1 ES cells, Smad2+/-ΔC, and Smad2ΔC/ΔCΔHyg were passaged three times in the absence of murine embryonic fibroblasts in ES medium. Protein lysates were prepared by lysing cells in buffer (10 mMTris pH 8.0, 1mM EDTA, 0.1% Tween-20 with protease inhibitors [Roche]). Lysates were then run on 8% PAGE gels and transferred to nitrocellulose using a Bio-Rad protein gel...
apparatus according to the manufacturer’s directions. Blots were probed with a mouse monoclonal antibody against Smad2/3 that recognizes the linker domain (Transduction Labs), washed, probed with an anti-mouse antibody coupled to alkaline phosphatase (Roche), and detected by Chemiluminescence using a kit from Amersham.

2.4.3 Embryonic analysis

*Smad2*+/ΔC and *Smad3*+/− mice were maintained on a mixed background of 50% 129Svev and 50% NIH Black Swiss. Embryos were dissected free of maternal tissues in 1xPBS and genotyped for *Smad2* and *Smad3* alleles as described [52, 59] using genomic DNA extracted from yolk sac or embryonic tissues. Whole mount *in situ* hybridization was performed as described [148] using the following probes: *Otx2* [149], *Fgf8* [150], *Shh* [151], and *Foxa2* [152]. Probes for *Hnf4*, *Afp*, and *Hex* were amplified from E13.5 liver cDNA using the following primer pairs: *Hnf4*: 5’- TTCTGCGAACTCCTTCTGGATG-3’ and 5’-GCTTCTTGCTTGGTGATCGTTG-3’; *Afp*: 5’- AAGAAGACTGCTCCGGCCTC-3’ and 5’-AGTGCCTGGCTCTCCTCGAT-3’; *Hex*: 5’-CCCTTCTACATCGACGACATCTTTGTGATTGATCGTTG-3’ and 5’-ACACTGCGAAGGATCCAAAGAG-3’.

After PCR amplification, probes were cloned into the RV site of pBluescript KS (Stratagene). The *Albumen* probe was a kind gift of Jim Wells of the Cincinnati Childrens Hospital. Preparation of histological sections was performed by standard procedures.
For the analysis on the A/J strain background, A/J males (Jackson labs) were bred with Smad2^{+/\Delta C} and Smad3^{+/\cdot} females. Offspring were genotyped for Smad2 and Smad3 alleles, and animals testing positive for Smad mutations were backcrossed to their A/J fathers for 5 or 6 generations for Smad2 and 10 generations for Smad3.

2.4.4 Xenopus embryo manipulations

Eggs were fertilized in vitro and reared in 0.1x Modified Barth's Saline (MBS). Microinjections were performed in 1xMBS, 4% ficoll. Embryos were injected in each of the four vegetal blastomeres at the 8 cell stage with 1.5 ng of synthetic mRNA encoding either wt murine Smad2 or the dominant negative Smad2^{S/A}. At mid gastrula, stage 11, embryos were frozen and subjected to RT-PCR as previously described [153] except that SYBR green was added to the reactions and amplification was monitored in real time with an Opticon PCR machine (MJ Research). For each experiment a serial dilution of whole embryo cDNA was used to generate a standard curve from which the amount of product in the experimental samples was determined at the log-linear amplification phase. The data for each sample is normalized to the total amount of RNA in that sample by comparing it to the expression level of the ubiquitously expressed gene ornathine decarboxylase (ODC) which were used as a loading control as previously described [154]. The data in the histogram are presented as a ratio of ODC expression. Each point in the analysis represents the results of 3 pooled embryos done in duplicate. The experiment was done twice.
<table>
<thead>
<tr>
<th>Age</th>
<th>wt</th>
<th>Smad2^{+/ΔC}</th>
<th>Smad3^{+/−}</th>
<th>Smad2;Smad3</th>
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<td>109</td>
<td>103(8)^{a}(8)^{c}</td>
<td>3</td>
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<td>96</td>
<td>106 (19)^{a}(28)^{b}(7)^{c}</td>
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<td>11</td>
<td>15</td>
<td>4 (1)^{a}</td>
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<td>55</td>
<td>79</td>
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</table>

^{a}Number of embryos exhibiting craniofacial defects
^{b}Number of embryos exhibiting midline defects
^{c}Number of embryos exhibiting gastrulation defects
^{d}Resorbed embryos

**Table 2.1.** Genotypic analysis of offspring resulting from crosses between Smad2^{+/ΔC} and Smad3^{+/−} mice.

<table>
<thead>
<tr>
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<th>wt</th>
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<th>Smad3^{+/−}</th>
<th>Smad2;Smad3</th>
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<td>9</td>
<td>7</td>
<td>3(0)^{b}</td>
<td>n/a</td>
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</table>

^{a}Resorbed embryos
^{b}Number of embryos or animals exhibiting craniofacial or midline defects

