INTERCELLULAR SIGNALING PATHWAYS IN THE INITIATION OF MAMMALIAN FOREBRAIN DEVELOPMENT

by

Yu-Ping Yang

Program in Genetics
Duke University

Date: March 16, 2007

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Elwood A. Linney

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David R. McClay

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Program in Genetics in the Graduate School of Duke University

2007
ABSTRACT
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Abstract

The Spemann organizer in amphibians gives rise to the anterior mesendoderm (AME) and is capable of inducing neural tissues. This inductive activity is thought to occur largely via the antagonism of Bone Morphogenetic Protein (BMP) signaling in the organizer. In the mouse, BMP antagonists Chordin and Noggin function redundantly in the AME and are required during forebrain maintenance. However, the timing of forebrain initiation and the function of BMP antagonism in forebrain initiation remained unclear prior to this study. In addition, the Transforming Growth Factor β (TGFβ) ligand Nodal patterns the forebrain via its function in the anterior primitive streak (APS), the precursor tissue of the AME. Whether BMP and Nodal signaling pathways interact has not been previously investigated.

The goal of this dissertation was to investigate the cellular and molecular mechanisms involved in early mammalian forebrain establishment by embryonic and genetic manipulations. This study determined that forebrain initiation occurs during early gastrulation and requires signals from the AVE and AME. The AVE was identified as a source of active BMP antagonism in vivo, and the BMP antagonism supplied by exogenous tissues was capable to promote forebrain initiation and maintenance in the murine ectoderm. It is likely that BMP antagonism enhances forebrain gene expression via inhibiting posteriorization. This study further identified a possible crosstalk between BMP and Nodal signaling. Loss of Chordin or Noggin in combination with heterozygosity for Nodal or Smad3 results in holoprosencephaly. Molecular analyses suggest that the BMP-Nodal interaction occurs in the APS and/or the AME. Failure of this interaction results in an imbalance of BMP and Nodal signal levels that devastate APS and AME patterning during early forebrain establishment, ultimately leading to holoprosencephaly in mid-gestation. This interaction is likely to occur extracellularly, possibly by formation
of a BMP-Nodal heteromeric complex. Furthermore, the spatiotemporal expression of phospho-Smad1/5/8, an effector of BMP signaling pathway, was characterized during early mouse embryogenesis. Distribution of phospho-Smad1/5/8 serves as a faithful readout of BMP signaling activity and helps to better understand how BMPs are involved in patterning early embryos. The implication of phospho-Smad1/5/8 expression in both wildtype and mutant embryos is also discussed.
Acknowledgements

It is finally the last part. I had never written this much English in my life, and the writing skill remains dull. I was thinking about trying to write very moving acknowledgements but right now it may be as plain as it can be.

I was really lucky and blessed to finish the dissertation work, and there are many to thank. Of course first you have to thank your advisor/mentor/PI to offer you an opportunity to work in this lab with these projects. That being said, I am grateful that I have this chance to work for Dr. John Klingensmith. Thanks for John’s assistance, advices, and patience during all these years. Sometimes I feel amazed how he could tolerate a stubborn and sometime unreasonably bullheaded person like me. I am glad (or I hope) that we survived from this advisor-grad student relationship. So the first thank goes to you John.

Next, I would like to thank my thesis committee: Drs. Dave McClay, Blanche Capel, Peggy Kirby, and Elwood Linney. They are the best committee members I could have been asked for. They are all very helpful and available for suggestions. Thanks for all the valuable advices in our meetings. I have to especially thank Uncle Dave for his understanding, encouragements and assistance. With his help, I get to have a chance to do TA this semester to support most of my stipend, which buys me a lot of time to finish this dissertation and eases my mind from a lot of worries. I probably would have adopted anxiety problem and have to go to therapy if it weren’t his help. I have been Dave’s TA for two semesters now, and yet I am still amazed by his teaching and enjoying teaching for him. Well, I do hope I am doing OK teaching for you, Dave.

Scientifically, I have to thank a lot of people for helping me with my projects. I have to thank Ryan Anderson for the collaboration of many projects we’re working on. Thanks to Xiao-Fan Wang for kindly sharing the Smad3 mutant mice with us. Thanks to
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whole time with the family two years ago when my grandmother passed away. Last month, my grandfather went into coma all of the sudden and is still in coma and is almost impossible to wake up again, but I couldn’t fly back or do anything to help. While I am writing this dissertation, I feel extremely sorrow the whole time. But all my family members are still encouraging me and cheering me up, while they are so sad and busy between their work and the hospital. Thank you Mom, Auntie Ching-Mei, Uncle Ta-Yu, my brother Chung-Fu, and my cousins Chia-Juei and Chia-Yuan. I also have to thank Simon’s mom for her prayers and support for my family and me. I owe them too much.

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This dissertation is dedicated to the loving memory of my grandparents,

Yong-Liang Sun-Lung and Shu-Chi Lung,

who have been and always will be the inspiration of my life.
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<th>Full Form</th>
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<tbody>
<tr>
<td>0B:</td>
<td>Zero-allantoic bud</td>
</tr>
<tr>
<td>A-P:</td>
<td>Anterior-posterior</td>
</tr>
<tr>
<td>ADE:</td>
<td>Anterior definitive endoderm</td>
</tr>
<tr>
<td>AME:</td>
<td>Anterior mesendoderm</td>
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<tr>
<td>APS:</td>
<td>Anterior primitive streak</td>
</tr>
<tr>
<td>AVE:</td>
<td>Anterior visceral endoderm</td>
</tr>
<tr>
<td>BA:</td>
<td>Branchial arches</td>
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<tr>
<td>BMP:</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BSA:</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CNS:</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>D-V:</td>
<td>Dorsal-vetral</td>
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<tr>
<td>DE:</td>
<td>Definitive endoderm</td>
</tr>
<tr>
<td>DVE:</td>
<td>Distal visceral endoderm</td>
</tr>
<tr>
<td>EB:</td>
<td>Early allantoic bud</td>
</tr>
<tr>
<td>EGO:</td>
<td>Early gastrula organizer</td>
</tr>
<tr>
<td>EHF:</td>
<td>Early headfold</td>
</tr>
<tr>
<td>ES:</td>
<td>Early streak</td>
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<tr>
<td>HPE:</td>
<td>Holoprosencephaly</td>
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<tr>
<td>IB:</td>
<td>Immunoblotting</td>
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<tr>
<td>IP:</td>
<td>Immunoprecipitation</td>
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<tr>
<td>L-R:</td>
<td>Left-Right</td>
</tr>
<tr>
<td>LB:</td>
<td>Late allantoic bud</td>
</tr>
<tr>
<td>LHF:</td>
<td>Late headfold</td>
</tr>
</tbody>
</table>
LS: Late-streak
MGO: Mid-gastrula organizer
MS: Mid-streak
PAF: posterior amniotic fold
PCP: Prechordal plate
pSmad1/5/8: Phosphorylated-Smad-1/-5/-8
qPCR: Quantitative RT-PCR
TGFβ: Transforming Growth Factor β
VE: Visceral endoderm
WMISH: Whole-mount in situ hybridization
Chapter 1:

Introduction
The head is one of the most complex and evolutionarily plastic parts of the vertebrate body. The complexities of building a head and face allow for the specialized functions of the head, and thus result in a functional anterior center. Craniofacial development involves a series of morphogenetic movements, epithelial-mesenchymal interactions, reciprocal tissue-to-tissue interactions, and neuron projections, all of which are orchestrated by crosstalk between signaling cascades. Elucidating these processes during craniofacial development, both morphologically and molecularly, provides the basis for understanding not only the evolutionary changes in craniofacial formation but also the mechanisms leading to craniofacial malformations.

The first step in craniofacial development is the formation of the central nervous system (CNS) precursors, which has long been the center of attention for vertebrate embryologists. This process is initiated by neural induction (the earliest step in the determination of ectodermal cell fates), followed by the patterning of the elaborate forebrain region. All of these processes involve morphogenetic movements and cell-to-cell interactions coordinated by signaling molecules. Specifically, the Transforming Growth Factor β (TGFβ) superfamily of secreted signaling molecules, Bone Morphogenetic Proteins (BMPs) and Nodal, are involved in these processes. This dissertation explores the role of the BMP signaling pathway during early mammalian neural induction and forebrain specification, as well as the interactions between BMP and Nodal signaling during early forebrain patterning.

**Neural Induction involves Signals From the Spemann Organizer**

In vertebrates, the neural plate is initially established during gastrulation, when the ectoderm becomes subdivided into neural and non-neural domains. In the
amphibian, the neural plate can be induced in the ectoderm by signals from the dorsal lip of the blastopore, the region referred to as the “organizer” (Figure 1.1). The organizer incorporates two biological properties: (1) the induction of neural differentiation, and (2) the patterning of induced tissues into regions with distinct polarities, reminiscent of embryonic body axes (reviewed by (Harland and Gerhart, 1997).

The identification of the organizer, which was the starting point towards our understanding of head induction, was the result of experiments carried out by Hans Spemann and Hilde Mangold. In their experiments, when the dorsal mesoderm from a light-colored newt embryo was transplanted to the presumptive ventral mesoderm of a dark-colored newt embryo, an ectopic axis was induced (Spemann and Mangold, 2001). This secondary axis included a notochord, somites and other axial components. Moreover, based on the pigmentation of the new axis, the grafted tissue not only contributed to the ectopic axis but also directed the patterning of host tissues into ectopic axial structures. This observation suggests the capability and the instructive properties of the organizer to induced neural fates (reviewed by (Harland and Gerhart, 1997). In addition, Spemann also established that the inductive ability of the organizer is dynamic. Transplants of early (younger) organizer tissue induced a secondary head, while transplants of older organizer tissue induced a secondary tail. Spemann thus proposed the existence of a “head organizer” and a “trunk organizer” (Harland and Gerhart, 1997).

Molecular Composition of the Organizer

The molecular composition of the organizer could not be identified until the technique of cDNA cloning became practical. The first investigations started with the
Figure 1.1: Proteins Secreted by the Spemann Organizer (Dorsal) or the Ventral Signaling Center in a Frog Gastrula. The Spemann organizer (purple) secretes BMP antagonists Chordin, Noggin, and Follistatin; Wnt antagonist Dickkopf, BMP/Wnt/Nodal antagonist Cerberurs; and *Xenopus* Nodal-related genes (Xnrs). The ventral center (green) is the source of BMP proteins, as well as Bambi, a BMP inhibitor induced by BMP signaling activation. D, dorsal; V, ventral.
Figure 1.1: Proteins Secreted by the Spemann Organizer (Dorsal) or the Ventral Signaling Center in a Frog Gastrula
cloning and characterization of the homeobox gene *goosecoid* (Cho et al., 1991), although subsequently many other genes were identified that have similar or even more potent activities than *goosecoid* (reviewed in (De Robertis, 1997). All these genes meet two minimal criteria: (1) they are present at the right time and place—within the organizer at the gastrula stage; and (2) the purified protein (or mRNA), when presented to normal responsive tissue at the right time, elicits a normal response (Harland and Gerhart, 1997). Discoveries of these organizer genes facilitated identification of homologous regions corresponding to the Spemann organizer in other vertebrates, which is known as the embryonic shield in zebrafish, Hensen’s node in the chicken, and the node in the mouse.

Several key secreted genes have been identified as components of the Spemann organizer, including Chordin (Chrd), Noggin (Nog), Follistatin, Dickkopf-1 (Dkk1), Cerberus, Frzb, and Xnr3 (Figure 1.1). Surprisingly, most of these proteins act as anti-ligands by binding to secreted growth factors, such as TGFβ and Wnt ligands, disrupting their activation of signaling (reviewed in (De Robertis et al., 2001). Molecular studies of these genes also suggest that the activity of the organizer in neural induction and patterning is in part determined by the spatiotemporal activation of these signaling pathways.

**Organizer Factors Chrd and Nog Are BMP Antagonists**

*Chrd* and *Nog* were first identified by their ability to induce a secondary axis after RNA injection in *Xenopus* (Sasai et al., 1994; Smith and Harland, 1992). They are expressed in the Spemann organizer in *Xenopus* gastrula and in the prechordal plate at slightly later stages. Expression of either *Chrd* or *Nog* induces neural tissue in animal cap explants (Sasai et al., 1995) (Lamb et al., 1993). *Chrd* and *Nog* encode secreted proteins
that form tight complexes with various BMPs, with higher affinities than that of BMPs binding to their receptors, thus sequestering BMPs in inactive complexes (Piccolo et al., 1996; Zimmerman et al., 1996). These observations provide supporting evidence that neural induction occurs via the inhibition of BMP signaling within the ectoderm by secreted antagonists from the organizer.

In mouse, the organizer is located at the anterior primitive streak (APS) of mid-streak (MS) embryos. The APS is the precursor of the axial tissues, including axial mesendoderm (AME) and the node. Chrd is expressed in the APS at early gastrula stages, and is later expressed in the axial mesendoderm (Bachiller et al., 2000). Chrd absence in a hybrid background causes only a small percentage of embryos to ventralize at gastrulation, suggesting that Chrd on its own does not suffice to block BMP signaling (Bachiller et al., 2003). Chrd<sup>−/−</sup> embryos display normal CNS development; however, they mimic human DiGeorge syndrome, which is due to a lack of Chrd function in the pharyngeal endoderm at later stages (Bachiller et al., 2003). Mouse Nog<sup>−/−</sup> embryos undergo normal gastrulation and neural plate patterning, but show abnormal skeletal and somite formation (McMahon et al., 1998; Stottmann et al., 2006). These observations suggest that Chrd and Nog have similar but redundant functions during mouse gastrulation. Indeed, Chrd;Nog double homozygous embryos display defects in forebrain patterning, formation of the anterior AME, and random left-right heart looping (Bachiller et al., 2000). Chrd<sup>−/−</sup>; Nog<sup>−/−</sup> compound mutant embryos display defects restricted to the head (Anderson et al., 2002). These observations suggest that Chrd and Nog synergize in patterning all 3 axes during mouse embryogenesis and their function in regulation of BMP signaling is specifically crucial during anterior development.