**Table 2.2.** Genotypic analysis of offspring resulting from crosses between A/J Smad2^{+/ΔC} and A/J Smad3^{+/−} mice.
Figure 2.1. Smad2ΔC is a null allele of Smad2 gene. (A) Wild-type Smad2 locus (top) and targeting vectors for generating the Smad2ΔC allele (middle and bottom). Black triangles represent loxP sites, and black boxes represent Smad2 exons. White boxes represent exons of Smad2 deleted in the Smad2ΔC mutation, with the corresponding protein region shown in white at the top. Hind, HindIII; Bm, BamHI; Sal, SalI; RI, EcoRI. (B) Southern blot of ES cell DNAs digested with HindIII and SalI and probed with the flanking probe shown in A. Lane 1 is from cells in which the hygromycin vector has targeted the Smad2ΔC allele (Smad2ΔChyg), generating a 6.5 Kb mutant band in addition to the wt 8.0 Kb band. Lane 2 is from cells carrying a Smad2ΔC allele, which show a 4.5 Kb mutant band. Lane 3 is from an ES line in which both alleles of Smad2 have been disrupted (Smad2ΔChyg/ΔC) and the wt 8.5 Kb band is lost. (C) Western blot of ES cell protein labeled with anti-Smad2 antibody showed diminished levels of Smad2 in the Smad2ΔC(neo)/+ (+/-) cells and undetectable amounts in the Smad2ΔC/ΔC (-/-) ES cells (top). Non-specific bands served as loading control (below). (D) Although 90% of the Smad2+/ΔC animals exhibited a wild-type phenotype (left), 5% of them showed severe defects of a tissue mass external to the yolk sac (right, arrow points to the embryo) at E9.5. (E) Otx2 staining suggests that these external embryos have an anterior (arrow). (F) Staining for Shh shows midline tissues (arrow) in the external embryos. em, embryo; ys, yolk sac. The bar in D equals 840 µm in D-F.
Figure 2.1
Figure 2.2. *Smad2\(^{+/\Delta C};Smad3^{+/−}\)* embryos exhibit craniofacial defects. In each case the *Smad2\(^{+/\Delta C};Smad3^{+/−}\)* mutant is on the right, while a normal sibling is shown on the left. (A) Severely affected mutant showing extreme midline defects with growth retardation, defective heart formation (H), irregular somites and underdeveloped cepahalic structures (arrow) at E9.5. (B) Mutant with defects of lesser severity, including craniofacial defects with cyclopia and proboscis (arrow) at E12.5. Note the reduced liver (arrowhead). (C) Mutant embryo exhibiting milder defect in craniofacial development (arrow) at E14.5. The arrowheads point to the liver, which is reduced in the mutant. The bar in C equals 2mm for A, and 4 mm for B and C.
Figure 2.3. Marker analysis of head and midline defects in Smad2\textsuperscript{+/}\textsuperscript{C};Smad3\textsuperscript{+/\textminus} embryos. Normal sibling controls are all shown on the left, while Smad2\textsuperscript{+/}\textsuperscript{C};Smad3\textsuperscript{+/\textminus} mutants are on the right. All embryos are E9.5. (A) Fgf8 staining is seen in the frontonasal process of the wt embryo (arrow), but is missing in the Smad2\textsuperscript{+/}\textsuperscript{C};Smad3\textsuperscript{+/\textminus} embryo. (B) Sagital section of wt embryo after Fgf8 staining. (B), Neural epithelia directly contacts epidermal cells where Fgf8 is expressed (arrow) in the frontal area of the wild-type embryos(C) In mutant embryos there are mesenchymal cells (arrow) separating epidermal and neural epithelial cells. Note that Fgf8 expression is still present in the mutant isthmus. f, forebrain; m, midbrain; i, isthmus; h, heart. (D) Whole-mount staining for Otx2. Note the reduction in the domain of Otx2 expression in the mutant. (E, F) Shh expression. Mildly affected Smad2\textsuperscript{+/}\textsuperscript{C};Smad3\textsuperscript{+/\textminus} embryos show rostral specific loss of Shh (arrow, E), while severely affected Smad2\textsuperscript{+/}\textsuperscript{C};Smad3\textsuperscript{+/\textminus} embryos exhibit more widespread loss of Shh (F). (G) Severely affected mutants exhibit decreased expression of Foxa2. The bar in E equals 820\textmu m for A, B, C, 1060\textmu m for D and E.
Figure 2.3
Figure 2.4. Endodermal defects in E8.5 Smad2^{+/C};Smad3^{+/C} embryos. (A) Foxa2 expression is seen in axial mesoderm (ax), prechordal plate (pr), and foregut endoderm (fe) of wild-type (top), but is reduced in the foregut of the Smad2^{+/C};Smad3^{+/C} (bottom). Arrows point to the foregut. (B, C) Transverse sections of the embryos shown in (A). While there is no difference in Foxa2 expression of the mutant (C) and wt (B) embryos in the floor plate (arrowhead), Foxa2 expression in the foregut (arrow) was greatly reduced in the Smad2^{+/C};Smad3^{+/C} mutant. (D) Injection of wild-type Smad2 (wt) can increase expression of Foxa2 in Xenopus embryos over uninjected controls (uninj.), while injection of dominant negative Smad2 (S/A) can slightly inhibit Foxa2 expression. (E) Albumin expression in the foregut endoderm was also reduced in Smad2^{+/C};Smad3^{+/C} embryos (bottom) at E8.5 compared with a wild-type sibling (top). The dashed lines in A indicates the plane of section in B and C. The bar in E equals 500\(\mu m\) for A and E, and 164\(\mu m\) for B and C.
Figure 2.4
Figure 2.5. Anterior ventral foregut defects in E8.5 Smad2+/ C;Smad3+/− embryos. (A, B) Hex expression is detected in the hepatogenic endoderm of the wt embryos (A, arrow), but is decreased in Smad2+/−;Smad3+/− embryos (B, arrow). (C, D) Ventral view of wt (C) and mutant (D) embryos within the yolk sac. Afp labels the visceral endoderm. (C) The revolute anterior ventral endoderm displaced visceral endoderm in wt embryos, although sporadic Afp positive visceral endodermal cells can be seen dispersed in the triangle-shaped definitive endoderm. (D) Definitive endoderm failed to displace visceral endoderm at the anterior intestinal portal of the Smad2+/− C;Smad3+/− embryos. The arrow points to the involution of the foregut. The bar in D equals 420μm for A and B, 270μm for C and D.
Figure 2.6. Lack of Hex expression in Smad2\(^{+/\Delta C};\)Smad3\(^{+/\Delta C}\) embryos. Embryos are shown at E9.5. (A, B) Wt (A) and Smad1\(^{/\Delta C}\) embryos (B) exhibited indistinguishable Hex expression (arrows). Anterior is on the right. (C) Wt embryos showed abundant expression of Hex within the liver bud (arrow). (D) Smad2\(^{+/\Delta C};Smad3^{+/\Delta C}\) siblings exhibit severely reduced Hex expression within the liver bud (arrow). Anterior is at the top. (E) Transverse section of the wt embryo shown in C with the plane of section indicated by a dashed line in C. Hex positive hepatogenic endoderm (arrow) has initiated dilation in advance of hepatogenesis in the wt embryo. (F) Transverse section of the Smad2\(^{+/\Delta C};Smad3^{+/\Delta C}\) embryo in D. The dilation of the foregut is less obvious (arrow), a reduced number of hepatogenic endodermal cells are seen, and they exhibit a reduced level of Hex expression. h, heart. The bar in F equals 430\(\mu\)m for A, B, C and D, 53\(\mu\)m for E and F.
Figure 2.6
Figure 2.7. Smad2^{+/ΔC};Smad3^{+/−} mutants showed defects in hepatoblast migration. (A) Normal sibling E9.5 embryos exhibit Hnf4 staining within the liver bud (arrow). (B) In Smad2^{+/ΔC};Smad3^{+/−} mutants, Hnf4 staining is severely reduced (arrow). (C) Transverse section through the wt embryo in A; the plane of section is illustrated with a dashed line in A. Hnf4^{+} hepatoblasts (arrow) are seen delaminating from the ventral foregut and migrating into the STM. (D) Transverse section of the embryo in B. The Hnf4^{+} domain of the foregut exhibits a slight dilation, but no migration of Hnf4^{+} endodermal cells is seen. (E) Afp staining of E10.5 sibling embryos illustrates numerous cells of the hepatogenic lineage within the developing liver (arrow). (F) Smad2^{+/ΔC};Smad3^{+/−} mutants exhibit much less staining with Afp. (G) Section of the embryo shown in E. Afp^{+} cells (arrow) are seen throughout the embryonic liver. (H) Section of the Smad2^{+/ΔC};Smad3^{+/−} embryo shown in F. Fewer Afp^{+} cells (arrow) are seen in the liver, despite its normal morphology. h, heart; l, liver. The bar in H equals 380μm for A and B, 58μm for C and D, 820μm for E and F, and 280μm for G and H.
Figure 2.8. Decreased Hex expression may cause liver and thyroid defects in Smad2\textsuperscript{+/ΔC};Smad3\textsuperscript{+/-} embryos. (A) Hex stains the liver (arrowhead) and thyroid (arrow) of E10.5 embryos. (B) Smad2\textsuperscript{+/ΔC};Smad3\textsuperscript{+/-} embryos exhibit reduced staining of both the liver and the thyroid. (C) Tranverse section of the embryo in A with the place of section indicated by a dashed line. The wt foregut does not exhibit Hex staining (arrowhead), and numerous Hex\textsuperscript{+} cells are seen within the developing lobes of the liver (white arrow). (D) The Smad2\textsuperscript{+/ΔC};Smad3\textsuperscript{+/-} mutant exhibits a severe decrease in the number of Hex\textsuperscript{+} cells in the liver (white arrow), although it shows Hex\textsuperscript{+} cells both in the gut (arrowhead) and migrating abnormally (arrow), suggesting delayed hepatic migration. (E, F) Transverse sections of embryos through the thyroid primordia after Hex staining. The plane of section in E and F is parallel to that in C and D, but is anterior and runs through the thyroid primordium (arrows in A and B). The thyroid (arrows) of the wt (E) is larger than that of the Smad2\textsuperscript{+/ΔC};Smad3\textsuperscript{+/-} (F). ph, pharynx. The bar in F equals 630\,µm for A and B, 130\,µm for C and D, and 140\,µm for E and F.
Figure 2.8
Figure 2.9. Model of Smad2 and Smad3 functions during endodermal development.
CHAPTER 3

GENERATION OF NOVEL CONDITIONAL AND HYPOMORPHIC ALLELES
OF THE SMAD2 GENE

3.1 Introduction

Smad2 and Smad3 are intracellular transmitters for TGF-β, Nodal, and Activin signaling ligands. Upon ligand binding, the receptors are activated, and in turn activate Smad2 and Smad3 via phosphorylation of the C-terminal serine residues in the MH2 domain. These activated mediators then bind with Smad4, the common Smad, and subsequently translocate to the nucleus where, through interactions with other transcription factors, regulate the expression of responsive genes [7, 9]. To elucidate the function of Smad2 during mammalian development, different loss of function alleles have been generated. Each of these Smad2 alleles results in embryonic lethal phenotypes [49, 51, 52, 121, 122]. In an effort to circumvent this early lethality, the Cre-loxP system [155] has been adopted to create Smad2 conditional alleles. A previously published Smad2 conditional allele [55] was designed to remove the first coding exon of the protein. However, a truncated Smad2 protein has been found in cells homozygous for a similar N-terminal Smad2 mutation [122] that can be
phosphorylated and translocated to the nucleus [53]. Therefore, we
designed a novel conditional allele, recombination of which results in a C-
terminal truncation that deletes the activation sites due to a frameshift in exon 11.
Thus, if a protein is created it will lack the majority of the MH2 domain, including
the C-terminal phosphorylation sites which are needed for function. Here we
also report the generation of this Smad2 conditional allele (Smad2\textsuperscript{flox}), which can
be excised by Cre-mediated recombination. The excision allele (Smad2\textsuperscript{\Delta E9,10})
phenocopies the previously reported Smad2\textsuperscript{\Delta C} mutation in the homozygous state
[52]. In addition, we report that Smad2\textsuperscript{3loxP}, the targeted mutation used to create
the Smad2\textsuperscript{floX} conditional allele, is itself hypomorphic. Smad2\textsuperscript{3loxP} can be
maintained in the homozygous state, but causes lethality when placed opposite a
Smad2 null mutation.

A targeting construct used to create the Smad2\textsuperscript{3loxP} allele was generated
by placing a loxP site into intron 8 of the Smad2 genomic sequence, and a floxed
PGK\textit{neo} cassette [156] into intron 10 (Fig. 3.1a). The construct was
electroporated into TC1 ES cells [157] and after G418 and gancyclovir double
selection, 10 homologous recombinants were identified by Southern blot analysis
using a 5’ external probe (Fig. 1a,b). Injection of targeted ES cells into C57BL/6
blastocysts generated chimeric mice, and when crossed to Black Swiss mice,
resulted in germline transmission of the Smad2\textsuperscript{3loxP} allele. Mice heterozygous for
the Smad2\textsuperscript{3loxP} allele are viable, healthy, and fertile. Heterozygous mice were
intercrossed and wildtype, heterozygous, and homozygous Smad2\textsuperscript{3loxP} mice were
generated at normal Mendelian ratios. The homozygotes are viable, healthy, and
Mice carrying a Smad2\textsuperscript{3loxP} allele were then mated with Ella-Cre transgenic mice [158] to create germline recombination between the three loxP sites. This cross generated the possible deletion alleles as follows: recombination between loxP1 and loxP2, deleting exons 9 and 10 only (Smad2\textsuperscript{2neoΔE9, 10}); between loxP2 and loxP3, removing the neo cassette only (Smad2\textsuperscript{flox}); and between loxP1 and loxP3, excising exons 9, 10, and the neo cassette (Smad2\textsuperscript{ΔE9, 10}) as shown in Figure 3.2a. The latter deletion introduces a frameshift in exon 11, which encodes part of the MH2 domain (Fig. 3.1a), resulting in an early stop codon. The offspring for each of the recombined alleles were identified by PCR analysis using the various sets of indicated primers (Fig. 3.2b–d) and by Southern blot using the 5' external probe. This demonstrates that the Smad2 founder targeted allele we generated can be recombined in the presence of Cre recombinase, producing all the expected products.

### 3.2 Results

Previous work with a deletion of the MH2 domain in Smad2 (Smad2\textsuperscript{ΔC}) resulted in recessive lethality before embryonic day 8.5 (E8.5). Smad2\textsuperscript{ΔC/ΔC} embryos observed at E6.5 were smaller than wildtype and lacked the extraembryonic portion of the egg cylinder [52]. In order to verify that the Smad2\textsuperscript{ΔE9,10} allele behaves in a similar fashion to Smad2\textsuperscript{ΔC}, Smad2\textsuperscript{ΔE9,10} mice were intercrossed and homozygous Smad2\textsuperscript{ΔE9,10} embryos were shown to be much smaller than their wildtype littermates (Fig. 3.3a), and appeared
indistinguishable from $Smad2^{ΔC/ΔC}$ embryos [52]. We and others have occasionally observed heterozygous Smad2 mutant embryos that are external to the yolk sac [51, 60], which were also seen in about 10% of the $Smad2^{ΔE9,10/+}$ embryos (Fig. 3.3a).

There has been controversy surrounding the various Smad2 deletion alleles, which may be at least partially due to the deletion of different regions of the Smad2 protein. N-terminal mutations of Smad2 lead to milder homozygous phenotypes than $Smad2^{ΔC}$ and $Smad2^{ΔE9,10}$ [49, 52, 55, 122]. It is possible that the $Smad2^{ΔE9,10}$ mutation creates a Smad2 protein with antimorphic effects on TGF-β signal transduction [159]. In order to address this possibility, a Smad2 cDNA corresponding to the transcript of the $Smad2^{ΔE9,10}$ allele ($Smad2^{ΔE9-11}$) was tested for dominant-negative activity in a Xenopus laevis embryo injection assay. The 32 uninjected and 19 LacZ injected embryos developed normally (Fig. 3.3b), with a DAI score of 5 [160], as compared to injection of the dominant-negative $Smad2^{3S→A}$ (n = 15), which exhibited anterior truncation (Fig. 3.3c) and a DAI score of 4.67 ± 0.7. However, no effects were seen on the development of the Xenopus embryos injected with $Smad2^{ΔE9-11}$ mRNA (Fig. 3.3d), as all 54 embryos injected exhibited a DAI score of 5, despite the presence of a truncated Smad2 protein (Fig. 3.3e). Therefore, deletion of exons 9 and 10 is likely to create a null allele of Smad2.
3.3 Discussion

As stated previously, deletion of the Smad2 N-terminus results in the production of a truncated protein [53]. In addition, a hypomorphic allele of Smad2 has been reported, which phenocopies N-terminal Smad2 mutations [123]. Therefore, the previously published Smad2 conditional allele [55] could result in a hypomorphic mutation after recombination. In contrast, the Smad2 conditional allele described here will be recombined into a null. This will provide a powerful tool to analyze Smad2 functions under a variety of physiological and pathological conditions.

It has been reported that the neo cassette contains a cryptic splice donor site and, therefore, insertion of a neo cassette intronically can result in a hypomorphic allele [161]. However, homozygous Smad2$^{3loxP}$ mice are obtained at Mendelian ratios and show no observable abnormality. In order to more completely characterize the Smad2$^{3loxP}$ allele, Smad2$^{3loxP}$ mutant mice were bred with Smad2$^{ΔC/ΔC}$ mice. From these crosses, no Smad2$^{3loxP/ΔC}$ offspring were found in 71 offspring, suggesting that this allele combination is lethal. The Smad2$^{ΔE9,10}$ mutant allele was also crossed with Smad2$^{3loxP}$, resulting in the same apparent lethality, with no Smad2$^{3loxP/ΔE9,10}$ animals being found in 138 offspring.

The timing of lethality was determined through crosses of Smad2$^{ΔE9,10/ΔC}$ with Smad2$^{3loxP/3loxP}$ animals. All of the Smad2$^{3loxP/ΔE9,10}$ embryos exhibited lethality before E11.5. Between E8.5 and E10.5 they displayed a range of phenotypes (Fig. 3.3f–i), which can be divided into three classes. Class I
mutants (Fig. 3.3f) consist of an empty yolk sac, phenocopying $\text{Smad}2^{\text{Robm1/Robm1}}$, $\text{Smad}2^{m1\text{Mag/m1Mag}}$, and $\text{Smad}2^{m1\text{Mag/Robm1}}$ embryos [49, 123]. Class II mutant embryos (Fig. 3.3g) have a mass of tissue attached to the distal tip of the yolk sac and, in some cases, this tissue is large enough to display obvious anterior/posterior polarity. Class III (Fig. 3h) consists of embryos with severe midline defects as compared to normal siblings (Fig. 3.3i). The phenotypic variability observed in the $\text{Smad}2^{3\text{loxP/\DeltaE9, 10}}$ embryos may be due to the outbred nature of the genetic background. To determine whether this hypomorphic effect is due to the presence of the intronic neo cassette, but not the inserted loxP sites, crosses were established to produce $\text{Smad}2^{\text{Flox/\DeltaE9, 10}}$ mice. These were found at normal Mendelian ratios, and are indistinguishable from littermates. The embryonic lethality and the developmental abnormalities observed in of $\text{Smad}2^{\DeltaE9, 10/\DeltaC}$ and $\text{Smad}2^{3\text{loxP/\DeltaE9, 10}}$ mutants supports the conclusion that the $\text{Smad}2^{3\text{loxP}}$ allele is a novel hypomorph. It is able to be maintained in the homozygous state, and exhibits no observable anomaly until it is placed opposite a null allele. This is a milder hypomorph than the previously reported $\text{Smad}2^{m1\text{Mag}}$ allele [123], since $\text{Smad}2^{3\text{loxP/3loxP}}$ mice are viable, while $\text{Smad}2^{2m1\text{Mag/m1Mag}}$ are not. This new hypomorphic allele should prove to be extremely useful for determining novel functions of Smad2 in different developmental processes.
3.4 Materials and Methods

3.4.1 Construction of the Targeting Vector and Generation of Germline Chimeras

A 9-kb Smad2 genomic fragment containing exons 6–11 was subcloned into pBluescript KS (Stratagene, La Jolla, CA) from a BAC clone. A loxP site with an upstream EcoRV site was inserted into the SalI site in intron 8. A 3.1-kb EcoRI/EcoRI fragment was placed downstream of the floxed neo cassette of pLoxpneo. A 5.9-EcoRI/Ngotl fragment was inserted upstream of the floxed neo. The NotI site was not genomic, but was carried over from the polylinker of pBluescript KS. These manipulations flank the genomic sequence of exons 9 and 10, as well as the neo cassette with loxP sites. This Smad23loxp vector was electroporated into 129/SvEv TC1 ES cells (Deng et al., 1996) and selected with G418 and gancyclovir; 192 resistant colonies were selected and further analyzed by Southern blot. Correctly targeted clones were microinjected into C57BL/6 blastocysts and implanted into foster mothers to obtain chimeras by standard procedures. The chimeric founders were crossed with Black Swiss mice to obtain Smad23loxp/+ offspring.

3.4.2 Xenopus Embryo Manipulations

Eggs were fertilized in vitro and raised in 0.1X modified Barth’s saline (MBS). Microinjections were performed in 1X MBS with 4% ficoll. Embryos were injected into the subequatorial dorsal marginal zone at the 4-cell stage with 1.5 ng of synthetic mRNA encoding either the dominant-negative Smad23S→A or
Smad2\(^{\Delta E9-11}\), along with LacZ. The Smad2\(^{3S\rightarrow A}\) has the C-terminal phosphorylatable serines replaced by alanines, while the Smad2\(^{\Delta E9-11}\) allele truncates after exon 8, thus removing a majority of the MH2 domain with the phosphorylation sites. Lineage tracing with β-galactosidase (with either Red-Gal or X-Gal) confirmed injection into dorsal areas of the embryos. Western blots on neurula stage embryos were performed using standard procedures and probed for the flag epitope attached to the N-terminus of Smad2 using an M2 anti-flag antibody from Sigma (St. Louis, MO).