The role of the BMPs and their antagonists during dorsal-ventral (D-V) patterning is evolutionarily conserved between fly and vertebrates (De Robertis and Sasai, 1996). The expression of *decapentaplegic* (*dpp*), a fly TGFβ homolog closely related
to vertebrate Bmp2 and Bmp4, is confined to the dorsal part of the embryo and specifies dorsal cell fates. short gastrulation (sog), the fly Chrd homolog, is expressed in the lateral part of the embryo and antagonizes the dorsalization activity of Dpp protein. Moreover, vertebrate Chrd or Nog can rescue sog mutant fly embryos, although Nog is not conserved in flies. Sog can also dorsalize the mesoderm and neutralize the ectoderm in the frog embryo (Holley et al., 1995; Holley et al., 1996). Another BMP/Dpp antagonist, twisted gastrulation (tsg), is also functionally conserved among species. In flies, tsg and sog synergistically inhibit Dpp signaling (Ross et al., 2001). Similarly, in frog and fish, Tsg acts to antagonize BMP signaling (Blitz et al., 2003) (Ross et al., 2001), while Chrd and Tsg synergize to enhance formation of a secondary dorsal axis in overexpression assays (Ross et al., 2001). In mouse, mutations in Twsg1, a Tsg homolog, result in craniofacial and foregut endoderm defects, which are similar to the phenotype found in Chrd+/−;Nog+/− embryos (Petryk et al., 2004; Anderson et al., 2002). This evidence confirmed the structural similarity and functional retention of signaling pathways between flies and vertebrates, which suggests that the same conserved mechanism is involved in the patterning of different organisms.

The BMP Signaling Paradigm

The BMPs belong to a family of secreted proteins related to the cytokine TGFβ and are involved in the development of multiple tissue types and organs at various stages in embryogenesis. BMPs were first identified in protein extracts derived from bone that could direct cartilage and bone formation, hence the name (Wozney et al., 1988). Years later, independent studies of mesoderm induction led to the discovery of a TGFβ-related class of mesoderm inducers, such as Activins and BMPs. Initially discovered along with Activin as mesoderm inducers, Bmp4, Bmp2 and Bmp7 were
subsequently found to be expressed in the gastrula ectoderm and also to be epidermal inducers (Wilson et al., 1997). Injection of Bmp4 mRNA resulted in the downregulation of organizer specific genes and the ventralization of the Xenopus embryos (Jones et al., 1996). Similar phenotypes were also found following injection of Bmp2 and Bmp7 mRNA (Dale et al., 1993; Hawley et al., 1995). Thus BMPs are revealed as having activities antagonistic to the organizer. Along with the discoveries of the BMP antagonists Chrd and Nog in the Spemann organizer, a neural default model was proposed whereby the inhibition of BMP signaling is required for the conversion of ectoderm to neuroectoderm in the development of the Xenopus embryo (Harland and Gerhart, 1997).

As members of TGFβ superfamily, BMP and TGFβ share a common paradigm (reviewed by Feng and Derynck, 2005) (Figure 1.2). Secreted BMP ligands are synthesized as pro-proteins, which are processed to form signaling-competent disulfide-linked dimers (Constam and Robertson, 1999). Secreted BMP dimers bind and signal through a complex of type I and type II receptors, which are structurally similar transmembrane serine/threonine kinases. Multiple different type I and type II subunits exist and are found in different combinations, but only the type I receptors have a conserved Gly/Ser-rich “GS sequence” upstream of the kinase domain. Following ligand binding, which permits the formation of a stable receptor complex, type II receptors trans-phosphorylate the GS sequences of type I receptors, which then propagate the signal. This phosphorylation activates the type I receptor kinases, leading to autophosphorylation of the type I receptor and subsequent phosphorylation of Smad proteins (Shi and Massague, 2003).

Known as receptor-associated (R) Smads, Smad1-3, 5, and 8 are intracellular effectors with specificities for particular signaling pathways. Smad1, 5, and 8 serve as substrates for BMP type I receptors specifically, whereas Smad2 and 3 are substrates
**Figure 1.2: Schematic Outline of the BMP Signaling Pathway.** BMP homodimers bind to hetero-multimeric receptors (type I and type II). The type II BMP receptor transphosphorylates the type I receptor, which activates Smad1, Smad5, and Smad8 by phosphorylation. Phospho-Smad1/5/8 heterodimerizes with Smad4 and translocates into the nucleus. There they act as transcriptional activators and evoke changes in target gene expression. Chrd and Nog are extracellular antagonists that inhibit BMP ligands by physically binding them and thus impeding the formation of the BMP-BMP Receptor complex.
Figure 1.2: Schematic Outline of the BMP Signaling Pathway
for TGFβ, Activin, and Nodal receptors. The RSmads detach from the receptor and oligomerize with the non-pathway restricted Co-Smad, Smad4, which serves as a common partner to all RSmads. The RSmad/Smad4 hetero-oligomers translocate from the cytoplasm to the nucleus and activate transcription of target genes either directly or in cooperation with unrelated DNA binding proteins (Figure 1.2). This signaling can be inhibited by a third class of Smads, the inhibitory (I) Smads, Smad6 and Smad7, at the cytoplasmic phase of the receptors. These ISmads serve as decoys, interfering with RSmad-receptor or RSmad-Smad4 interactions (Massague, 1998). As mentioned in the previous section, one method of regulating BMP signaling activity is the extracellular inhibition of ligand-receptor complex formation via a group of soluble proteins. These antagonistic proteins (including Chrd, Nog, Twsg, Follistatin ad Cerberus) bind to the BMP ligand physically to bar its access to membrane receptors.

During early mouse gastrulation, Bmp4 is expressed throughout the extraembryonic ectoderm, as well as the primitive streak before and during gastrulation. Knockout and chimeric studies suggest that Bmp4 in the extraembryonic ectoderm, but not in the primitive streak, is required for normal primitive streak formation. The function of Bmp4 in the epiblast is crucial for the early establishment of the left-right (L-R) axis (Fujiwara et al., 2002). Bmp2, which is the most closely related member in the TGFβ family, is expressed in the extraembryonic region of the E6.0 mouse embryo. Bmp2 mutant embryos show normal gastrulation but possess defects in the morphogenesis of the extraembryonic region (Zhang and Bradley, 1996); however, the significance of Bmp2 expression in the epiblast, specifically in the APS and the node, is unclear. Interestingly, in Foxa2−/− embryos, ectopic Bmp2 expression, as well as ectopic BMP signaling activation, is found in these tissues. This observation suggests that BMP2 is likely the ligand responsible for the ectopic BMP signaling activity, which was proposed as an inhibitory cue during anterior specification (Yang and Klingensmith, 2006).
Studies involving disruption of the BMP receptor Bmpr1a, which encodes a type I receptor for BMP4 and BMP2, demonstrate the functional importance of BMP signaling in both extraembryonic and embryonic regions. Bmpr1a is expressed ubiquitously in the mouse gastrula, and embryos carrying a Bmpr1a mutation die due to lack of mesoderm formation (Mishina et al., 1995). However, unlike the Bmpr1a null embryos, embryos with mosaic inactivation of Bmpr1a in the epiblast show an initiation of gastrulation, suggesting that BMP signaling through BMPRIA in the extraembryonic region is critical for gastrulation (Davis et al., 2004; Miura et al., 2006). Interestingly, these embryos show expansion of the prechordal plate (PCP), the anterior visceral endoderm (AVE), the anterior definitive endoderm (ADE) and the underlying anterior neuroectoderm, suggesting that BMP signaling through BMPRIA in the epiblast negatively regulates the patterning of the anterior neuroectoderm, as well as the patterning of the AVE and AME that promote and maintains neuroectoderm specification (Davis et al., 2004).

**Staging the Mouse during Gastrulation**

The classification of gastrulating mouse embryos is especially important for studies in early mouse embryogenesis. Visible morphological landmarks were used to stage the embryos with greater precision and accuracy than day of embryonic development alone. Embryos between E6.25-E8.0 were used in this dissertation and were staged based on Downs and Davies (1993), as summarized below (see also Figure 1.3).

Three main stages are defined during gastrulation, namely primitive streak stage, neural plate stage and headfold stage. The primitive streak stage is then subdivided into early-streak (ES), mid-streak (MS) and late-streak (LS) stages by location of the anterior tip of the streak during its elongation (Figure 1.3). At ES stage, the mesoderm is newly
Figure 1.3: The Morphology of Gastrulating Mouse Embryos. This figure denotes the key stages during mouse gastrulation from MS (E6.5) to LHF (E7.5) and is also serves as the staging guide used in this dissertation. See text for detailed description. This figure is adapted from Down and Davies, 1993. PS, primitive streak; PAF, posterior amniotic fold; n, node; etc, ectoplacental cavity; ec, exocoelomic cavity; am, amnion; hp, head process; al, allantoic bud; hf, headfold. Stages: MS, mid-streak; LS, late-streak; 0B, zero allantoic bud; EB, early bud; LB, late bud; EHF, early headfold; LHF, late headfold.
Figure 1.3: The Morphology of Gastrulating Mouse Embryos
formed and starting to elongate in the posterior epiblast around the embryonic-extraembryonic junction. At MS stage, the primitive streak is about halfway towards the distal tip of the epiblast, and its spreading mesodermal wings extend laterally from the midline. This gastrula organizer at MS stage (i.e. MGO) has the capability to induce a complete axis in transplantation experiments (Kinder et al., 2001b). At LS stage, the mesoderm is condensed in the distal tip of the egg cylinder. This structure is the node and is first morphologically distinguishable at LS stage. In addition, the other feature of LS embryos is the appearance of the posterior amniotic fold (PAF), which has closed to form the amnion at this stage.

The neural plate stage is divided into 3 stages based on the appearance of the allantoic bud: zero allantoic bud (0B), early bud (EB), and late bud (LB). At 0B, the PAF has fused with the anterior amniotic fold to form three complete cavities: the amniotic cavity, the exocoelomic cavity, and the ectoplacental cavity. At this stage, the allantoic bud is not visible yet, and the head process is starting to extend anteriorly. At EB, a small allantoic bud can be seen at the posterior junction, and the head process is more extended. At LB, a large allantoic bud is projecting into the exocoelomic cavity, and the anterior neuroectoderm is thickening (but not yet forming headfolds). In addition, the node is visible as a slight depression at the distal tip.

The headfold stage is divided into early headfold (EHF) and late headfold (LHF) stages. At EHF, a distinct headfold formation can be seen and the early neural groove can be found anteriorly (although this stage does not show foregut invagination). At LHF, the headfolds are well defined and the neural groove is clearly present in the anterior midline. The foregut invagination is also seen, and the node is conspicuous distally.

These landmarks are visible under microscope in intact embryos during embryo collection and help precise staging, which is essential for this study. Adoption of these
criteria facilitates identification of morphological differentiation essential for embryonic manipulation and interpretation of gene expression during gastrulation (Downs and Davies, 1993).

The Gastrula Organizer in the Mouse

The organizer is conserved in vertebrates; it is known as the embryonic shield in zebrafish, Hensen’s node in the chicken, and the node in the mouse. The node of the late-streak (LS) stage embryo is the first morphologically recognizable organizer. Ectopic transplantation of the node generates a secondary axis that lacks anterior structures (Beddington, 1994). Elucidation of the expression and mutation phenotypes of organizer genes, such as Foxa2 (Ang and Rossant, 1994) and Gsc (Belo et al., 1998; Blum et al., 1992), helped to identify a region of organizer activity in ES stage embryos. This group of cells is located at the anterior tip of the primitive streak and is referred to as the early gastrula organizer (EGO). When transplanted, the EGO can induce an ectopic secondary axis but cannot induce anterior structures. Induction of the anterior neural genes in the ectopic axis was achieved only when the AVE and anterior epiblast were transplanted along with the EGO (Tam and Steiner, 1999). These data suggest that neither the node nor the EGO does possess the full activities needed to induce rostral neural structures. Signals outside of these tissues, possibly in the AVE and anterior epiblast, are required to induce anterior neural structures.

Interestingly, in the mid-streak (MS) stage embryo, expression of several organizer factors, including Foxa2, Chrd, Cerl, and Gsc, overlap in the anterior tip of the elongating primitive streak and adjacent endoderm (Camus and Tam, 1999; Davidson and Tam, 2000) (APS, Figure 1.4B). A graft of this group of cells is able to induce a full secondary axis with expression of anterior neural genes in the host embryo.
Figure 1.4: Cell Movement and Signaling Molecules during A-P Axis Patterning in the Mouse Gastrula. (A) Before and at early primitive streak formation (PS/ES), the DVE located at the distal epiblast is marked by Hex. Asymmetrical expression of some VE markers, such as Lefty1 and Cerl, is found within the sub-domain of the DVE. The DVE moves towards the presumptive anterior side and gives rise to the AVE. The location of the early gastrula organizer is indicated. (B) At mid-streak (MS) stage, the AVE expresses several key genes that are involved in the anterior patterning (red box). Posteriorly, the primitive streak (purple) forms and elongates towards the distal epiblast. The APS expresses several key genes that are involved in patterning its derivatives (purple box). Notice that the axis has rotated from proximal-distal (P-D) to anterior-posterior (A-P). (C) At early bud (EB) stage, the AVE has been replaced by the APS derived AME, which expresses key genes involved in forebrain patterning (orange box). The prospective forebrain ectoderm is indicated. The node (N) is located at distal end of the embryo and is morphologically distinguishable. ExE, extraembryonic ectoderm; Epi, epiblast; VE, visceral endoderm; DVE, distal visceral endoderm; EGO, early gastrula organizer; AVE, anterior visceral endoderm; APS, anterior primitive streak; AME, axial mesendoderm; N, node; Fb, forebrain.
Figure 1.4: Cell Movement and Signaling Molecules during A-P Axis Patterning in the Mouse Gastrula
(Kinder et al., 2001b). This observation suggests that the APS (also referred as the mid-gastrula organizer or MGO), which displays full patterning capacity, is the functional equivalent tissue of the early Spemann organizer. The APS gives rise to the AME, which expresses several genes essential for forebrain patterning (Figure 1.4C). What could be causing the difference in induction ability between the EGO and MGO? The results from fate mapping of the epiblast suggests that additional progenitors of the head process of the AME are found in the epiblast but are anterior to the EGO. It is likely that the MGO incorporates both the EGO and the anterior regions containing the progenitors, and that head inducing ability is associated with the AME (Kinder et al., 2001b), which comprises the PCP and the ADE (Camus et al., 2000; Lawson, 1999). These observations also suggest that the gastrula organizer, similar to the Spemann organizer, is composed of a transitory population of tissue precursors.

The requirement of the node in axis patterning in the mouse embryo is still unclear. Embryonic manipulations such as ablation of the node have little effect on A-P axis patterning but disrupt D-V and L-R axis patterning (Davidson et al., 1999). Similar results were found in chick embryos with an ablated Hensen’s node (Yuan and Schoenwolf, 1998). Embryos with homozygous deletion of Foxa2 do not develop a morphologically recognizable node or axial tissues, but they do express a full range of region-specific neural markers (except for forebrain markers) (Ang and Rossant, 1994; Klingensmith et al., 1999). Chimeric and other studies suggest that Foxa2 functions in the AVE and promotes forebrain gene initiation (Dufort et al., 1998; Yang and Klingensmith, 2006), which explains why expression of forebrain markers is often lost in Foxa2−/− embryos. Similarly, embryos carrying a Foxa2 conditional knockout in the epiblast have normal A-P neural markers, including initial forebrain gene expression, in the absence of a node or notochord. These studies suggest that Foxa2 is required autonomously for node and axial tissue formation (Hallonet et al., 2002). Given that A-P patterning occurs
in the absence of a node, it is possible that the cues required for patterning have been provided by the EGO or MGO prior to the formation of the node.

**The DVE Migrates Anteriorly to Become the AVE**

Prior to gastrulation, the mouse embryo is composed of two layers: the epiblast, which will give rise to the embryo; and the visceral endoderm (VE), which will be replaced by definitive endoderm (DE) and is destined to become the extraembryonic visceral yolk sac (Lawson, 1999) (Figure 1.4A). The expression of the homeobox gene *Hex* is dynamic but restricted to the VE before gastrulation commences, and thus it labels the path of VE movements (Rivera-Perez et al., 2003; Thomas et al., 1998). The distal VE (DVE), which gives rise only to the anterior descendents, moves towards the presumptive anterior side and promotes anterior fates in the underlying epiblast (Beddington and Robertson, 1999). Therefore, the directional migration of DVE cells determines the orientation of the A-P axis. Two potential mechanisms for directing DVE migration have been put forward by a couple of studies. First, Nodal signaling is required for cell proliferation in the VE, and asymmetric inhibition of Nodal ligands by *Cerl* and *Lefty1* in the AVE results in an imbalance in cell proliferation in the posterior vs anterior VE (Yamamoto et al., 2004). Second, *mDkk1* expression in the AVE can rescue the A-P axis rotation defects of *Otx2*–/– embryos. In addition, *mDkk1* may also direct DVE migration by down-regulating canonical Wnt signaling, which prevents axis conversion when misexpressed (Kimura-Yoshida et al., 2005). These two mechanisms are not mutually exclusive, and others remain to be discovered (reviewed in (Srinivas, 2006).

The migratory movement of DVE cells, which results in their becoming the AVE, initiates the biological process of A-P axis establishment. What makes the same group of
DVE cells change from DVE into AVE? Morphological differences between columnar DVE cells and squamous AVE cells are detectable with specialized microscopy and techniques (Rivera-Perez et al., 2003; Srinivas et al., 2004), and the AVE can be easily distinguished molecularly. The AVE expresses transcription factors, such as Otx2 (Ang et al., 1994), Lim1 (Shawlot and Behringer, 1995), Foxa2 (Ang et al., 1993) and Hex (Thomas et al., 1998). It also produces several secreted molecules that may act on the adjacent epiblast, such as TGFβ family proteins Nodal (Conlon et al., 1994) and Lefty1 (Oulad-Abdelghani et al., 1998), the Nodal antagonist Cerl (Belo et al., 1997), and the Wnt antagonist mDkk1 (Glinka et al., 1998) (Figure 1.4B). The molecular composition of the AVE, which is surprisingly similar to genes found in the Spemann organizer (Harland and Gerhart, 1997), suggests that the AVE is possibly a discrete head organizer which acts as an inductive center that forms separately from the gastrula organizer.

The AVE Promotes Forebrain Patterning But is Not a Head Organizer

The patterning function of the AVE has been unraveled using both embryological and genetic manipulations. Ablation of the AVE leads to loss of forebrain markers, and this experiment demonstrated a role for the AVE in contributing to neuroectoderm specification (Thomas and Beddington, 1996). The tetraploid chimera technique, which allows for production of embryos in which the extraembryonic tissues, including the AVE, have a genotype different than the epiblast, is another method used to unveil the function of the AVE. Mice or embryos lacking Foxa2, Lim1 or Otx2 function have a trunk and tail with normal A-P patterning but lack head structures (Acampora et al., 1995; Ang and Rossant, 1994; Ang et al., 1996; Shawlot and Behringer, 1995). However, the anterior deficits are partially rescued in tetraploid chimeras with their wildtype gene
functions in the AVE (Dufort et al., 1998; Rhinn et al., 1998; Shawlot et al., 1999). These chimeric studies also suggest an essential role for the AVE in forebrain patterning.

Even though it is necessary during forebrain formation, the AVE alone is not sufficient to act as a direct head organizer. Mouse AVE fails to induce neural tissue when grafted to a lateral region of a mouse epiblast, and it has only been shown to pattern anterior structure in conjunction with signals from both the EGO and the anterior epiblast (Tam and Steiner, 1999). Similarly, chick hypoblast (which is equivalent to the AVE) could only induce transient expression of forebrain and neural markers when transplanted (Foley et al., 2000). Therefore, while it is evident that it is crucial in A-P patterning, the AVE does not act as a separate organizer.

The function of the AVE (as it is understood to date) is that it promotes anterior specification by inhibiting posteriorizing cues from the primitive streak. Previous studies demonstrate that the VE defects in Otx2\(^{-}\) embryos are associated with abnormal primitive streak gene expression in the epiblast, suggesting posteriorization of the embryo (Perea-Gomez et al., 2001). Explant studies show that the AVE is able to repress the posterior genes Brachyury and Cripto (Kimura et al., 2000; Yang and Klingensmith, 2006), whereas the defective VE from Otx2\(^{-}\) embryos lacks such ability (Kimura et al., 2000). In addition, embryos carrying compound mutations in Cerl and Lefty1 possess patterning defects including primitive streak duplication (Perea-Gomez et al., 2001). While Nodal, Wnt3, and BMP4 are required for primitive streak formation and maintenance (Conlon et al., 1994; Liu et al., 1999; Winnier et al., 1995), the AVE produces secreted molecules that are Nodal and Wnt3 antagonists. All these observations suggest that the AVE restricts the influence of posteriorization signals, but there is still much that remains to be clarified (see also Chapter 2).
Head Induction Requires Inhibition of Wnt and BMP Signals

Spemann proposed the presence of separate head and trunk organizers, suggesting a difference of the molecular nature in these two region-specific organizers. Indeed, the findings that co-expression of Wnt inhibitors and BMP inhibitors leads to ectopic head induction, while expression of BMP inhibitors alone leads to trunk duplication (Glinka et al., 1997) supports this idea (Figure 1.5). This led to the proposal of a dual inhibition model, which states that the differential inhibition of Wnt and BMP leads to formation of either a head or a trunk specifically. Regional specification in the head or trunk organizer is mediated by differential expression of key organizer genes between mesendoderm and chordamesoderm. The former expresses both Wnt and BMP antagonists while the latter only expresses BMP antagonists (Figure 1.5). One of the Wnt antagonists, *Xenopus Dkk1*, encodes a cysteine-rich secreted protein expressed in the dorsal mesendoderm (Glinka et al., 1998). Dkk1 selectively inhibits the action of Wnt (in the canonical β-catenin-mediated Wnt pathway) by binding to LRP5/6 and Kremen, and thus depleting the availability of the Wnt co-receptor LRP5/6 required for β-catenin stabilization (Mao et al., 2002). Expression of Dkk1 anteriorizes embryos, while co-expression of Dkk1 with BMP antagonists (either Chrd or Nog) induces complete ectopic axes, including heads (Glinka et al., 1998). These data suggest that head induction requires inhibition of Wnt and BMP signaling simultaneously. Interestingly, other studies demonstrate that the early regionalization of the forebrain involves repression of Wnt and BMP4 by Six3, a forebrain specific gene, within the neuroectoderm (Gestri et al., 2005; Lagutin et al., 2003). Therefore, the low activity of Wnt and BMP required for forebrain patterning is generated not only by their own antagonists, but also by Six3, which plays a dual role in inhibiting both Wnt and BMP signaling.
Figure 1.5: The Dual Inhibition Model for Head Induction. While formation of the trunk requires inhibition of BMP signals, formation of the head requires inhibition of both Wnt and BMP signals. Regional specification by the head or trunk organizer is mediated by differential expression of key organizer genes in the mesendoderm and chordamesoderm. These genes include both Wnt antagonists (Dkk1, Cerberus and Frzb) and BMP antagonists (Chordin, Noggin and Follistain) (adapted from Glinka et al., 1997 and del Barco Barrantes et al., 2003).
Figure 1.5: The Dual Inhibition Model for Head Induction
The requirement for inhibition of both BMP and Wnt during anterior patterning is also implicated in the mouse. Much evidence suggests that BMP signaling and its antagonists play an important role in vertebrate brain development. *Chrd* and *Nog* are known to promote the inductive activities of rostral organizing centers (Anderson et al., 2002). A decrease of BMP signaling in the epiblast results in expansion of anterior neuroectoderm (Davis et al., 2004). These observations suggest the inhibition of BMP activity promotes forebrain initiation and subsequent neural patterning. In the case of Wnt inhibition, mouse *Dkk1* (*mDkk1*) is expressed in the axial tissues and head mesenchyme (Glinka et al., 1998). *mDkk1*−/− embryos lack structures rostral to the midbrain, demonstrating the requirement of *mDkk1* in forebrain patterning (Mukhopadhyay et al., 2001). Notably, *mDkk1* synergizes with *Nog* in forebrain formation, possibly via their interaction in the AME (del Barco Barrantes et al., 2003), which is also consistent with the dual inhibition model for head development (Glinka et al., 1997). Altogether, these findings not only suggest that anti-BMP or anti-Wnt signals on their own are crucial but they also collaborate to promote anterior patterning.

Another head-inducing factor is *Cerberus*. *Xenopus Cerberus* encodes a secreted protein that prevents signaling by Nodal, BMP and Wnt8 through binding to these ligands (Piccolo et al., 1999). Endogenous *Cerberus* is expressed in the anterior dorsal endoderm. It is able to dramatically induce ectopic head structures in the absence of trunk formation, suggesting its function in head induction (Bouwmeester et al., 1996). The head inducing ability of *Xenopus Cerberus* is supported by findings of the *Cerberus* homolog in mouse and chick embryos. In mouse, *Cerberus-like* (*Cerl*) is expressed in the AVE (Belo et al., 1997), which as discussed above promotes forebrain patterning in mouse gastrula (reviewed by Beddington and Robertson, 1999). *Cerl*−/− embryos do not have a gastrulation phenotype (Belo et al., 2000). However, *Cerl* and another Nodal antagonist *Lefty1* synergize to antagonize Nodal signaling. *Cerl* and *Lefty1* exert their
function from the AVE to determine the direction of VE cell migration by asymmetrically inhibiting Nodal activity at the future anterior side (Yamamoto et al., 2004), and by restricting primitive streak formation to the posterior end of mouse embryos through antagonizing Nodal signaling anteriorly (Perea-Gomez et al., 2002). In chick, Cerberus is expressed in the hypoblast, and similar to the findings in mouse, it prevents ectopic primitive streak formation by inhibiting Nodal activity (Bertocchini and Stern, 2002). These studies highlight a role for anti-Nodal activity via Cerl and Lefty1 to initiate establishment of A-P axis and to ensure only single axis formation. All of these findings suggest that Cerberus antagonizes Wnt, BMP and Nodal signaling to promote anterior formation and repress posterior trunk-tail development. They also implicate a requirement for tight regulation of the spatiotemporal activities of these signaling cascades for anterior formation.

**Nodal Signaling Paradigm**

Ligands of the Nodal signaling pathway are members of the TGFβ superfamily. While there is only one Nodal ligand found in mouse, human and chick, there are multiple Nodal-related ligands in frogs and fish. These Nodal-related genes appear to perform the same core functions as Nodal, except for Xnr3, which may act independently of the Nodal pathway (Schier, 2003).