3.4.3 Genotyping Analysis

Mice were genotyped either by Southern blot or PCR. For PCR analysis, the following primers were used to detect both the wildtype and conditional alleles. For detecting the loxP1, SM2-16 (5’-GAG CTG CGC AGA CCT TGT TAC-3’) and R2 (5’-TGC CTG ACA AAC AGT CCT GG-3’) amplify a 370-bp product from the wt allele and a 400-bp product from the conditional allele (Fig. 3.2a, c). The neo cassette is detected with 3JNEO (5’-GTT CTA ATT CCA TCA GAA GCT GAC TCA AG-3’) and WTR (5’-GAA GGG GAT CCC ATC TGA GT-3’) (Fig. 3.2a,b). The Smad2\(^{\Delta E9, 10}\) allele was amplified using SM2-16 and WTR, which generates a 950-bp product from the mutant allele (Fig.3.2a, d). The Smad2\(^{neo\Delta E9,10}\) allele was amplified using SM2-16 and RINA (5’-CCA GAC TGC CTT GGG AAA AGC-3’), which generates a 400-bp product (Fig. 3.2a, and data not shown). The Smad2\(^{foxx}\) allele can also be detected using LEFT (5’-TAC TTG GGG CAA TCT TTT CG -3’) and SM2AA (5’-GTC ACT CCC TGA ACC TGA AG-3’).
-3'), which generates a 470-bp wt product and a 500-bp mutant product (Fig. 2a, and data not shown).
Figure 3.1. Generation of the Smad2$^{3\text{loxP}}$ allele. (a) A genomic sequence containing exons 6–11 was subcloned for vector construction. The Smad2 genomic locus is shown to scale at top while, the targeting vector is shown in the middle, and the targeted allele ($3\text{loxP}$) at the bottom. Exons 6–11 are indicated by solid boxes; regions of the protein corresponding to the different exons are indicated by dashed lines. (b) Homologous recombinants were identified by Southern blot analysis. Genomic DNA from ES cell clones were digested with EcoRI and EcoRV and hybridized with a 5' external probe, which detects a wildtype 11.4 kb fragment and a targeted 8.5 kb fragment.
Figure 3.1
Figure. 3.2. Deletion alleles from Cre-mediated recombination of Smad2\textsuperscript{3loxP} and the PCR strategy used to identify them. (a) Founder mice heterozygous for the \textit{3loxP} allele were bred with \textit{Ela-Cre} mice to obtain offspring, which were mosaic for the different recombination alleles. Black arrowheads indicate the location and direction of the primer sets used for identification of specific alleles. (b–d) Expected lengths of the PCR products used in genotyping.
Figure 3.3. Analysis of Smad2 ΔE9, 10 and Smad2 3loxP alleles. (a) Embryos from Smad2 ΔE9, 10/+ intercrosses were observed at E7.5. Smad2 ΔE9, 10/ΔE9, 10 embryos (right, top, and bottom) are much smaller than their wildtype littermate (left). The middle embryo is Smad2 ΔE9, 10/+, which has embryonic tissue external to the yolk sac. This defect is observed in less than 10% of the heterozygous using the Smad2 ΔE9, 10/+ or Smad2 ΔC/ΔC. (b–d) Xenopus injections were used to compare the function of Smad2 mutant alleles. (b) Uninjected embryo control. (c) Injection of the dominant-negative Smad2 allele, Smad2 3S→A. (d) Injection of Smad2 ΔE9, 10. (e) Western blot of injected Xenopus embryos showing the presence of Smad2: flag fusion proteins. The lower panel shows a nonspecific band used for a loading control. (f–h) Smad2 3loxPΔE9, 10 embryos display a range of phenotypes at E9.5. (f) Class I. (g) Class II. (h) Class III. (j) Normal Smad2 3loxP/+ littermate. Scale bar in i=303 um for a; 1.6 mm for f,g; 565 um for h; and 943 um for i.
Figure 3.3
4.1 Introduction

Control of mRNA turnover is very important in the regulation of gene expression. A critical step in eukaryotic mRNA degradation is the removal of the 5’ cap, so called mRNA decapping. Two decapping enzymes (or enzyme complexes) have been identified in eukaryotes; each is involved in distinct exonucleolytic pathways. The first is the scavenger decapping enzyme (DcpS), which mediated 3’ to 5’ decay of the mRNA. And the second is Smif (Dcp1a)/Dcp2 protein complex, which specifically removes the 5’ cap from the deadenylated mRNAs, followed by 5’ to 3’ mRNA degradation (reviewed in [162-165]).

In contrast to the nuclear localization of DcpS, Smif and Dcp2 are enriched at specific cytoplasmic foci termed the processing bodies (P-bodies, also referred to as Dcp bodies or GW bodies) where active mRNA decay occurs.
Just like the P-bodies are found in both yeast and human cells, Smif and Dcp2 are evolutionarily conserved from yeast to human (reviewed in [167]). Of the two proteins, Dcp2 is the actual decapping enzyme, catalyzing the hydrolysis of the methylated cap on an mRNA [19-21]. Smif, on the other hand, doesn’t have enzyme activity, rather it facilitates Dcp2 mediated decapping by bringing other decapping activators into the protein complex [20, 25]. Smif/Dcp2 not only functions in the general mRNA turnover pathway, but also plays important roles in NMD and the decay of ARE-containing mRNAs (a group of highly unstable mRNAs) by interacting with the ARE-binding protein tristetraprolin (TTP) or its homolog BRF-1 [21, 168].

In addition to the role of Dcp1 in eukaryotic mRNA decapping, recent discoveries suggest Dcp1 has other activities which may or may not relate to the decapping pathway. The Drosophila decapping protein 1, dSmif, has been found to be part of a RNP complex essential for the subcellular localization of oskar mRNA in oocytes and this function of dSmif is irrelevant of its decapping activity [169]. Moreover, human Smif (hSmif) has been identified as an interacting protein of Smad4, an intracellular mediator of the TGF-β/BMP pathways. Ablation of Smif expression in zebrafish by the morpholino antisense approach results in shortening of the body axis of the fish embryos, indicating the potential roles of Smif in vertebrate embryonic development [14].

Despite the well studied biochemical mechanisms of hSmif in mRNA decay, little is know about its roles in biological and physiological processes such as development and disease progression. To learn more about Smif function in
this aspect we have generated a loss-of-function mutation of *Smif* in mice using gene targeting approach. Disruption of *Smif* expression results in recessive embryonic lethality at E11.5 due to the malformation of the umbilicus and the dysfunctional placenta. These defects, however, can be traced back to the abnormal development of the allantoic vasculature, which appears in the embryo at much earlier stages, E8.0-E8.5.

4.2 Results

4.2.1 *Smif* expression pattern in postgastrulation stage mouse embryos

To examine the spatiotemporal expression of *Smif* in mouse embryos, section in situ hybridization against *Smif* was performed on embryos at E8.5 and E9.0. It showed that *Smif* is ubiquitously expressed in all tissues examined, including all embryonic structures, yolk sac, allantois and chorionic plate at E8.5 (Fig. 4.1). This is consistent with the assumption that Smif, as a mammalian homologue of yeast Dcp1, may be involved in mRNA decapping.

4.2.2 Targeted disruption of the murin *Smif* gene

The mouse *Smif* gene consists of eleven exons (http://www.ensembl.org ENSMUSG00000021962). A BLASTN search of the *Smif* genomic sequence against the dbEST database didn’t identify any potential 5’ UTR upstream of exon 1, suggesting exon 1 is located adjacent to the promoter region. The targeting vector was designed to replace *Smif* exon1 and a 2 kb putative promoter sequence with a reverse-orientated PGK-neo cassette to disrupt *Smif*
transcription (Fig. 4.2A). Because exon 1 also contains translational start codon, even if there is cryptic transcription initiation site, the targeted allele is still predicted to be a null allele due to the lack of translation. In addition, since none of the downstream ATGs is in an appropriate Kozak sequence, the mutant allele is unlikely to produce any truncated protein. The targeting vector was electroporated into ES cells and after drug screening, 1% of the surviving clones were found to be correctly targeted (3 out of 288) (Fig 4.2.B). All three clones were injected into C57Bl/6J blastocysts and all gave rise to germline chimeras (Fig 4.2.C).

To confirm that the introduced mutation abolishes Smif mRNA transcription, total RNA was extracted from wildtype, Smif\textsuperscript{+/−}, and Smif\textsuperscript{−/−} embryos and analyzed by RT-PCR. The result showed that Smif homozygous mutants exhibited reductions in the level of mRNA (no band after 30 cycles and only a weak band after 35 cycles) as compared to the wildtype and heterozygous siblings (Fig. 4.2. D). We hypothesized that the expression of the truncated transcript from the targeted allele is likely due to a weak promoter in the reverse-oriented PGK-neo cassette. Indeed, when Neo insertion was deleted by EIIa-Cre (PGK-Neo is flanked by two LoxP sites), the resulted Smif\textsuperscript{ΔN} allele produced no transcript at all, as shown by no band for Smif\textsuperscript{ΔN/ΔN} embryos by RT-PCR analysis even after extensive PCR amplification (Fig. 4.2. F). The Smif\textsuperscript{ΔN/ΔN} mice phenocopied Smif\textsuperscript{−/−} animals, suggesting that the trace amount of transcript which arises from the Smif allele is initiated from the PGK-neo cassette and therefore nonfunctional as predicted.
4.2.3 Loss of Smif results in embryonic lethality

Although mice heterozygous for Smif or Smif$^{\Delta N}$ were viable, healthy, and fertile, no live born Smif$^{-/-}$ or Smif$^{\Delta N}$ mice were recovered from heterozygous crosses (Table 4.1). In addition, homozygosity for both alleles resulted in identical abnormalities which later leaded to embryonic lethality. Therefore, in the following experiments, only the original targeted allele was analyzed and homozygous embryos harboring this allele were refered to as mutants unless otherwise specified.