Nodal ligands signal through the type I serine-threonine kinase receptor ALK4 (ActRIB), and the type II receptors ActRIIA or ActRIIB (Reissmann et al., 2001; Yan et al., 2002; Yeo and Whitman, 2001), in a similar mechanism as other TGFβ ligands transduce their signals (Figure 1.6). What is unique to the Nodal pathway is that it requires co-receptors of the EGF-CFC family, which are small cysteine-rich extracellular proteins attached to the plasma membrane through a glycosyl-phosphatidylinositol
(GPI) linkage (Shen and Schier, 2000). Nodal ligands do not have signaling activity in the absence of EGF-CFC co-receptors, which is different from the way in which signaling by the TGFβ ligand Activin is activated, even though both Nodal and Activin elicit similar responses in gain-of-function studies (Shen, 2007). EGF-CFC proteins bestow specificity for ALK4 through protein interactions (Yan et al., 2002; Yeo and Whitman, 2001). There are 2 EGF-CFC co-receptors in mammals (Cripto and Cryptic), three in frogs and a single gene in fish (one-eyed pinhead, oep) (Shen and Schier, 2000). Zebrafish embryos that lack both maternal and zygotic oep phenocopy double mutants for the Nodal ligands, suggesting that oep is essential for Nodal function (Gritsman et al., 1999). In mouse, Cripto acts as a secreted transacting factor to mediate Nodal signaling (Chu et al., 2005; Yan et al., 2002) and is required for A-P axis rotation (Ding et al., 1998).

Downstream of the receptors, Smad2 and Smad3 both function as RSmads and are phosphorylated by the activated type I receptor (Massague et al., 2005) (Figure 1.6). Smad2 and Smad3 share about 92% similarity in amino acid identity (Dunn et al., 2005); however, endogenously their functions are different. Mouse Smad2 is required in the extraembryonic tissue to establish the A-P identity within the epiblast (Waldrip et al., 1998), while Smad3 is not essential for embryogenesis (Datto et al., 1999). Nonetheless, they synergize in mesoderm and endoderm patterning (Dunn et al., 2004; Liu et al., 2004). Although Smad2 and Smad3 appear to differ in their abilities to regulate transcription of downstream target genes, Smad3 can functionally replace Smad2 by gene substitution experiments in mouse (Dunn et al., 2005). Overall, these observations suggest that Smad2 and Smad3 are significantly diverged and are non-redundant. The activated Smad2 or Smad3 oligomerizes with the Co-Smad Smad4 and together they translocate into the nucleus to regulate the transcription of target genes.

Inside the nucleus, Nodal signaling functions via the transcription factors FoxH1 and Mixer, and several target genes have been are identified, such as Nodal, Lefty2, Pitx2,
and Foxa2 (Shen, 2007). Similar to the BMP signaling pathway, Nodal signaling is negatively regulated by multiple extracellular inhibitors. Lefty proteins are diverged members of the TGFβ superfamily, and they can antagonize Nodal signaling by blocking EGF-CFC co-receptor activity and by interacting with Nodal itself (Chen and Shen, 2004; Cheng et al., 2004) (Figure 1.6). Interestingly, Lefty proteins may function as competitive inhibitors of Nodal receptors, because they have not been found to interact with Nodal receptors (Shen, 2007). Notably, Lefty genes are transcriptional targets for Nodal signaling; therefore, this extracellular regulation is also a negative-feedback for this pathway. Another Nodal inhibitor is Cerberus, which can antagonize Nodal signaling by physically blocking Nodal from its the receptors physically (Piccolo et al., 1999). In Xenopus, Cerberus have been shown to act as antagonists of BMP, Wnt and Nodal (Silva et al., 2003), while the mouse Cerl proteins are primarily Nodal antagonists (Marques et al., 2004; Perea-Gomez et al., 2002).

**Nodal-Smad2/Smad3 Signaling Regulates Anterior Patterning**

The versatile, dose-dependent Nodal signaling pathway plays a central role in pattern formation during early embryogenesis, including mesoderm and endoderm induction, A-P and L-R axis patterning, as well as a newly discovered role in the maintenance of mouse embryonic stem cell pluripotency (reviewed in Shen, 2007). Here, we focus on its function in A-P patterning the mouse embryo.

Nodal signaling plays a dual role in the neural patterning of A-P axis. First, inhibition of Nodal is required for the initiation of the A-P axis and anterior identity. It was proposed that the morphogenetic force which propels the asymmetrical movement of the DVE cells is in part generated by regional differences in cell proliferation. Asymmetrical DVE expression of Cerl and Lefty1 in the presumptive anterior side
Figure 1.6: Schematic Outline of the Nodal Signaling Pathway. Homodimeric Nodal ligands bind to the EGF-CFC co-receptor in a complex with the type I receptor ALK4 and the type II receptor ActRII. This activates intracellular signals by transphosphorylation of ALK4, and the subsequent phosphorylation of Smad2 and Smad3. Phospho-Smad2 (or phospho-Smad3) dimerizes with Smad4, and the complex translocates into the nucleus. Smad2/3-Smad4 complexes interact with FoxH1 on target promoters, leading to transcriptional activation of target genes. Extracellularly, Cerberus (Cer) and Lefty (Lef) are both antagonists that interact with Nodal ligands to inhibit Nodal from binding to its receptors. Lefty additionally interacts with the EGF-CFC co-receptor to inhibit its function.
Figure 1.6: Schematic Outline of the Nodal Signaling Pathway
generates a region of low Nodal signaling, as well as a region of low proliferation. As a result DVE cells are displaced away from the prospective posterior, which is a region of higher Nodal signaling activity and thus higher cell proliferation (Yamamoto et al., 2004). In addition, the synergistic function of Cerl and Lefty1 in the AVE is also required to promote anterior identity, possibly via inhibition of posteriorization (Perea-Gomez et al., 2002).

A second, high Nodal signaling activity is required posteriorly in the APS and its derivatives in the subsequent patterning of anterior structures (see also Figure 1.4B). Prior to gastrulation, Nodal expression is detected throughout the epiblast; it is then becomes rapidly confined to the posterior epiblast, marking the site of primitive streak formation (Conlon et al., 1994; Varlet et al., 1997). Genetic studies suggest that Nodal in the epiblast signals to the overlying VE to activate Smad2 in the VE, and this reciprocal tissue interaction is required for the expression of AVE genes and for the establishment of the A-P axis (Brennan et al., 2001). Notably, graded Nodal-Smad2 activity in the epiblast and the primitive streak governs the specification of the AME, which is comprised of the PCP and ADE and is crucial for anterior patterning. In these studies, embryos carrying loss-of-function alleles of Nodal or Smad2 selectively disrupt anterior AME patterning, which leads to an anterior truncation phenotype (Vincent et al., 2003). In addition, sequential reduction of Nodal-Smad2/3 activity (by removal of Smad2 and Smad3 gene dose in the epiblast) sequentially eliminates APS derivatives, impacting first the anterior AME, then the node and remaining AME, and finally the tissues originating from the middle of the primitive streak (Dunn et al., 2004). All these observations suggest that the dose-dependent Nodal-Smad2/3 signaling activity in the epiblast is tightly regulated and is essential for anterior patterning. In addition, analyses of human holoprosencephaly patients have identified genetic loci that include both Nodal and regulators of the Nodal pathway such as Cripto (reviewed in (Roessler and Muenke,
2001) that further support the function of Nodal signaling in anterior patterning in mammals.

Overview

This dissertation seeks to elucidate the cellular and molecular mechanisms underlying the initiation and patterning of the early mammalian forebrain. Firstly, utilizing explantation and Foxa2 mutant embryos, the timing during which cues sufficient to initiate forebrain are received by anterior epiblast was determined. How BMP signaling and the exogenous tissues modify forebrain initiation was also analyzed. Next, the genetic interaction between BMP and Nodal signaling pathways in anterior patterning was investigated. Failure of this interaction results in anterior malformations; analysis of the malformations provides spatiotemporal information regarding BMP-Nodal interactions. The possible molecular mechanisms of BMP-Nodal interaction were proposed based on explantation and biochemical analyses. This study not only reveals the regulation of these two signaling pathways during forebrain patterning, but also provides a model of genetic etiology of human holoprosencephaly. Lastly, this work provides an spatiotemporal atlas of endogenous BMP signaling activity during mouse gastrulation and neurulation. The identification of key domains of sustained BMP signaling in intact mouse embryos provides insights into the regulation of this signaling in vivo.
Chapter 2:

Roles of Organizer Factors and BMP Antagonism in Mammalian Forebrain Establishment

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Summary

A critical question in mammalian development is how the forebrain is established. In amphibians, Bone Morphogenetic Protein (BMP) antagonism emanating from the gastrula organizer is key. Roles of BMP antagonism and the organizer in mammals remain unclear. Anterior visceral endoderm (AVE) promotes early mouse head development, but its function is controversial. Here we explore the timing and regulation of forebrain establishment in the mouse. Forebrain specification requires tissue interaction through the late streak stage of gastrulation. Foxa2−/− embryos lack both the organizer and its BMP antagonists, yet about 25% show weak forebrain gene expression. A similar percentage shows ectopic AVE gene expression distally. The distal VE may thus be a source of forebrain promoting signals in these embryos. In wild-type ectoderm explants, AVE promoted forebrain specification, while anterior mesendoderm provided maintenance signals. Embryological and molecular data suggest that the AVE is a source of active BMP antagonism in vivo. In pre-specification ectoderm explants, exogenous BMP antagonists triggered forebrain gene expression and inhibited posterior gene expression. Conversely, BMP inhibited forebrain gene expression, an effect that could be antagonized by anterior mesendoderm, and promoted expression of some posterior genes. These results lead to a model in which BMP antagonism supplied by exogenous tissues promotes forebrain establishment and maintenance in the murine ectoderm.
Introduction

The forebrain gives rise to many of the specialized structures that perform the higher neural processes characteristic of mammals. The forebrain itself arises from the anterior ectoderm of the epiblast. Molecular signaling within and between anterior tissues of the early postimplantation embryo regulates formation and patterning of the forebrain, but the timing, origins and identities of these signals are poorly understood.

Classic experiments in amphibian embryos demonstrated that anterior brain structures could be induced and organized in the ectoderm by signals from the dorsal lip of the blastopore, a region subsequently referred to as “the organizer” (Gilbert and Saxen, 1993). The early organizer induced head structures, including forebrain. The mammalian organizer is the node (Beddington and Smith, 1993). The node forms at the anterior end of the primitive streak, and node precursors are located at the advancing anterior tip of the primitive streak as it elongates during gastrulation. This precursor of the node is more potent than the node itself in promoting early anterior neural development (Klingensmith et al., 1999; Tam and Steiner, 1999). At the mid-streak (MS) stage, when the primitive streak is elongated midway, grafts of the anterior primitive streak are able to induce anterior brain markers. In contrast, earlier and later grafts induce more posterior markers (Kinder et al., 2001b). These observations suggest that the mammalian functional equivalent of the early amphibian organizer is this “mid-gastrula organizer”.

Genetic studies of the organizer in mouse have confirmed an important role for the organizer and its derivatives in forebrain formation. A mutation in Foxa2, which eliminates the organizer and its precursors from at least MS stages, results in a large percentage of homozygotes which show forebrain truncations at early neurulation stages and lack forebrain-specific gene expression anywhere along the neural axis (Ang and
Rossant, 1994; Klingensmith et al., 1999). A critical role for the node-derived axial mesendoderm (AME) of the ventral midline was revealed by a genetic deletion of Foxa2 restricted to the AME (Hallonet et al., 2002). These embryos formed a forebrain with expression of early forebrain-specific markers, but this tissue was not maintained after neurulation. Thus the early organizer and its derivatives play major roles in promoting development of the early forebrain. The potential role of the mouse organizer in the initiation of forebrain gene expression is unknown.

Extraembryonic tissues also play an essential role in early mammalian forebrain development. For example, Foxa2 is required in extraembryonic as well as embryonic tissues for normal forebrain gene expression and structure (Dufort et al., 1998). There is strong evidence that the AVE promotes early forebrain formation (Beddington and Robertson, 1999). The AVE is formed from columnar visceral endoderm (VE) initially located at the distal tip of the egg cylinder; upon gastrulation these cells migrate anteriorly (Rivera-Perez et al., 2003; Srinivas et al., 2004). The AVE overlies the portion of the epiblast fated to give rise to anterior neural ectoderm, and appears to provide cues that support forebrain fates in this tissue (Beddington and Robertson, 1999). Nevertheless, at least in mouse, the AVE doesn’t appear to act as a direct “organizer” of the forebrain. Mouse AVE fails to induce neural tissue when grafted to a lateral region, but rather acts synergistically with the gastrula organizer to induce forebrain character (Tam and Steiner, 1999; Thomas and Beddington, 1996). Evidence suggests that the AVE functions in part by suppressing posterior gene expression in underlying ectoderm (Kimura et al., 2000; Perea-Gomez et al., 2002; Robb and Tam, 2004). The extent to which it might promote forebrain gene expression directly is unclear.

The mouse forebrain precursors have been mapped to the distal epiblast at early gastrulation (Quinlan et al., 1995). The AVE is in close proximity to the forebrain precursors during gastrulation, whereas the organizer is somewhat removed. However,
as AME forms from the organizer and migrates anteriorly, it comes to underlie the midline of the neural plate by headfold stages. Although the expression of early forebrain-specific genes such as Six3 has been shown to occur by headfold stages (Acampora et al., 2001), the timing of forebrain initiation in mouse has not been addressed. Thus, it remains unknown when the signals leading to forebrain initiation occur, and when forebrain genes are first expressed as a consequence.

The molecular mechanisms mediating the inductive influence of various tissues on forebrain fate in the anterior ectoderm are not fully elucidated, but a few key factors have been identified. Inhibition of BMP signaling appears to be an essential prerequisite in Xenopus. The Spemann organizer BMP antagonist proteins Chordin and Noggin can each mediate the induction of forebrain genes in cultured frog ectoderm (De Robertis and Kuroda, 2004). Noggin (Brunet et al., 1998) and Chordin (Klingensmith et al., 1999) are also expressed in the mouse node. Absence of both factors in mouse results in embryos with defects in all three axes, including an absence of the forebrain in many mutants (Bachiller et al., 2000). Reduced gene dosage of these BMP antagonists results in forebrain truncations (Anderson et al., 2002). In contrast, decreased BMP signaling in the mouse epiblast results in a greatly expanded prospective forebrain (Davis et al., 2004). These results are consistent with the hypothesis that BMP inhibits forebrain gene expression in the ectoderm, while BMP antagonists promote it. However, it is also possible that the effects of the presumed increase in BMP signaling on forebrain development in Chordin; Noggin embryos are indirect; for example, the forebrain truncations may be due to the lack of underlying prechordal plate rather than a local requirement for BMP antagonism.