To determine the timing of the lethality, embryos from heterozygous intercrosses were analyzed at different stages. By E10.5, homozygous mutant concepti were found at 26% (close to the medelian ratio). However, at E11.5 only 10% Smif$^{-/-}$ embryos were recovered with some decidua containing remnants of dead feti, of which the genotypes were unanimously Smif$^{-/-}$ and from E12.5 onward no live mutant was discovered, suggesting that death occured around E11.5 (Table 4.1).

At E10.5, while the embryos per se looked grossly normal, the umbilical cords of mutant embryos were much thinner as compared to wild type littermates. In rare cases, there was no umbilical cord formation; instead a spherical structure was formed at the caudal region, presumably the derivative of unattached allantois (Fig. 4.3 A-C). Similar phenotypes have been reported in other mutations affecting chorioallantoic fusion such as Smad1 [102]. The defects in mutant umbilical cord formation were further manifested by whole mount immunostaining of the placentae against Pecam1, a marker for endothelial cells
[170]. Consistent with the previous observation, the caliber of mutant umbilical cord was much smaller in comparison to that of the wild type. In addition, the fetal surface vessels were also abnormal in mutant placental disk. Unlike wild-type placenta, of which the surface artery and vein were exhibited as tree-like structures, mutant counterparts displayed no major blood vessel, and instead only a network of fine vessels was observed (Fig. 4.3 D-F). Together, these data suggested that the ablation of Smif caused defective formation of the umbilical cord and fetal placental vasculature, both of which are of allantoic origin.

4.2.4 Mutation of Smif gene affects placental labyrinth development

The presence of abnormal placental vascular development led us to question whether there were other placental defects associated with Smif ablation. For this, extensive histological analyses were carried out to compare mutant and wildtype placentae at various developmental stages raging from E8.5 to E10.5. The placentae of Smif−/− embryos were found to develop normally until E8.75 when in most cases, the allantois had already fused with the chorionic plate (data not shown). But at later stages, the chorionic plate of mutant placentae remained flat; no interdigitation of allantoic mesodermal cells with the chorionic trophoblast cells ever occurred, whereas wildtype placentae at this stage developed a complicated labyrinthine structure due to the invasion of fetal vasculature and subsequent growth and branching (Fig. 4.4).

Similar defects has been reported in ablation of Gcm-1, which is essential for spongiotrophoblast differentiation and loss of which causes failure in
branching morphogenesis at the chorioallantoic interface [105, 106]. Therefore, we set out to determine whether Gcm-1 expression is affected in Smif mutant placenta. Although it was obvious that no fetal vascular intrusion occurred at the Smif−/− placenta as shown by a flat unbroken line of Gcm-1 positive cells, the strength of Gcm-1 expression was comparable to that of the wildtype, indicating the syncitiotrophoblast cells in Smif mutant placenta was specified even though the labyrinth failed to develop (Fig. 4.5 A,B). Furthermore, the expression of Hand-1, encoding a basic helix-loop-helix transcription factor essential for trophoblast giant cell differentiation [108], also appeared normal in Smif null placentae (Fig. 4.5 C, D). In conclusion, while the specification of different trophoblast lineages in the chorioallantoic placenta was unaffected by Smif mutation, placental architecture was heavily perturbed.

To probe the mechanistic basis of the placental abnormality, cellular proliferation and apoptosis were assessed in Smif−/− placentae as abnormalities in these could easily affect placental structure. The proliferation rate of the undifferentiated chorionic trophoblast cells in mutant was not significantly different from that in control placenta as determined by BrdU incorporation assay. In addition, other trophoblast subtypes such as the spongiotrophoblast and trophoblast giant cells in mutant were also comparable with wildtype placenta (Fig. 4.6 G-K). E10.5 placentae were also examined for apoptotic cells by TUNEL assay, which showed that although for most cell types in chorioallantoic placentae there was no detectable difference between mutant and wildtype, the mutant fetal erythrocytes underwent extensive apoptosis within the placental
compartment (Fig. 4.6 A-F). This was somewhat surprising as at this stage the 
Smif⁻/⁻ embryos per se including erythorocytes circulating within the embryos 
displayed no increased cell death compared to control counterparts (data not 
shown). Nevertheless, the high apoptotic rate of fetal erythrocyte together with 
the failure of labyrinth formation in the placenta is likely the reason for mid-
igestation lethality of Smif mutant embryos.

### 4.2.5 Defects in allantoic vascular formation

In addition to placental vascular defects, we have also seen abnormalities 
in the umbilicus. Both are derivatives of the allantois. For this reason we decided 
to examine the allantoic vasculature. It has been suggested that the allantoic 
vessels are originally formed through vasculogenesis, just as the yolk sac and 
dorsal aorta (reviewed in [87]). Around head fold stage, shortly after the 
appearance of the allantoic bud, a nascent blood vessel starts to form at the 
distal tip of the allantois. With the growth of the allantois, the central vessel while 
undergoing extensive branching, extends proximally, and eventually fuses with 
the dorsal aortae. To determine whether there is any defect in the 
vasculogenesis within the Smif mutant allantois, embryos from E8.0 to D8.5 were 
examined by Pecam1 staining and carefully staged according to somite numbers. 
At S2, there was no sign of blood vessel formation in the mutant allantois, in 
contrast to the wildtype littermates of the same age, which already exhibited a 
major blood vessel along the center of the nascent allantois, suggesting delayed 
vasculogenesis due to Smif deletion (Fig. 4.7 A, B). As development continued,
small and short blood vessels of similar caliber were observed all over mutant allantois instead of one major central vessel, whereas in the wildtype fine vessels were formed as branches from one obvious major vessel (Fig. 4.7 C, D). The allantoic defects were further analyzed by sectioning after whole mount staining against Pecam1. Consistent with previous observations, only short vessels or clusters of angioblasts were found sporadically in the mutant allantois while there was one major vessel persisting throughout the whole-length of wildtype allantois (Fig. 4.7 E, F). This suggests that not only there was delayed allantoic vasculogenesis but the major vessel never formed in Smif mutants. The early defects in allantoic vascular development in the Smif null embryos were likely the reasons for the later abnormalities found in the umbilical artery and vein as well as the fetal surface vessels in the placenta disk, all being derivatives of the allantoic vasculature.

Nascent blood vessels form through two processes: vasculogenesis, which is the de novo formation of blood vessels, and angiogenesis, which defines the processes of branching and remodeling of the pre-existing blood vessels (reviewed in [171]). Therefore, an eminent question would be whether the defective vasculogenesis is accompanied by compromised angiogenesis in Smif mutant allantois. To determine this, an in vitro culture system was employed, in which the mesodermal cells of the cultured allantois spread out into a disc of cells, old blood vessels remain in the center, and newly branched blood vessels appear at the periphery region [172]. Interestingly, there was about 50% reduction of newly formed vascular branches in cultured Smif null allantois as
compared to that of the wt, suggesting that angiogenesis is likely compromised in the mutant allantois (Fig. 4.7 G-I).

Smif has been suggested to be a transcription activator in BMP pathway. The allantoic defects in Smif mutants indeed were reminiscent of the knockout phenotype of Smad1 (intracellular mediator for BMP signaling), which displayed chorioallantoic fusion defects or poorly formed umbilical chord connection [101, 102]. In addition, it has been shown that the expression of Vcam-1, an allantoic mesothelium marker important for chorioallantoic fusion, is absent in Smad1 mutant allantois [102]. In order to determine whether the loss of Smif might affect allantoic development in a similar fashion to the absence of Smad1, whole mount immunostaining of Vcam-1 was performed on E8.5 embryos. In contrast to Smad1 mutant, there was no change of Vcam-1 expression in Smif mutant allantois, indicating that the mechanism for Smif in chorioallantoic placental formation is different from BMP pathway (Fig. 4.8 A, B).

4.2.6 Reduced primordial germ cells (PGC) in Smif mutants

The PGCs are derived from precursors in the proximal epiblast, which also give rise to the allantois, the amnion, and the yolk sac blood islands. After gastrulation, PGCs can first be identified as a cluster of AP positive cells located posterior to the primitive streak at the base of the allantois. Later on, PGCs migrate through developing hindgut and finally reside into the genital ridges [173, 174]. Mutations affecting allantoic development have often been shown cause reductions in PGC numbers [101, 175, 176]. To determine whether deletion of
Smif also affects PGC development, Smif−/−, Smif+/+, and wild-type embryos ranging from S2-S5 were stained for alkaline phosphatase and PGCs were counted. While wt embryos and Smif heterozygotes show similar PGC numbers, there is greatly reduced PGC numbers was found in Smif homozygotes (Fig. 4.8 C-E). These results suggest that loss of Smif affects either the initial specification of the PGC precursor population in the proximal epiblast or survival and/or proliferation of the allocated PGC population.