In this study, we analyze the early events of forebrain development in the mouse. We determine when the cues sufficient to initiate forebrain development are received by the anterior epiblast, and when the initial expression of forebrain genes begins. These
results allow us to consider how BMP signaling and exogenous tissues, such as the AVE and AME, modify forebrain initiation. We also assess the consequences for the initiation of forebrain development in embryos lacking the key organizer transcription factor *Foxa2* and the organizer BMP antagonists, Chordin and Noggin.
Materials and methods

Embryo Collection

Foxa2 (Foxa2\textsuperscript{tm1Jrt}) heterozygotes (Ang and Rossant, 1994) were maintained on an outbred ICR (Harlan) background. Mutant embryos and control littermates were generated by timed matings of Foxa2\textsuperscript{+/−} stocks. Wildtype embryos for explant studies were from ICR matings. Embryos were staged as described (Downs and Davies, 1993).

Tg(Hex-eGFP)ARbe heterozygotes (Rodriguez et al., 2001) was crossed to Foxa2 heterozygotes to generate double heterozygotes. These were crossed to Foxa2\textsuperscript{−/−} to generate Foxa2\textsuperscript{−/−}; Tg(Hex-eGFP)ARbe/+ embryos. All embryos in this study were genotyped by PCR for Foxa2 (Ang and Rossant, 1994) and eGFP (primers: 5′-ACGTAAACGGCCACAAGTTC and 5′-AAGTCGTGCTGCTTCATGTG).

In situ Hybridization, Immunohistochemistry and Histological Sectioning

Whole-mount in situ hybridization (WMISH) followed an established protocol (Belo et al., 1997). Whole-mount immunohistochemistry for anti-phosphorylated-Smad-1/-5/-8 (anti-pSmad1/5/8, Cell Signaling) was performed as described (Faure et al., 2002) with the following modifications. Anti-pSmad1/5/8 antibody was used at a dilution of 1:50 in blocking solution for immunohistochemistry (Hogan, 1994). After primary antibody incubation overnight, embryos were washed in PBS/Triton (PBS with 0.1% TritonX-100), and then incubated with biotinylated goat anti-rabbit antibody (Vector Lab), diluted 1:200 in blocking solution. After several washes in PBS/Triton, embryos were incubated with HRP-conjugated biotin-streptavidin system (Vector Lab). Staining was then developed with the ImmunoPure Metal-enhanced DAB substrate kit (Pierce). Stained embryos were cryo-sectioned and counter stained with histo-green based on standard protocols (Hogan, 1994).
Explant Culture and Protein Treatment

Anterior ectoderm explants were prepared and cultured as described (Ang et al., 1994; Klingensmith et al., 1999). Putative anterior neural ectoderm and adhering tissues were isolated from streak to headfold stage embryos using glass needles. Needles were used to make a straight cut from one landmark to another, resulting in a single piece of ectoderm (and adhering tissues) and the remainder of the conceptus. For pre-streak/early-streak (PS/ES) embryo, the distal tip of the epiblast was isolated. For mid to late-streak (MS to LS) embryos, epiblast tissue from well anterior to the distal extent of the primitive streak to a point midway between the distal tip and the anterior extraembryonic border was isolated. For no-bud (0B) to later stages, the putative anterior neural ectoderm isolated was that portion that generally overlapped with the Six3 or Hesx1 expression domains (Figure 2.1). Proximal extraembryonic tissue and primitive streak were always excluded in the isolated fragment. Germ layer fragments were then treated with a trypsin/pancreatin solution (0.5/2.5%) for germ layer separation for 5 minutes at 4°C, then physically dissociated by teasing apart with glass needles. Explants were cultured in 96-well plates for 24 hours at 37°C. Recombinant BMP2 (Genetics Institute) was used at a final concentration of 100 ng/mL. Recombinant mouse Chordin and Noggin (R&D Systems) were used at 2 µg/mL. BSA (Sigma) concentration was 1 µg/mL.

Quantitative RT-PCR (qPCR)

Total RNA was prepared from 3 explants using Trizol (Invitrogen) and glycogen carrier (Ambion). The entire sample was used for RT reaction with Taqman RT-PCR kits (Applied Biosystems) after treatment with DNase I (DNA-free, Ambion). qPCR was performed using a MyiQ real-time PCR system and iQ SYBR Green mix (Bio-Rad).
Results were normalized to the mouse “house-keeping” gene *Peptidylprolyl isomerase C* (*Ppic*) (Gauthier et al., 2004), then calculated to determine the difference between threshold cycle of the sample and that of the control.
Figure 2.1: Abnormal Forebrain Gene Expression in Foxa2<sup>+/−</sup> Embryos (E7.25-E8.5).

(A1-A4, C1-C4) Six3 and Hesx1 expression in wildtype. Six3 was detected in some embryos at the EB stage (A2, arrowhead) and in all embryos at later stages (A3-A4). Hesx1 was expressed in AVE at LS stage (C1), in the ectoderm at EB stage (C2, arrowhead), and in all embryos at later stages (C3-C4). (B1-B2, D1-D2) Foxa2 mutants at headfold stage show either no (B1,D1) or weak Six3 and Hesx1 expression (B2,D2, arrowheads). (E-H) Six3 and Hesx1 were either not expressed (F1,H1) or weakly expressed at the rostral end (F2,H2, arrowheads) of Foxa2<sup>+/−</sup> embryos, compared to wildtype (E,G). Scale bar: 100 and 500 µm in A1 and E respectively. (I) Endogenous Six3 and Hesx1 expression in the ectoderm detected by qPCR is shown.
Figure 2.1: Abnormal Forebrain Gene Expression in Foxa2<sup>−/−</sup> Embryos
Results

The initiation of forebrain gene expression in wildtype and Foxa2 mutants

Previous studies concluded that a small proportion of Foxa2−/− embryos express forebrain genes in their normal domain at neurulation stages, despite a total lack of the organizer and its constellation of secreted factors, such as the BMP antagonists Chordin and Noggin (Ang and Rossant, 1994; Klingensmith et al., 1999). Restoration of Foxa2 function to the extraembryonic tissues, while the embryo proper is still devoid of all Foxa2, greatly increases the proportion of embryos expressing forebrain markers, and improves the structural malformations of the rostral neural tube in the mutants (Dufort et al., 1998). These studies did not address the key issue of whether forebrain gene expression is initiated in all Foxa2 embryos but isn’t maintained in most of them, or whether it is initiated in some but not the majority. This is important to determine because it is a test of the need for the organizer and/or extraembryonic tissues in the initiation of forebrain gene expression in the intact embryo.

To resolve this issue, we studied the onset of expression of two key forebrain genes, Six3 and Hesx1. Six3 is specifically expressed in the anterior neural ectoderm (Oliver et al., 1995). Hesx1 is expressed in the anterior neural ectoderm, and also is expressed initially in the AVE and the ADE (Hermesz et al., 1996; Thomas and Beddington, 1996). Six3 and specifically the ectodermal function of Hesx1 are essential for forebrain formation (Lagutin et al., 2003; Martinez-Barbera et al., 2000).

We first determined when the forebrain fate is first manifest in normal, wildtype embryos by systematic examination of Six3 and Hesx1 expression from the MS stage to the late-headfold (LHF) stage via whole-mount in situ hybridization (WMISH). Six3 expression was first detected at the early-bud (EB) stage in the anterior ectoderm (6/12, Figure 2.1A2). Robust expression was detected in every embryo by the late-bud (LB)
stage (n=7, Figure 2.1A3,A4). *Hesx1* was expressed in the AVE at the LS stage (Figure 2.1C1). The earliest ectodermal expression was detected at EB stage (9/15, Figure 2.1C2), and was robust from the LB stage in all embryos examined (n=8, Figure 2.1C3,C4). Therefore, the onset of expression for both genes in the epiblast, as judged by in situ hybridization, is around the EB stage. To further assay the onset of transcription in the future neurectoderm, we used quantitative polymerase chain reaction amplification of cDNA (qPCR) to determine the transcript levels of these two forebrain genes at early stages. Ectoderm from EB, 0B, LB and EHF embryos was isolated and prepared for reverse transcription and qPCR. We compared expression levels at these stages to that at the early streak stage, well before any neural-specific gene is known to be significantly expressed. We detected an abruptly increased transcription level of both genes in EB ectoderm, while the 0B ectoderm showed no difference from the background level (Figure 2.1I). This data is consistent with the finding by WMISH that onset of *Six3* and *Hesx1* transcription occurs at around EB stage. In summary, these data show that the anterior ectoderm expresses both *Six3* and *Hesx1* beginning at the EB stage, with stable expression from the LB stage. At the EB stage, the AVE has moved toward the proximal extraembryonic portion of the conceptus, while the organizer is located at the distal tip of the embryo and forming into a distinct morphological node.

In contrast to wildtype embryos, we did not detect *Six3* (n=5) or *Hesx1* (n=4) expression in bud stage *Foxa2* mutants (Figure 2.1B1,D1). At headfold stages, rare mutants showed weak expression (*Six3*, 1/4; *Hesx1*, 1/3; Figure 2.1B2,D2). As previously observed (Ang and Rossant, 1994; Klingensmith et al., 1999), we found that a few E8.0-8.5 *Foxa2* embryos have some forebrain gene expression. In these mutants, we detected small patches of weak expression of both genes (*Six3*, 3/12; *Hesx1*, 3/10; Figure 2.1F2,H2) at the rostral extreme of the thickened neural plate. In all cases, however, rostral neurulation is highly abnormal. Taken together, these results suggest
that forebrain initiation is abnormal in all Foxa2 mutants. In a majority of mutants, forebrain fate may never be initiated at all, since there is never a sign of forebrain character. However, in approximately 27% of mutants, forebrain markers are eventually expressed, indicating that forebrain initiation may be delayed but did occur in these mutants.

**Deficient anterior visceral endoderm in Foxa2 mutants**

Extraembryonic Foxa2 function greatly improves the proportion of mutant embryos with forebrain gene expression and structures (Dufort et al., 1998). Foxa2 is expressed in the AVE (Belo et al., 1997), which promotes early forebrain development (Beddington and Robertson, 1999). This suggests that the AVE might have abnormal function in Foxa2−/− embryos; perhaps it is missing entirely, lacks expression of key genes, or fails to migrate anteriorly, any of which could potentially preclude normal activity. To address these points, we assayed the spatiotemporal expression of several AVE markers in Foxa2 mutants during early gastrulation (E6.5). The transgenic line Tg(Hex-GFP)ARbe (Hex-GFP), which expresses the eGFP reporter gene under the control of the regulatory regions of Hex gene (Rodriguez et al., 2001), was introduced into Foxa2 heterozygotes to generate Foxa2 homozygous embryos with a copy of Hex- GFP. Hex expression was visualized by GFP fluorescence. In wildtype, Hex-GFP is detected in the AVE at E6.5 (Figure 2.2A). However, in mutants, GFP expression in the VE was either undetectable (3/4, Figure 2.2B) or localized at distal tip of the epiblast (1/4, Figure 2.2C). By WMISH, we saw no Hex expression in Foxa2 mutants (n=3, Figure 2.3A,B). Although Hex does not serve an essential function in the AVE for forebrain formation (Martinez Barbera et al., 2000), its absent or distally localized expression reveals that the AVE is deficient in these mutants.
Figure 2.2: Foxa2<sup>−/−</sup> Embryos Reveal Abnormal Hex Expression in the VE. (A) In Hex-GFP/+ embryos, marker expression was detected in the AVE (A2, arrowhead). (B-C) In Foxa2<sup>−/−</sup>; Hex-GFP/+ embryos, Hex-GFP showed either no (B2) or weak expression at distal epiblast (C2, arrowhead).
Figure 2.2: *Foa2*−/− Embryos Reveal Abnormal Hex Expression in the VE
Figure 2.3: The AVE is Not Correctly Specified in Foxa2<sup>−/−</sup> Embryos. (A-B) Hex was not expressed in the AVE of the Foxa2<sup>−/−</sup> embryo (B) compared to wildtype (A). (C-E) Cer1 expression was either absent (D) or distally localized in the Foxa2<sup>−/−</sup> embryos (E, arrowhead) compared to wildtype (C). (F-G) Normal AVE expression of mDKK1 (F) was absent in the Foxa2<sup>−/−</sup> embryo (G). (H-J) Lefty1 expression was either absent (I) or distally localized in the Foxa2<sup>−/−</sup> embryos (J, arrowhead) compared to wildtype (H). Scale bar: 100 µm.
Figure 2.3: The AVE is Not Correctly Specified in Foxa2⁻⁄⁻ Embryos
We also examined by in situ hybridization the expression of other AVE genes implicated in forebrain patterning. *mDkk1*, a Wnt antagonist required for head development (Mukhopadhyay et al., 2001) that is expressed in the AVE (Glinka et al., 1998), was absent in *Foxa2<sup>−/−</sup>* embryos (n=10, Figure 2.3F,G). Two Nodal antagonists expressed in the AVE, *Cer1* and *Lefty1*, have been reported to regulate anteroposterior axis formation (Yamamoto et al., 2004). Interestingly, we observed a few mutants with *Cer1* and/or *Lefty1* expression in distal VE (*Cer1*, 1/5; *Lefty1*, 3/8; Figure 3.3E,J) while most show no expression (Figure 3.3D,I). *Cer1* expression was also seen in a subset of E7.5 *Foxa2* mutant embryos in anterior endoderm tissue (Klingensmith et al., 1999). Whether the expression observed in the earlier study was in residual visceral endoderm or in anterior definitive endoderm (ADE) was not determined.