4.2.7 Smif affects mRNA degradation

Smif has been reported as mammalian homologue of yeast decapping enzyme. However, its function in mRNA degradation remains unclear as purified Smif protein didn’t have any decapping activity nor did it change the decapping rate of Dcp2, a bona fide decapping enzyme in in vitro decapping assay [19-21]. To determine the function of Smif in mammalian mRNA degradation, MEFs derived from E10.5 mutant and control embryos were immortalized and subjected to pathway specific mRNA decay analysis. Basically, both mutant and control MEFs were treated with ligands to stimulate the expression of direct target genes prior to the addition of DRB (5,6-dichloro-1-b-D-ribofuranosylbenzimidazole), which suppress further transcription [177]. Subsequently, cells were harvested at different time points and the expression of immediate response genes were monitored by real-time PCR. For TGF-β treatment, the decay of its immediate response gene PAI-1 was examined. We found that PAI-1 degradation in mutant cells was slower than in control,
suggesting that Smif functions as a positive regulator of PAI-1 mRNA decay. In contrast the degradation of RARB, target gene for ATRA, was negatively regulated by Smif as deletion of Smif accelerated the degradation of RARB mRNA. Therefore, our data suggested that the role of Smif (Dcp1a) in mRNA decapping may be pathway dependent.

4.3 Discussion

Smif (Dcp1a) as a mammalian decapping protein has been suggested to be involved in mRNA degradation [19-21]. The turnover of mRNA plays an essential role in the regulation of gene expression; mutation of Dcp1 in yeast results in lethality [178]. However, the actual function of Smif in mammalian mRNA decapping remains elusive. It has been shown that the purified Smif protein doesn’t have enzymatic activity, nor does it seem to affect the dynamics of Dcp2 (the true mammalian decapping enzyme) mediated hydrolysis of 5’ cap structure [19]. Recently, it has been found that Smif helps to bring decapping-enhancing proteins into the Smif/Dcp2 protein complex. Thus it is likely that Smif positively regulates Dcp2 mediated mRNA decapping in mammals [25]. Meanwhile, Smif has also been identified as a Smad4 interacting protein, under the regulation of TGF-β/BMP signaling [14]. Therefore, the question becomes whether Smif functions as a connector between the two pathways or whether the two roles of Smif are actually independent of each other [19].
4.3.1 Smif in mRNA degradation

Our lab has established Smif mutant and control MEF lines. By using cell extracts in a decapping essay, our collaborators failed to identify any difference in mRNA decapping between the mutant and control MEFs (data not shown). However, there is a limit of sensitivity associated with this in vitro decapping assay, therefore we cannot exclude the possibility that Smif is involved in certain aspects of mRNA decapping or more generally, degradation. In addition, if Smif functions in the regulation instead of the general decapping reaction, the decapping assay is simply not suitable to examine this.

Thus we are trying to address this question using an in vivo mRNA degradation assay, in which the expression of target genes is stimulated in cells by ligand addition followed by scrutinizing transcript level over time. Interestingly, our preliminary data suggest that the degradation rate of target genes is different between mutant and control cell lines. In addition, it is pathway/target gene dependent. Notably, the basal level of target genes as well as fold of induction is also different between mutant and control cell lines in both ATRA and TGF-β stimulation, which suggests Smif may also play a role in determining the peak value of mRNA upon ligand induction. However, whether this putative function of Smif is related to its role in mRNA degradation or not are still too early to predict.

The expression pattern of Smif suggests it is a general housingkeeping gene (so is Smad2 and Smad4) as Smif is uniformly expressed in all tissues examined ([14] and our data). Therefore, it is somewhat surprising that mice homozygous for the Smif null allele, although embryonic lethal, can still develop
into mid-gestation stages with the majority of the tissues unaffected. Several possibilities remain open for examination. There is a second homologue of yeast Dcp1 in mammalian genomes Dcp1b. It is possible that Dcp1b can partially compensate for the loss of Smif (Dcp1a). Targeted deletion of Dcp1b can be generated and thus compound mutations of both genes can be achieved by mouse crosses to test the hypothesis.

Another possibility is that the Smif/Dcp2 mediated mRNA decapping plays lesser roles in higher organisms than in the yeast, in which both Dcp1 and Dcp2 are essential genes. In fact, in addition to Smif/Dcp2 complex, there is another decapping enzyme named scavenger decapping protein (DcpS), which has been suggested to play a major role in mRNA turnover in mammals [179].

It is also possible that Dcp1a, which is not directly involved in the hydrolysis of 5’ cap structure, functions as an adaptor protein so that mRNA decapping can be specifically regulated by certain signaling pathways. This notion is supported by the report that Smif is a Smad4 interacting protein [14]. However, more evidence is needed to cement this claim.

4.3.2 Smif and ARE mediated mRNA decay

It is well known that many unstable mRNAs contain the adenine-uracil-rich (ARE) elements, which target the mRNA for exosome mediated mRNA degradation (reviewed in [167]). Recently, it has been discovered that the Smif/Dcp2 protein complex is recruited to the ARE by the ARE-binding protein tristetraprolin (TTP) and its close homologue BRF1 [25]. Both TTP and BRF1
(Zfp36L2) belong to the tristetraprolin (TTP) family. There are four TTP family members identified in mammals, the other two being Zfp36L1 and Zfp36L3 [180, 181]. All four proteins contain the highly conserved CCCH tandem zinc-finger (TZF) domains, which are the ARE-binding domains. The family members have similar activities in ARE-mediated mRNA decay, but are expressed differently in tissues and respond to various stimuli, suggesting that they may control the stability of specific mRNAs in a highly regulated manner.

TTP, the best studied family member, has been shown to destabilize tumor necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNAs. TTP deletion mice display a severe inflammatory phenotype due to the excessive secretion of the cytokines [181]. Zfp36L1, however, is highly expressed in murine allantois at E8.0 and deletion of Zfp36L1 leads to failure in chorioallantoic fusion in two thirds of the embryos while the rest of concepti display decrease cell division in the placenta at E10.5 [182]. This is interesting as it suggests a model in that ARE-mediated mRNA degradation is highly active in the allantois before chorioallantoic fusion. This mRNA degradation event requires both Smif/Dcp2 protein complex as the constitutive mRNA decapping machinery and the ARE-binding protein Zfp36L1 which functions to confer tissue as well target gene specificity to the pathway. Therefore, loss of either protein can result in severe consequences for allantoic development.
4.3.3 Smif and TGF-β/BMP signaling

Smif has previously been suggested to translocate into the nucleus in response to TGF-β/BMP activation [14]. However, we found that upon TGF-β stimulation, the transfected GFP-Smif fusion protein remained punctuately distributed in the cytoplasm (which is consistent with the previously reported Dcp1a cellular localization at quiescent state) and does not translocate into the nucleus as reported (data not shown). In a parallel experiment, Smad4 was shown to translocate into the nucleus with the addition of TGF-β in both mutant and control MEF cells (data now shown). Our results suggest that even if Smif is involved in certain aspect of TGF-β/BMP pathways, it is unlikely Smif would translocate into the nucleus and directly regulate mRNA transcription and that the previous reported Smif and Smad4 interaction may need to be more carefully re-examined [14].

4.3.4 Smif is important for allantoic vascular patterning

The most prevalent phenotype of Smif knockout embryos is the defective vascular development in the allantois. The allantoic core vessel is first developed as a cord of aggregated angioblast, which then undergoes tubulogenesis to form one major blood vessel (reviewed in [183, 184]). In contrast, Smif mutant allantois only forms multiple small vessels instead of a large one. This indicates that the angioblasts in mutant allantois are still differentiated, as there is still vascular formation, but vascular patterning is defective. Real-time PCR was performed for several genes important for
vasculogenesis or angiogenesis in an attempt to identify the target genes affected by Smif mutation. However, none of them seems to be affected (data not shown). Although the allantoic defects have been reported in knockout of many genes (reviewed in [83]), little has been done to analyze the vasculature in mutant allantois, which makes it difficult to narrow down potential Smif targets.

Recently the Down’s lab has reported that Brachyury (T), a T-box gene which has previously been shown to be involved in mesodermal formation and posterior development, is essential for allantois growth, survival of allantoic core cells and allantoic vasculogenesis. Embryos homozygous for T mutation display absence of Pecam1 during the initial stage of allantoic formation, resembling Smif mutant allantoic phenotype, although more severe. Notably, T displays haploinsufficiency with partial penetrance, indicating the dosage of T is critical to murine embryogenesis [185]. Therefore, it will be interesting to examine whether there is reduction of T expression in Smif mutant allantois.

Another question is whether the vasculogenesis defect is cell autonomous or non-autonomous; in other words, whether it is due to the intrinsic nature of Smif mutant angioblasts or the failure in the communication with dysfunctional stroma cells. Notably, the function of the stroma cells in allantoic vasculogenesis remains largely unknown. To dissect the interaction between the two cell types would be facilitated by specifically deleting Smif only in one cell type using conditional knockout approaches.

Disruption of genes in the same pathway often results in similar phenotypes. In the case of TGF-β pathway, none of the TGF-β family ligands or
the intracellular mediator Smad2/Smad3, when mutated, have exhibited any allantoic/placental dysmorphogenesis [2, 47, 186, 187], indicating it is unlikely Smif functions in TGF-β pathway in the development of the allantois/placenta. For the BMP pathway, however, on the first glance, it seems that Smif may function in the BMP pathway in that targeted deletion of numerous BMP components have given rise to allantoic/placental/PGC defects [99-102]. However, on close examination, placental deformation is a fairly prevalent phenotype; more than 50 genes have been reported to display placental defects when mutated, which is no surprise as many major pathways such as WNT, FGF, EGF, BMP, MAP kinease and VEGF signaling have been reported to be involved in some aspect of placental development (reviewed in [83]). In addition, it is found that the expression of Vcam-1 is missing in Smad1/BMP mutant allantois but not in Smif mutant, indicating BMP signaling may affect allantoic development through the specification of the mesothelium while Smif is not [100, 102]. Together, these suggest Smif may function through separate mechanisms from BMP signaling during mammalian embryogenesis.

4.4 Materials and Methods

4.4.1 Construction of the Targeting Vector and Generation of Germline Chimeras

A 16kb KpnI/KpnI Smif genomic fragment containing promoter sequence and exons 1-3 was subcloned into pBluescript KS (Stratagene, La Jolla, CA) from a BAC clone. A 5kb KpnI/XhoI fragment containing Smif promoter region
was placed downstream of the floxed neo cassette of pLoxpneo. A 7.5 kb HpaI/HpaI fragment containing exon2 and exon 3 was inserted upstream of the floxed neo. These manipulations replaced the genomic sequence encompassing exons 1 with the reverse oriented PGK-neo cassette in the vector (Fig. 4.2A). This Smif targeting vector was electroporated into 129/SvEv TC1 ES cells and selected with G418 and gancyclovir; 288 resistant colonies were selected and further analyzed by Southern blot using a 1kb 3’ external probe (Fig. 4.2A). Correctly targeted clones were microinjected into C57BL/6 blastocysts and implanted into foster mothers to obtain chimeras by standard procedures. The chimeric founders were crossed with Black Swiss mice to obtain Smif\textsuperscript{+/−} offspring. To generate Smif null allele onto C57/BL6 background, C57/BL6 males (Harlan) were bred with Smif\textsuperscript{+/−} females. Offspring were genotyped for Smif deletion allele, and animals testing positive for Smif mutation were backcrossed to their C57/BL6 fathers for 5 or 6 generations. To obtain Smif\textsuperscript{WW} allele, Smif\textsuperscript{+/−} mice were bred to EIIA-Cre [158] mice in order to excise the neomycin cassette.

4.4.2 Genotype analysis of Smif\textsuperscript{+/−} mice

Smif\textsuperscript{+/−} mice were genotyped using following primer combinations. For detecting the wildtype allele, PL (5’-GCA TCC CTG CAC ACT TAT GTC AGC ATA-3’) and PR (5’-ACT AGC CTG CCT GGA ACT ACA ATA GAC-3’) amplify a 1.2kb product. For the targeted mutation allele, PL and TW (5’-ATC GCC TTC TAT CGC CTT CTT GAC-3’) amplify a 600bp fragment.
4.4.3 Histology and in situ hybridization

Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4°C overnight, followed by dehydration through a graded ethanol series, cleared in xylene, embedded in paraffin wax Paraplast (Oxford Labware, USA), and sectioned at 8um. Sections were collected on Superfrost plus glass slides (Fisher Scientific, USA) and stained with hematoxylin and eosin using standard procedures.

Radioactive in situ hybridization was performed using standard procedures. S\textsuperscript{35}-labeled probes were prepared for Hand-1\cite{108}, Gcm-1\cite{105}, and Smif (exon1-6, generated by cloning RT-PCR product into pBluescript KS).

4.4.4 BrdU incorporation assay and TUNEL analysis

BrdU incorporation assays were performed using BrdU In-Situ Detection Kit II from BD Pharmingen (San Diego, CA) according to the manufacture’s protocol.

The FragEL™ DNA Fragmentation Detection Kit was purchased from Calbiochem (San Diego, CA) to assay the level of programmed cell death in placenta sections.

4.4.5 Whole-mount immunostaining for Pecam1

Embryos were fixed in 4% paraformaldehyde (2 hours, 4°C), rinsed three times in PBS, and specimens were processed and stained according to published methods \cite{170}. After color reaction, embryos were washed in PBS, post-fixed in PFA, rinsed in PBS again, equilibrated first in 50% and then 50%
glycerol, gently squashed beneath a cover slip and viewed and photographed using a compound microscope. Some of embryos, after Pecam1 staining, were embedded in paraffin and sectioned at 8um.

4.4.6 Allantoic explant culture

Allantoises were dissected from E8.0-E8.5 embryos, carefully staged, cultured and processed according to published methods [172]. Genomic DNA from the rest of the embryonic tissues were extracted and used for PCR genotyping.

4.4.7 Detecting and counting PGCs

Embryos between E8.0 and E8.5 were dissected from the deciduas, fixed in 4% PFA for 2 hours at 4°C, washed in PBS, and transferred into freshly prepared NBT/BCIP solution (Roche, IN) to stain for 10 minutes. Color reactions were stopped by washing embryos with PBS first and then post-fixation in PFA for 2 hours. The counting of PGCs was performed according to the published method [175].

4.4.8 RNA isolation and Real Time RT-PCR

Total RNA was isolated using the total RNA kit (Bio-Rad laboratories, Hercules, CA, USA). cDNA was synthesized using 1µg of total RNA (DNase-treated) in a 20µl reaction mixture with random primers using Superscriптase III First-Strand Synthesis Systems for RT-PCR kit (Invitrogen, Carlsbad, CA, USA). Serial tenfold dilutions (104 to 109 molecules) of pBactin-231 were used as a reference molecule for the standard curve calculation (Figure 2). All Real-Time PCR
reactions were performed in a 20µl mixture containing 1/20 volume of cDNA preparation, 1X iQ SYBR Green Supermix, and 0.5µl of each primers (0.2µg/µl). Real-Time quantitations were performed using the BIO-RAD iCycler iQ system. The fluorescence threshold value was calculated using the iCycle iQ system software.
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<sup>a</sup> Embryos in the process of being resorbed