Altogether, of 27 *Foxa2<sup>−/−</sup>* embryos examined for these markers, none showed a normal AVE expression domain: 81% showed no expression at all, and 19% showed expression confined to the distal VE. In addition, we examined several other AVE markers, such as *Gsc*, *Lim1*, and *Hesx1*, in *Foxa2* mutants; most showed no expression, but a low percentage exhibited weak distal expression (data not shown). Such distal expression represents the temporal precursor of the AVE, the distal VE, which normally migrates anteriorly to form the AVE and underlie the epiblast fated to form forebrain (Quinlan et al., 1995; Rivera-Perez et al., 2003; Srinivas et al., 2004). These data show that most *Foxa2* mutants fail to express AVE genes, but some show distally localized expression in a domain of VE that failed to migrate anteriorly.

**An explant culture system to study forebrain specification in the mouse**

The results above demonstrate that the AVE is defective or absent in *Foxa2* mutants, while previous studies have shown an absence of the gastrula organizer (Ang and Rossant, 1994) and the organizer BMP antagonists (Klingensmith et al., 1999). To
assess the potential relevance of deficient AVE, absent AME or reduced BMP antagonism to the forebrain defects in Foxa2 mutants, we used an explant culture approach to analyze their roles in forebrain establishment in mouse ectoderm. It was first necessary to determine the timing of forebrain initiation, and the need for any exogenous signals for early forebrain gene expression, in the ectoderm normally fated to become forebrain.

Specification is defined as the behavior of a developing tissue when grown in isolation from other tissues (Slack, 1991). Therefore, specification assays provide an experimental means of determining when the signals sufficient for imparting fate to a target tissue have been received. The point at which anterior ectoderm tissue, fated in the normal embryo to become forebrain, actually achieves the necessary cues to express forebrain-specific genes remains unknown. Kimura et al. (2000) found that anterior ectoderm is not yet specified to express forebrain specific markers Six3 and Hesx1 at the MS stage; however, the authors did not determine when expression of these genes does become specified. To clarify this ambiguity regarding the timing of forebrain specification, we addressed forebrain specification via systematically analyzing forebrain-specific markers in cultured explants.

Anterior ectoderm from different stages was isolated from other tissues and cultured for 24 hours, then assayed for Six3 or Hesx1 expression by WMISH and/or qPCR. As a control to detect potential contamination by underlying tissues, explants were assayed for expression of an axial mesoderm gene, Brachyury, and an endodermal gene, Hex (expressed in both visceral and definitive endoderm). Neither was expressed in any explant sample (data not shown), confirming complete isolation of ectoderm from other tissue layers. Six3 was virtually undetectable in explants isolated from MS (13/13) or LS (8/9) stages, but rather was first observed in those isolated from 0B stage embryos (9/44, Figure 2.4A1,A2,E). Similar timing pertained to the specification assay
Figure 2.4: Six3 and Hesx1 Expression in Anterior Ectoderm Explants. (A1-A3) Six3 expression was detected in explants from 0B stage (A2, arrowhead) and EHF stage (A3), but not in those from LS stage (A1). (B1-B3) Hesx1 expression was detected in the explants from 0B stage (B2, arrowhead) and EHF stage (B3) but not in those from MS stage (B1). (C1) Explants from the LS stage showed Six3 expression (arrowhead) when cultured with AVE. (C2-C3) More explants from the 0B and EHF stages showed Six3 expression when cultured with the AME. (D1) Explants from the MS stage showed Hesx1 expression (arrowhead) when cultured with AVE. (D2-D3) More explants from the 0B and EHF stages showed Hesx1 when cultured with the AME. (E) Six3 expression levels and the numbers of explants examined. Explants were either cultured alone (ecto) or cocultured (+AVE or +AME). Frequency of expression was significantly different at MSLB stages (by Chi-square). (F) Hesx1 expression levels and the numbers of explants examined. Explants were either cultured alone (ecto) or co-cultured (+AVE or +AME). Frequency of expression was significantly different at MS, 0B-0-5s stages (by Chisquare). Asterisks denote P value: *-P<0.05, **-P<0.005, ***-P<0.001. Asterisks in (A3, B3, D2 and D3) denote control embryos for WMISH.
Figure 2.4: Six3 and Hesx1 Expression in Anterior Ectoderm Explants
for Hesx1 expression in anterior ectoderm. No expression was detected in explants from MS and LS stages (MS, 8/8; LS, 10/10; Figure 2.4B1,F). The expression was first detected in explants isolated from 0B stage (4/23, Figure 2.4B2), though in vivo Hesx1 is not expressed in 0B embryos (n=10). More explants expressed Six3 and/or Hesx1 when isolated from EB and LB embryos (Figure 2.4). Therefore, the anterior ectoderm is specified to express Six3 and Hesx1 by the 0B stage.

**Maintenance of forebrain gene expression requires exogenous signals**

Whether the forebrain fate needs to be maintained after it is specified is unclear. Ablation of the organizer gene Foxa2 from the axial mesoderm after gastrulation resulted in a failure to maintain forebrain gene expression in the mutants (Hallonet et al., 2002). This suggests that axial mesoderm provides signals necessary for the maintenance of forebrain gene expression. On the other hand, studies on the specification of the midbrain and forebrain gene Otx2 suggest that any maintenance cues needed for its expression are autonomous to the ectoderm after specification at MS (Ang et al., 1994).

To address the issue of maintenance of forebrain-specific gene expression in the absence of exogenous tissues, we isolated and cultured anterior ectoderm after forebrain specification, then assayed for forebrain gene expression. Because both Six3 and Hesx1 are expressed in the anterior ectoderm from the LB stage, a failure of expression in ectoderm explants isolated from this stage onward would suggest that forebrain identity requires external maintenance signals.

Hesx1 expression is poorly maintained after forebrain specification in the absence of other tissues. Overall, no more than 65% of explants at any stage expressed Hesx1, weakly in most cases (Figure 2.4B3). This failure of Hesx1 maintenance was found in explants from all stages tested, from LB to the 0-5 somite (0-5s) stage (Figure 2.4F). These observations suggest that exogenous signals are required to maintain Hesx1 expression.
expression at least between the LB to 0-5s stages. Although the percentage of explants expressing Six3 increased at later stages of isolation (Figure 2.4E), it did not reach 100% until LHF. As the LHF stage is well past the time of forebrain specification, this finding suggests the need for an exogenous maintenance signal for Six3 as well.

We hypothesized that the anterior mesoderm and endoderm, which normally underlie Six3- or Hesx1-positive ectoderm, are a source of the maintenance signals required for forebrain gene expression. To test this, anterior ectoderm and underlying germ layers were isolated, recombined and co-cultured. We then assayed for Six3 or Hesx1 expression, which are not expressed in non-ectodermal tissues at the stages tested.

When cultured with underlying tissues, a significantly increased proportion of explants showed Six3 expression at 0B, EB, and LB stages, but not at EHF or LHF stages (Figure 2.4E). The fraction of anterior ectoderm explants expressing Hesx1 was significantly increased at all stages when cultured with underlying tissues (0B to 0-5s, Figure 2.4F). These results suggest that underlying germ layers provide direct maintenance signals. Hesx1 expression, which is relatively less stable than Six3 expression after specification, was particularly enhanced by the presence of other tissues.

To confirm that this enhanced expression is not a general feature of better cell health when more tissue mass is present, we recombined anterior ectoderm with mesodermal wings from EB stage and assayed for Hesx1 expression. We found about 28% (12/42) of recombined explants showed weak Hesx1 expression, similar to the result when EB anterior ectoderm was cultured alone (Figure 2.4F). Therefore, the enhanced expression is not a general result of increased mass or the juxtaposition of tissues. These data also suggest that Hesx1 expression cannot be maintained by non-
axial mesodermal tissue, implying the maintenance signals we detected are likely provided by the AME.

**Ectopic BMP signaling in Foxa2 mutants**

The molecular basis for the loss of forebrain gene expression and development in Foxa2 mutants has not been addressed. In *Xenopus*, organizer BMP antagonists are necessary for neural plate formation in vivo, and promote forebrain gene expression in naïve animal caps cultured in vitro (De Robertis and Kuroda, 2004; Khokha et al., 2005). Foxa2 mutants lack organizer-associated expression of the BMP antagonists Chordin and Noggin (Klingensmith et al., 1999), but mouse embryos lacking Chordin and Noggin per se often show substantial development of the forebrain, despite dorsoventral patterning defects (Anderson et al., 2002; Bachiller et al., 2000). However, it is likely that in mouse as in frogs, there are additional BMP antagonists expressed in the organizer besides Chordin and Noggin. We therefore hypothesized that the forebrain defects in Foxa2 mutants result at least in part from increased BMP signaling activity.

To test this hypothesis, we examined the status of BMP signaling in Foxa2 mutants at gastrulation. *Bmp4*, encoding a BMP ligand critical for mesoderm formation (Winnier et al., 1995), is expressed predominantly in extraembryonic tissues in wildtype gastrula. A similar pattern is seen in Foxa2 mutants (data not shown; Klingensmith et al., 1999). *Bmp2* is expressed in the extraembryonic tissues, the proximal part of the AVE located at the anterior-proximal region of the epiblast, and weakly in the vicinity of the node (Figure 2.5A, arrow and asterisk). Interestingly, we found expanded *Bmp2* expression in the distal half of Foxa2 mutants, including the VE and the primitive streak (3/3, Figure 2.5B). *Bmp7*, encoding the other BMP known to be relevant at these stages and antagonized by Chordin or Noggin, is not expressed in Foxa2 mutants (Klingensmith et al., 1999).
Figure 2.5: Ectopic BMP Activity in Foxa2<sup>+/</sup> Embryos. (A-B) Expanded Bmp2 in the Foxa2<sup>+/</sup> embryo was detected at distal epiblast, including the VE and primitive streak (B, arrowheads). (C-D) Similar expression pattern of Msx2 was found in wildtype and Foxa2<sup>+/</sup> embryos. (E-F') Immunostaining of pSmad1/5/8 was shown in EB (E,E') and LB (F,F') and the section (sagittal). (G-G') Ectopic pSmad1/5/8 protein distribution was shown in the Foxa2<sup>+/</sup> embryo and in its section (sagittal). In (E-F'), arrowheads denote the boundary of staining; red bars denote staining in primitive streak; asterisks denote the VE. (H-I') Msx2 was ectopically expressed in Foxa2<sup>+/</sup> embryos (I) compared to wildtype (H). Transverse sections (H',I') show the ectopic Msx2 in the neuroectoderm of the Foxa2<sup>+/</sup> embryos. (J-K') Ectopic pSmad1/5/8 staining was found in anterior region of the Foxa2<sup>+/</sup> embryo (K) compared to wildtype (J). The arrowhead in (J) denotes the anterior boundary of staining. Transverse sections (J',K') reveal ectopic pSmad1/5/8 in the neuroectoderm and the heart in the Foxa2<sup>+/</sup> embryo. Abbreviation: ch, chorion; am, amnion; al, allantois; ps, primitive streak; ht, heart; ne, neuroectoderm; se, surface ectoderm. Scale bar: 25 µm (A) and 100µm (H).
Figure 2.5: Ectopic BMP Activity in Foxa2+ Embryos
In chick and frog embryos, anti-pSmad1/5/8 antibody has been used to reveal BMP signaling patterns during gastrulation and neurulation (Faure et al., 2002; Faure et al., 2000). We used this approach to study patterns of BMP signaling activation in mouse embryos. In wildtype EB stage embryos, when forebrain specification has just occurred, pSmad1/5/8 staining is mainly distributed at the extraembryonic portion of the conceptus and is restricted to the proximal half of the embryo proper (Figure 2.5E). In sectioned embryos, pSmad1/5/8 is detected in the extraembryonic endoderm, allantois, proximal primitive streak, and extraembryonic mesoderm at the anterior-proximal part of the epiblast (Figure 2.5E'). We found that pSmad1/5/8 staining is absent in visceral endoderm and/or definitive endoderm underlying the prospective forebrain ectoderm (Figure 2.5E' asterisk). Interestingly, we have detected weak pSmad1/5/8 staining in embryonic ectoderm adjacent to the extraembryonic tissues. This area of anterior ectoderm appears to correspond to the anterior/proximal quarter of the prospective forebrain ectoderm as marked by Six3 and/or Hesx1 expression at this stage (Figure 2.1A2,C2). Later, at LB to head-fold stages, pSmad1/5/8 staining is excluded from the anterior ectoderm area (Figure 2.5F, F'). These results suggest that BMP signaling activation, as detected by pSmad1/5/8 staining, is low if not absent in forebrain ectoderm at these stages, though it may overlap briefly with forebrain fates at the anterior/proximal margin. This pattern also reflects expression of Bmp4 and known BMP transcriptional targets, such as Msx2 (Figure 2.5C).

In Foxa2<sup>−/−</sup>, the pSmad1/5/8 staining pattern in the extraembryonic tissues is similar to that seen in wildtype. In sharp contrast, however, we found strong ectopic pSmad1/5/8 staining in the distal region of EB-LB stage mutant epiblast (n=5, Figure 2.5G). Due to abnormal anteroposterior axis orientation in Foxa2<sup>−/−</sup> embryos, the prospective anterior ectoderm region is located in the distal to distal-anterior portion of the epiblast (Kinder et al., 2001a). We find that this region is just anterior to the most
anterior reaches of the primitive streak in Foxa2+/− embryos. We observed pSmad1/5/8 staining at this distal region (Figure 2.5G,G'). Specifically, ectopic pSmad1/5/8 was found in the elongating streak, the distal ectoderm, and distal VE (Figure 2.5G', asterisk). These VE cells are likely those that should have migrated to form AVE. Bmp2 expression also occurred ectopically in this area (Figure 2.5B), and thus BMP2 is likely the ligand responsible for ectopic BMP signaling activity in the distal portion of Foxa2+/− embryos.

To further characterize the alteration of BMP signaling in Foxa2 mutants, we assessed pSmad1/5/8 reactivity at E8.5. In wildtype embryos, we observed staining in the neural folds posterior to the optic vesicles (Figure 2.5J), consistent with our finding that BMP signaling activation is low if not absent in forebrain ectoderm. We also observed staining in the surface ectoderm, the presomitic mesoderm and the allantois (n=5, Figure 2.5J,J'). While the posterior staining domains in Foxa2 mutants are very similar to those in the wildtype at E8.5 (Figure 2.5K), we found very strong expression anteriorly in the mis-folded neuroectoderm region and in the heart (Figure 2.7K,K'). These data indicate that BMP signaling occurs ectopically in the most anterior neuroectoderm of Foxa2 mutants at E8.5, and in the precursors of this tissue at E7.5.

Among BMP signaling target genes assayed at E7.5 and E8.5 (including Msx1, Msx2, BAMBI, Dlx5, Smad6), only Msx2 showed a different expression pattern in E8.5 mutants. We found that Msx2 expression was expanded in anterior neuroectoderm and head mesenchyme (5/5; Figure 2.5H-I'), although we did not see different expression pattern in E7.5 mutants (n=8, Figure 2.5C,D). Thus at least one positive transcriptional target of BMP signaling, Msx2, is upregulated in domains relevant to forebrain formation in Foxa2+/− embryos.

**BMP2 inhibits expression of early forebrain genes**
The presence of ectopic BMP signaling in the prospective anterior neuroectoderm of *Foxa2* mutants, during both gastrulation and neurulation stages, suggests that BMP signaling potentially inhibits expression of genes required for forebrain fate, such as *Six3* and *Hesx1*. We therefore tested the effects of purified BMP on their expression in cultured explants.

Anterior ectoderm was cultured in medium supplemented with recombinant BMP2 protein, or with bovine serum albumin (BSA) as a negative control. We examined the mRNA level of the BMP target gene *Msx2* to validate the effectiveness of BMP2 protein treatment. *Msx2* expression was significantly increased in BMP-treated explants relative to the BSA control at the tested stages (EB, LB, and EHF), shown by both qPCR (Figure 2.6A) and WMISH (data not shown). *Six3* expression, measured by qPCR, was decreased significantly in BMP-treated explants from the EB and LB stages, but the decrease was not significant in those isolated from the EHF stage (Figure 2.6B). Similarly, *Hesx1* mRNA level was significantly decreased in BMP-treated explants prepared from EB and LB stages, but not changed in those prepared from the EHF stage (Figure 2.6C). To further confirm that BMP is inhibitory to forebrain gene expression, we performed WMISH to detect *Six3* expression in the same culture system. Explants cultured with BMP2 showed significantly weaker expression than control explants from EB to EHF stages, and a much greater proportion showed no expression at all (Figure 2.7A-C). However, it is worth noting explicitly that some of the explants still express *Six3* and *Hesx1* even after being exposed to relatively high concentrations of BMP protein, suggesting that anterior neural genes and BMP signaling are not necessarily mutually exclusive. The decrease in *Six3* expression was not significant in stages later than MHF. Taken together, these results indicate that BMP activity has an inhibitory effect on forebrain gene expression in mouse anterior ectoderm through the MHF stage, but does not preclude such expression entirely.
Figure 2.6: Exogenous BMP2 Inhibits Forebrain Gene Expression and Promotes Hoxb1 Expression. (A) Msx2 mRNA level was increased in BMP2-treated explants from EB, LB and EHF stages significantly (P<0.05). (B) Six3 mRNA level was significantly reduced in BMP2-treated explants from the EB and LB stages (P<0.05), but less so at EHF stage. (C) Hesx1 mRNA level was significantly reduced in BMP2-treated explants from the EB and LB stages (P<0.005). (D-E) Gbx2 (D) and Pax2 (E) mRNA level is significantly reduced in BMP2-treated explants in EB stage (p<0.05) but not at EHF stage. (F) Hoxb1 mRNA level was significantly increased in BMP2-treated explants at LB and EHF stage (p<0.05) but not at EB stage. All samples were normalized to Ppic mRNA level in BSA-treated explants from EB stage. Asterisks denote P value (by Chi-square): *-P<0.05, **-P<0.005.
Figure 2.6: Exogenous BMP2 Inhibits Forebrain Gene Expression and Promotes Hoxb1 Expression
Figure 2.7: BMP2 is Inhibitory to Six3 Expression, and Underlying Tissue Relieves the Inhibition. (A-B) Decreased Six3 expression was found in BMP2-treated explants (A) compared to BSA-treated explants (B). (C) Six3 expression levels and the numbers of explants (ectoderm) examined. Significant decrease of expression was found in BMP2-treated explants at EB-EHF stages (by Chi-square; asterisks denote P value: *-P<0.05, **-P<0.005, ***-P<0.001). (D-E) No difference in Six3 expression was detected in BMP2-treated (D) or BSA-treated (E) explants when cultured with underlying tissues. (F) Six3 expression levels and the number of explants (ectoderm plus underlying tissues) examined. Asterisks in (A, B, D and E) denote control embryos for WMISH.
Figure 2.7: BMP2 is Inhibitory to Six3 Expression, and Underlying Tissue Relieves the Inhibition
Underlying germ layers antagonize BMP inhibition of forebrain gene expression

Given our finding that BMP inhibits forebrain gene expression in mouse, the cues from underlying tissues that promote such expression might act by antagonizing the inhibitory effect of BMPs. To test this hypothesis, anterior ectoderm was recombined with underlying tissues (endoderm and mesoderm) and cultured in either BMP2- or BSA-containing medium. In marked contrast to its effects on explants of ectoderm only, BMP caused no significant difference in Six3 expression at any stage tested when underlying tissues were included (Figure 2.7D-F). This suggests that signals from other germ layers antagonize the repression of Six3 by BMP2.

BMP has differential effects on expression of the posterior neural genes

The prevalent “Nieuwkoop model” for neural patterning in vertebrate embryos posits that initial neural patterning is accomplished via activation signals, which give rise to neural ectoderm of anterior character, followed by transforming signals that act in a graded fashion to specify posterior cell fates in the induced neural ectoderm (Nieuwkoop, 1952). One of such signals for the second step “posteriorization” of the neural ectoderm found in Xenopus is BMP signaling (reviewed in (De Robertis and Kuroda, 2004). Whether BMP posteriorizes neural ectoderm in mice is not clear from existing studies. Since we observed that BMP2 protein inhibits expression of the most anterior neural genes, an interesting question is whether BMP simultaneously promotes expression of more posterior genes.

To test this hypothesis, we examined transcript levels of Gbx2, Pax2, Krox20 and Hoxb1, genes involved in mid/hindbrain development (Studer et al., 1998; Urbanek et al., 1997; Voiculescu et al., 2001; Wassarman et al., 1997), by qPCR in anterior ectoderm explants. Although not as dramatic as the effect on anterior genes, Gbx2 and Pax2 transcription is significantly decreased at the EB stage when treated with BMP2, while
showing no significant difference at LB and EHF (Figure 2.6D,E). We did not detect significant changes in mRNA levels of the hindbrain-specific $Krox20$ gene at any stage (data not shown). Interestingly, we found that $Hoxb1$ expression responds differently to BMP treatment. $Hoxb1$ mRNA level is significantly increased in anterior ectoderm from the LB and EHF stages after treated with BMP protein, while showing no difference at the EB stage (Figure 2.6F). Importantly, both $Gbx2$ and $Hoxb1$ are expressed similarly in the presumptive posterior spinal cord in addition to more anterior domains. Thus, while inhibiting forebrain gene expression, BMP has differential effects on more posterior neural genes; it promotes the expression of some while inhibiting the expression of others transcribed in the same tissue.

The AVE promotes forebrain gene expression in unspecified ectoderm

Previous studies suggest that the AVE plays an essential role in forebrain induction (reviewed in (Beddington and Robertson, 1999), but does so by acting synergistically with the gastrula organizer (Tam and Steiner, 1999). Given our finding that about a quarter of $Foxa2$ mutants expresses AVE markers and a similar fraction expresses forebrain markers, an interesting possibility is that the AVE is the source of the signals that promote forebrain gene expression in this subset of mutant embryos lacking the gastrula organizer. Accordingly, we asked whether AVE can serve as a source of signals to initiate forebrain gene expression in anterior ectoderm.

Anterior ectoderm from streak stage embryos was explanted and cultured with or without the AVE intact, then assayed for anterior gene expression. Explants from PS-ES stages test the sufficiency of AVE in inducing forebrain gene expression since the ectoderm fragment has not yet been specified, nor has it been in direct contact with tissues other than the AVE, nor has the gastrula organizer begun to form. At MS-LS stage, the ectoderm might have been exposed to potential long range signals from the
early and mid gastrula organizer (Tam and Steiner, 1999); therefore, these explants provide information as to whether the AVE promotes forebrain specification, but not whether it is sufficient. By qPCR, we did not detect significant changes of Six3 expression in explants of PS/ES ectoderm cultured together with AVE compared to PS/ES ectoderm alone (data not shown), suggesting that the AVE is not sufficient to induce forebrain gene expression.

Although very few explants expressed Six3 when ectoderm isolated from mid-to-late streak stages was cultured alone (Figure 2.4A1), Six3 was expressed in a significantly increased number of explants cultured together with AVE tissue at MS (6/20) and LS (13/15, Figure 2.4C1,E) stages. Similarly, Hesx1 was expressed in a significant number of MS explants cultured with AVE (6/17, Figure 2.4D1,F) when compared to MS explants (n=8, Figure 2.4B1). We obtained similar results using qPCR (data not shown). One might argue that the Hesx1 expression we detected is from AVE tissue; however, the Six3 expression we observed is certainly from the ectoderm since Six3 is not expressed in the AVE (Figure 2.1A1). In addition, in terms of percentage of positive explants, the effect of AVE on Six3 expression was considerably greater than its effect on Hesx1; this is a further example of the differences in regulation between these two early forebrain genes. These data suggest that, though probably not sufficient to induce forebrain gene expression, the AVE serves to promote the initial expression of forebrain genes in underlying ectoderm.

The AVE is a probable source of BMP antagonism

In amphibians, simultaneous inhibition of two posteriorizing signals, BMP and Wnt, is sufficient for head induction (Glinka et al., 1997). In the mouse, the AVE is a source of signals that antagonize posteriorizing signals: mDkk1 antagonizes Wnt signaling (Glinka et al., 1998), while Cerl and Lefty1 antagonize Nodal signaling (Perea-
However, it is unknown whether the AVE is also a source of BMP antagonism. Based on our findings that BMP signaling is ectopically active in the defective visceral endoderm and the adjacent neural ectoderm in Foxa2 mutants, while also promoting the initiation of forebrain gene expression in wildtype ectoderm explants, we hypothesize that the AVE is a source of BMP antagonism that inhibits endogenous BMP signaling in the neuroectoderm to allow proper forebrain specification.

To test this hypothesis, we first examined whether there is endogenous BMP signaling activity in the naive anterior ectoderm before forebrain specification. Although we did not detect significant pSmad1/5/8 staining in the early to mid-streak ectoderm (data not shown), it is possible that any such expression is below the range of detection by this assay. We therefore assayed for early expression of the BMP positive transcriptional target gene Msx2 by isolating PS/ES to LS anterior ectoderm and performing RT-PCR. Msx2 expression was detected at each stage (Figure 2.8E), suggesting that there is indeed BMP signaling in the naive ectoderm. To confirm that the Msx2 expression in naive ectoderm is responding to BMP signaling regulation, we cultured the ectoderm with recombinant Chordin and Noggin proteins, or with BMP protein. We found that Msx2 transcription is repressed by Chordin/Noggin treatment and increased by BMP treatment (Figure 2.8A). Because the added BMP antagonists reduce Msx2 expression and BMP increases it, the Msx2 expression in the pre-specification anterior ectoderm is regulated positively by BMP signal transduction.

We then examined the effect of AVE on this early ectoderm Msx2 expression to test whether the AVE might influence BMP signaling in this context. Msx2 transcript levels were assayed by qPCR in ES and MS ectoderm cultured with or without the AVE. We found that Msx2 expression level is significantly decreased at both stages in ectoderm explants cultured with AVE (Figure 2.8A). These data suggest that the AVE is
Figure 2.8: The BMP Antagonists Chordin and Noggin Promote Forebrain Gene Expression in Naïve Ectoderm. (A) Msx2 mRNA level was significantly reduced when the ES or MS stage ectoderm explants were cultured with the AVE intact or treated with Chordin/Noggin protein. In contrast, Msx2 mRNA was increased by BMP2 treatment. (B) Cripto mRNA level was significantly reduced when the ES or MS ectoderm explants was cultured with the AVE or treated with Chordin/Noggin protein. Cripto mRNA was increased by BMP2 treatment. (C) A significant increase in expression of Six3, Hesx1, and Sox2 was found in Chordin/Noggin-treated explants of naïve ectoderm from the MS stage. (D) Increased Six3 and Hesx1 was detected after Chordin/Noggin treatment in naïve ectoderm explants. (E) Msx2 mRNA was detected in ectoderm from the PS/ES stages through the LS stage by RT-PCR after 40 cycles of amplification. Expression of Ppic serves as a positive control, while the negative control reaction included no reverse transcriptase and thus no cDNA.
Figure 2.8: The BMP Antagonists Chordin and Noggin Promote Forebrain Gene Expression in Naïve Ectoderm
a source of BMP signaling antagonism that inhibits endogenous BMP signaling in the anterior ectoderm prior to forebrain specification.