**Table 4.I.** Genotypic analysis of offspring resulting from crosses of 50:50 Smif^{+/-} mice.
Figure 4.1. In situ hybridization to detect Smif expression during early embryonic development. (A and C) Bright-field photomicrographs of sections hybridized with Smif antisense probe. (B and D) dark-field photomicrographs of the same sections. (A and B) Sagittal section of E8.5 wildtype murine embryo within maternal decidua. (C and D) Transverse section of E9.0 wildtype murine embryo. ch, chorion; al, allantois; br, brain; ht, heart; sm, somite; ys, yolk sac.
Figure. 4.2. Targeted deletion of the Smif gene in mouse embryos. (A) Gene targeting strategy for generating the Smif knockout allele. Smif exon 1 and its flanking sequence is replaced with a reverse-oriented PGK-neo cassette in the targeting vector. The PGK-neo cassette is floxed with loxp sites which are indicated as grey triangles. The Smif genomic sequence is shown schematically with the exons indicated by grey boxes. The PCR primers for genotyping are shown as black arrowhead: PL, PR, and TW. (B) Southern blot analysis of the targeted allele in ES cells. Genomic DNA from ES cells is digested with EcoR V and detected by a 3' external probe. A 17.7 kb fragment is detected for the wild-type allele, and a 13.1 kb fragment is detected for the Smif- allele. (C) PCR genotype analysis of embryos generated from Smif"/" crosses. Although no homozygotes (-/-) mouse is found at weaning stage, they could be recovered at a normal Mendelian ratio at E8.5 and E9.5. (D) The genomic structure of wildtype, Smif deletion, and ΔN loci. The primers for RT-PCR are shown as arrows: E2 and E6. They are located in exon 2 (E2) and exon 6 (E6) respectively. (E) RT-PCR for Smif and Hprt expression in Smif embryos. (F) RT-PCR for Smif and Hprt expression in SmifΔN/ΔN embryo.
Figure 4.2
Figure. 4.3. Homozygous disruption of Smif leads to abnormal formation of umbilical cord as well as placental fetal vasculature. (A and B) The umbilical cord (outlined by dotted lines and indicated by a black arrow) of control and a less severely affected mutant embryo at E10.5. Mutant umbilical cord is much thinner than control. (C) In more severe cases, the umbilical cord fails to form, instead, the allantois grows into a sphere of cells (black arrow). (D-E) Pecam taining of placenta from A-C, respectively. (D) In control placenta, the invasion and branching of the umbilical artery and vein (red arrow) forms fetal vascular tree (red arrowhead). (E) In mutant placenta, however, not only is the umbilical chord (red arrow) much thinner, but no obvious vascular branching patterns are observed. Red arrowhead pointed to vascular plexus. (F) In the case when the allantois failed to fuse with chorionic plate, there are still blood vessels formed, which are presumably derived from a thin layer of extra-embryonic mesodermal cells on the surface of the chorionic plate.
Figure 4.4 Haemotoxylin and eosin staining of representative histological sections from Smif control (A and C) and mut (B and D) placentas at E9.5. C and D represent magnified chorioallantoic interface of A and B, respectively. (A and C) In control conceptuses, the fetal blood vessels invade into chorionic plate to create villi (red arrow in A and C). Extensive villous branching generates the densely packed labyrinth zone. (B and D) In contrast, the labyrinth zone is completely absent in the mutant placenta. Mutant chorionic plate remains flat at this stage without any sign of villous formation. The dashed line in A and B separates trophoblast giant cells from maternal tissue. The black dotted line in C and D indicates chorioallantoic interface. The red dotted line indicates endothelial lining of fetal blood vessels. Black arrows in C and D indicate nucleated fetal erythrocytes. al, allantois; ch, chorionic trophoblast cells; lz, labyrinthine zone; sp, spongiotrophoblast; m, maternal erythrocytes.
Figure 4.5 Expression of molecular markers in E9.5 placentas from control (A and C) and mutant (B and D) littermates analyzed by in situ hybridization. (A) Gcm1 expression in syncytiotrophoblast outlined chorionic villi. (B) In mutant placenta, Gcm1 is expressed in a subset of trophoblast cells in the flat chorionic plate. (C) Hand1 transcripts are present in trophoblast giant cells, spongiotrophoblasts and labyrinthine trophoblasts. Similar expression level of Hand1 is detected in mutant placenta section (D).
Figure 4.6 Cellular proliferation and Apoptosis. (A-D) TUNEL assay on representative sections of control (A and C) vs. mutant (B and D) placenta at E9.5. Note that although there are more apoptotic cells (black arrow) in mutant placenta (B) than control (A), on close up view, almost all apoptotic cells in mutant are fetal erythrocytes (red arrow). (E and F) H&E staining of fetal erythrocytes (red arrow). Note that many mutant erythrocytes display fragmented nuclei (F). (G-K) Proliferating cells in control (G and I) vs. Smif mutant (H and J) E9.5 placenta are quantified using BrdU incorporation analysis. Chorionic trophoblasts, which exhibit higher BrdU incorporation (brown nuclei) rate than spongiotrophoblasts (also see statistics in K), undergoes extensive folding ( black arrow) in control placenta (G). In contrast, mutant chorionic plate remains flat (H). The dashed line indicates the outer surface of placenta lined by trophoblast giant cells.( I and J) Magnified chorioallantoic interface of G and H, respectively. The dotted lines separate chorionic trophoblast from other cell types. al, allantois; ch, chorionic trophoblast cells; ly, labyrinthine trophoblast cells; sp, spongiotrophoblast. (K) Quantification of BrdU positive nuclei in control vs mutant placenta sections. Results are expressed as percentage of BrdU-immunoreactive cells as compared to total cells (pro rate). mut cho, mutant chorionic trophoblast; mut spo,mutant spongiotrophoblast; mut avr, mutant average; wt avr, wt average.
Figure 4.6
Figure 4.7  (A-D) Whole mount Pecam staining of E8.0-E8.5 embryos. At S2, a central vessel (black arrow) appears in control allantois (A), while no vaculogenesis occurs in mut allantois (B). At S5, in control allantois (C), the central vessel (black arrow) persists and starts to branch out (black arrowhead). No central vessel is detected in mut allantois (D) although there are fine blood vessels (black arrowhead) formed at this stage. (E and F) Representative cross-sections of control and mutant allantois after Pecam staining (left/right: proximal/distal). The central vessel (white arrow) is throughout the whole length of control allantois (E). But no such vessel is found in mutant allantoic sections in F, instead, there are sporadic short vessels or angioblasts (white arrowhead) in mutant allantois (F). Fine blood vessels other than the central allantoic vessel are pointed by white arrowheads. Dorsal aortae at the root of allantois (first from left in E and F) are indicated by black arrows. The black arrowhead in the second from left section of F indicates remnant yolk sac. (G-I) PECAM staining of control (G) and mut (H) allantoic culture. The black arrows indicates newly formed vessel branchings spreading to the peripheral region, magnified in inset at right. (I) Numbers of nascent peripheral blood vessel branchings (as indicated in G and H) of control and mut allantois are compared.
Figure 4.8 Allantoic mesothelium and primordial germ cells in mutant vs. control allantois. (A and B) Whole mount staining of Vcam1 after chorioallantoic fusion (E8.5, S7). Vcam1 staining (white arrow) in mutant allantois (B) is similar compared with that of control (A), even though mutant allantois is often shorter and failed to form the “L” shaped contact with chorionic plate as seen in control at this stage. (C-E) Alkaline phosphate staining for PGCs (red arrow) in both control (C) and mutant (D) embryos. (E) Although there is no difference in PGC numbers between Smif<sup>++</sup> and Smif<sup>++/−</sup> embryos, Smif<sup>−/−</sup> embryos exhibit significant reduction of PGCs around E8.5.
Figure. 4.9 Comparison of the rate of pathway-specific mRNA decay in immortalized Smif<sup>−/−</sup> (mutant) vs. Smif<sup>+/−</sup> (control) MEF cultures by quantitative real time RT-PCR. Cells were initially exposed to all-trans RA(ATRA) (A) or TGFβ (B) for 2 hrs, and then changed to regular media with DRB (100 µM) to suppress new mRNA synthesis. Total RNA, obtained at different time points before or after DRB addition, was subjected to real time RT-PCR analysis. (A) Realtime PCR for ATRA responsive gene, RARB showed in Smif mutant cells, RARB decayed faster than in control cells. (B) The degradation rate of PAI-1, a TGFβ direct target, was slower in Smif<sup>−/−</sup> vs. Smif<sup>+/−</sup> cells.
CHAPTER 5

DISCUSSION

Embryonic development is a very complicated and highly coordinated process; about one in thirty babies is affected by birth defects. Using mouse as a model system helps to elucidate the molecular mechanisms in human development. In addition, since many signaling pathways are involved in both embryogenesis and disease progression, discoveries in developmental biology certainly help to understand the abnormalities under pathological conditions.

The TGF-β family ligand Nodal is involved in multiple processes during early embryogenesis. The susceptibility of each process to the reduction of Nodal also varies [44]. One way to address this question is the attenuation of Smad2 and Smad3, intracellular mediators for Nodal signaling [48]. Here we deleted one allele each of Smad2 and Smad3 to determine which of the three germ layers is more susceptible to the reduction of Nodal signaling. Our results show that the specification of the anterior endoderm is affected in all of the double heterozygous embryos, suggesting that it is the differentiation of anterior definitive endoderm that requires the highest level of Nodal signaling. About half
of the double heterozygous embryos also exhibit AME defects, indicating AME requires the second-highest Nodal level for its specification.

We have also shown in this study attenuation of Smad and Smad3 leads to decreased expression of Hex, a gene needed for hepatogenic development, which is likely the cause for failure in hepatocyte specification in the mutant. Our model for the effects of Smad2 and Smad3 on Hex expression involves both direct and indirect mechanisms. First, the definitive endoderm fails to cover the heart in the mutant embryos, and is therefore unavailable to receive hepatic inductive signals. This defect appears to be due to a failure in endodermal migration and is indirect. Second, Hex expression from the hepatogenic endoderm is lost due to a direct mechanism. Smad2 and Smad3 likely transactivate the Hex promoter directly. Hex has been shown to be a Nodal target gene in *Xenopus laevis* [140], and the murine Hex promoter has Smad binding sites [133].

Formation of the placenta is a unique feature of mammalian embryonic development. Therefore mouse provides a good model system to study placental organogenesis. More than 50 genes have been reported to display placental defects when mutated, which is no surprise as many major pathways such as WNT, FGF, EGF, BMP, MAP kinease and VEGF signaling have been reported to be involved in some aspect of placental development (reviewed in [83]).

The first evidence that mRNA decay pathway is also involved in placental formation comes from targeted disruption of *Zfp36L1*, a TTP family protein functioning in ARE-binding. AREs are present in many unstable mRNAs and
recognized by mRNA degradation machineries. Zfp36L1 is expressed at high levels in murine allantois at E8.0 and is important for chorioallantoic fusion [182].

To further elucidate the role of mRNA decay in embryonic development, we generated deletion allele of Smif gene, which encodes a mammalian homolog of yeast decapping protein 1 [19-21]. Disruption of Smif expression results in recessive embryonic lethality at E11.5 due to the malformation of the umbilicus and the dysfunctional placenta. In addition, allantoic vascular formation is also severely impaired, which is likely the cause of the later umbilical and placental defects. Smif has been recently shown to associate with two of the TTP family member proteins. Therefore, it would not be a surprise that Smif also interacts with Zfp36L1 in ARE-mediated mRNA decay. In addition, the similarity between the mutational phenotypes of the two genes strongly suggests that they function in the same pathway during allantoic development. The fact that Smif is ubiquitously expressed in all embryonic tissues while Zfp36L1 is specifically upregulated in pre-fusion allantois is interesting. It suggests that Smif/Dcp2 protein complex functions in the ARE-mediated mRNA degradation event as the constitutive mRNA decapping machinery while the ARE-binding protein Zfp36L1 is to confer tissue as well as target gene specificity to the pathway.
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