It has been proposed that the AVE promotes forebrain character by inhibiting posteriorizing influences, such as *Cripto* expression (Kimura et al., 2000; Perea-Gomez et al., 2002). Since we found that the AVE inhibits expression of the BMP signaling target gene *Msx2*, we wondered whether the anti-posteriorizing nature of the AVE involves its anti-BMP activities. We therefore examined *Cripto* expression levels in ectoderm explants cultured with AVE tissue intact relative to ectoderm cultured without AVE, and found that *Cripto* was significantly reduced (Figure 2.8B). This result is consistent with previous findings (Kimura et al., 2000). In ectoderm explants treated with Chordin and Noggin proteins, Cripto expression was significantly reduced compared to explants treated with BSA (as a negative control). In contrast, its expression was increased when the ectoderm was treated with BMP protein (Figure 2.8B). Thus, BMP acts as a posteriorization signal, inducing *Cripto* in ES-MS ectoderm, as well as *Hoxb1* in LB/EHF ectoderm (see above). The AVE is therefore not only a probable source of BMP antagonism, but also appears to inhibit posteriorization in part by inhibiting endogenous BMP signaling.

**BMP antagonists directly trigger forebrain gene expression in isolated ectoderm**

Our data raise the possibility that in naïve mouse ectoderm, the antagonism of BMP signaling may be able to trigger forebrain gene expression. We therefore sought to determine whether BMP antagonists could promote forebrain genes in naïve mouse ectoderm in the absence of other tissues, as has been observed in *Xenopus* animal caps (Sasai and De Robertis, 1997). We cultured ectoderm from the MS stage in medium supplemented with Chordin and Noggin proteins or with BSA. Ectoderm isolated from MS stages is considered naïve with respect to forebrain specification because we did not
detect *Six3* (Figure 2.4E) or *Hesx1* (Figure 2.4F) expression after prolonged culture. We examined the *Msx2* mRNA level by qPCR to validate the effectiveness of the Chordin/Noggin protein treatment. The expression was significantly decreased in explants cultured with Chordin/Noggin versus BSA (Figure 2.8A, C). We found that, in BSA-treated explants, *Six3* and *Hesx1* expression levels were very low. However, Chordin/Noggin-treated explants showed significantly increased expression of both forebrain genes (Figure 2.8C). Levels of *Sox2* mRNA, a pan-neural gene, are also greatly increased. Increased *Six3* and *Hesx1* expression in explants with Chordin/Noggin protein treatment was also detected by WMISH (Figure 2.8D). Our findings support the hypothesis that antagonism of BMP signaling directly promotes forebrain specification. They further suggest that the low level of BMP signaling activity in the pre-specification anterior ectoderm must be overcome for forebrain establishment to occur.
Discussion

In order to probe the mechanisms of forebrain initiation, we performed a variety of experiments in normal embryos and in mutants lacking the organizer factor Foxa2. We focused particularly on the relevance of BMP signaling to the functions of the anterior visceral endoderm and the gastrula organizer, two tissues previously implicated in forebrain induction. Our results reveal that the forebrain is specified in the anterior ectoderm by the 0B stage of development, and that signals from underlying tissues are required for forebrain maintenance. BMP is inhibitory to the onset of forebrain gene expression, while BMP antagonists promote it. Our results also suggest important roles for AVE in the initiation of forebrain gene expression, and for organizer derivatives in maintaining appropriate gene expression through headfold stages. The AVE and the axial mesendoderm are both probable sources of active BMP antagonism that serves to promote forebrain gene expression. Our data also suggest a possible molecular explanation for the cause of forebrain defects observed in Foxa2 null embryos, which are deficient in both the organizer and the AVE.

Initiation and maintenance of forebrain gene expression in the mouse

We used Six3 and Hesx1 expression as markers of presumptive forebrain character because they are the earliest known genes expressed specifically in the forebrain rudiments. A previous study using a similar explant assay assayed Otx2 expression as a marker for specification of neuroectoderm; however, Otx2 expression is not forebrain-specific (Ang et al., 1994). While Otx2 is expressed throughout the epiblast prior to gastrulation, the first detectable ectodermal expression of either Six3 or Hesx1 was found at the EB stage, with persistent expression from the LB stage, in the portion of the epiblast fated to become forebrain (Quinlan et al., 1995). Therefore, the
forebrain specific gene expression program has been initiated by the EB stage. Any exogenous signals or cues required for initiation must have been received by this time.

Our anterior ectoderm explant culture system enabled us to study specification of the forebrain fate. Anterior ectoderm explants from MS and LS stages did not go on to express either Six3 or Hesx1. We found explants from the 0B stage expressed both Six3 and Hesx1, even though these genes were not expressed at detectable level at the stage of separation in intact embryos. The anterior ectoderm is specified to express Otx2 from the MS stage (Ang et al., 1994). This is similar to the timing of specification determined for engrailed expression (MS-LS), a marker of the midbrain fate in the mouse anterior ectoderm (Klingensmith et al., 1999). The forebrain fate appears to be specified slightly but consistently later, at the LS to 0B stage, as judged by forebrain-specific markers.

Although expression of both Six3 and Hesx1 was robust in the ectoderm of all intact embryos from the LB stage, both genes were expressed in a subset of anterior ectoderm explants isolated from embryos after the EB stage. The proportion of explants expressing Six3 increased gradually from 70% at EB to 100% at LHF, after which it was stable. In contrast, Hesx1 was not expressed in more than 65% of explants at any given stage. This demonstrates a requirement for maintenance signals for forebrain gene expression in the anterior ectoderm after the onset of expression. Interestingly, Otx2 expression does not require such maintenance signals after specification (Ang et al., 1994). Moreover, despite very similar spatiotemporal expression patterns and requirements in forebrain development, Six3 and Hesx1 expression shows markedly different dependence on maintenance signals.

Role of the AVE in forebrain initiation
Genetic and embryological studies suggest an important role for the AVE in forebrain induction (reviewed in Beddington and Robertson, 1999). AVE alone is insufficient to induce forebrain in grafting experiments, but can do so in concert with other tissues (Tam Steiner, 1999). AVE functions to inhibit the expression of posterior genes (Yamamoto et al., 2004), but much remains unknown about how it might promote forebrain fates. Our results offer further insight into the role of the AVE in relation to BMP signaling in forebrain initiation.

Although we found that the AVE is not sufficient to induce forebrain character in explants from the ES stage, it does promote forebrain gene expression in explants from the MS and LS stages. This is consistent with findings from other species: the chick equivalent of the AVE, the hypoblast (Foley et al., 2000) and the rabbit AVE (Knoetgen et al., 1999) induces transient and ectopic neural character respectively. In our explant system, the AVE is not sufficient to promote forebrain character at ES stage; however, we found that it is able to promote Six3 in MS explants. An explanation for why we observed anterior neural character promoted by AVE in explants from the MS stage, but not earlier, is that the ectoderm may have already been exposed to signals from the so-called “early-gastrula organizer” (EGO, Tam and Steiner, 1999). These authors found that the AVE and EGO had a synergistic effect on the induction of forebrain structures in grafting experiments. Nonetheless, many explants isolated from MS or LS failed to express forebrain markers when cultured with the AVE, suggesting that such explants had experienced insufficient exposure to factors from other sources, including the EGO, that augment the initiation of forebrain gene expression.

Kimura and colleagues (2000) cultured MS total epiblast with or without AVE, and observed no expression of Six3 in either case. We also observed a total absence of Six3 expression when MS epiblast was cultured alone, but a significant percentage of explants expressed Six3 when AVE was included. Possibly this difference reflects
experimental design; for example, we used anterior epiblast explants, while Kimura et al. used the entire epiblast. Alternatively, since we observed a lack of expression in cultures of ES ectoderm regardless of AVE, a slight difference in staging designation might account for the different observations.

Our data provide evidence that the AVE is a probable source of BMP antagonism during early gastrulation. We found that there is not only an endogenous level of BMP signaling in the naïve ectoderm shown by Msx2, but also that this BMP signaling activation can be inhibited by AVE tissue, as well as by exogenous Chordin/Noggin protein. In addition, Chordin/Noggin protein triggered inhibition of the posterior gene Cripto, which was also inhibited when the AVE is cultured with ectoderm explants (this study; (Kimura et al., 2000). In contrast, when naïve ectoderm explants were treated with BMP, both Msx2 and Cripto expression was increased. These data suggest that the AVE patterns the epiblast by inhibition of posteriorizing signals (Kimura et al., 2000; Perea-Gomez et al., 2002), and the molecular mechanism of this “posteriorization inhibition” involves inhibition of BMP signaling.

**Forebrain initiation and BMP antagonism**

Given the evidence for endogenous BMP signaling in the pre-specification anterior ectoderm, does forebrain induction require inhibition of such signaling? In our naïve ectoderm explants, Chordin/Noggin protein treatment was able to inhibit a substantial amount of endogenous BMP signaling, and also trigger expression of forebrain markers. Therefore, the AVE tissue underlying the presumptive anterior ectoderm in intact embryos prior to forebrain specification is likely to supply at least some of this BMP inhibition function. This is also suggested by the finding that distal visceral endoderm and underlying ectoderm in Foxa2 mutants undergo ectopic BMP signaling. There is evidence for at least one such BMP antagonist: Cer1, a secreted factor that can
antagonize BMP as well as other signals, is expressed in the AVE (reviewed by (Balemans et al., 2002). Therefore, our data suggest that at least one way for the AVE to promote forebrain gene expression is by inhibiting BMP signaling, which may promote posterior fates in mouse ectoderm (e.g. (Zakin et al., 2005); this study).

While we found that Chordin/Noggin proteins induce forebrain gene expression, it is intriguing that in chick embryos Chordin is not sufficient to induce neural identity (Streit et al., 1998). There are several reasons that may explain this discrepancy. It is possible that a Chordin/Noggin combination provides a more effective way of inhibiting endogenous BMPs. By combining with other endogenous BMP antagonists, such as Follistatin, this may provide a more complete spectrum of BMP inhibition. A role for multiple different BMP antagonists is suggested by studies in Xenopus (Khokha et al., 2005). It is also possible that differences in experimental design account for the different results, such as Chordin-expressing cells used by Streit et al. versus concentrated Chordin protein used in our study. Moreover, in addition to WMISH, we also used qPCR as a more sensitive assay for changes in gene expression.

We showed that BMP inhibits early forebrain gene expression, which is also supported by the recent finding of mutual repression of Six3 and BMP4 in Xenopus (Gestri et al., 2005). While BMP is inhibitory to anterior neural gene expression, an interesting question is whether the neural ectoderm is posteriorized when exposed to BMP. We found that Gbx2 and Pax2 expression was significantly reduced after BMP treatment, but at the EB stage only. In contrast, Hoxb1 expression was increased at LB and EHF when explants were treated with BMP protein. Taken together, these data suggest that BMP compromises forebrain specification by decreasing forebrain gene expression, and perhaps also by increasing expression of some posterior neural genes.

While our data indicate that BMP signaling inhibits forebrain specification in explants, they often expressed decreased Six3/Hesx1 levels, rather than none, even after
exposure to a relatively high concentration of BMP protein. Similarly, we observed that in embryos, pSmad1/5/8 staining briefly overlapped with about the proximal quarter of the domain of forebrain gene expression at the EB stage. Therefore, it appears that BMP signaling and anterior neural genes are not necessarily mutually exclusive in vivo.

Reduction of the organizer BMP antagonists Chordin and Noggin, presumably resulting in increased BMP signaling, is associated with forebrain truncation (Anderson et al., 2002; Bachiller et al., 2000). The double null mutants also showed reduced expression of AVE markers, suggesting AVE is deficient in the absence of Chordin and Noggin, though neither gene is known to be expressed there. Our data suggest that forebrain-promoting BMP antagonists are likely to achieve their inhibition of BMP signaling via both the AVE and the organizer.

**Anterior mesendoderm and the maintenance of forebrain gene expression**

At the stages after forebrain has been specified, we observed that loss of Hesx1 expression in anterior ectoderm explants can be rescued by culturing with underlying anterior mesendoderm, but not with mesodermal wings. This strongly suggests that the AME and/or the ADE, both derived from the anterior primitive streak, provided the rescuing activity. This in turn implies that these tissues provide maintenance signals for forebrain development in intact embryos, consistent with genetic studies (Hallonet et al., 2002; Martinez-Barbera et al., 2000; Shawlot et al., 1999). Such signals are not required for the maintenance of Otx2 gene expression, and Six3 was less sensitive than Hesx1. These findings reveal a variable dependence for expression of anterior neural genes on maintenance signals from underlying tissues.

Forebrain specification is completed around the 0B stage, which implies that the period of active tissue interactions that specify the forebrain occurs just prior; i.e., at mid-to-late streak stages. The AVE is immediately subjacent to the future forebrain.
rudiments at these stages (Srinivas et al., 2004; Thomas and Beddington, 1996). The organizer precursors are at the advancing anterior end of the primitive streak, which is located from midway to fully extended along the proximodistal axis of the embryo (Tam and Steiner, 1999). Thus the organizer per se is somewhat remote from the forebrain precursors at the time of specification. However, derivatives of the anterior streak, such as the ADE and the AME that displace the AVE, begin to underlie anterior ectoderm at the LS stage (Thomas et al., 1998). In summary, both the AVE and the early organizer derivatives are in the right place at the right time to be the sources of signals that specify the ectoderm to a forebrain fate.

At the time forebrain genes are first expressed in embryos, at the EB stage, the organizer precursors have begun to form the node at the distal extreme of the embryo, the AME and ADE have extended far to the anterior, and the AVE has already moved away from the region of ectoderm fated to be forebrain. The AME continues to underlie the midline of the anterior neuroectoderm thereafter. Thus, although the AVE is no longer relevant, organizer and anterior primitive streak derivatives are positioned appropriately to provide maintenance cues for the nascent forebrain rudiments.

**Forebrain development in the absence of the organizer gene Foxa2**

The structure of the rostral neural tube and the expression of forebrain genes in a minority of Foxa2 mutants indicate that forebrain establishment occurs in some Foxa2 mutants. We found that 27% of Foxa2 mutant embryos show some forebrain character while the rest show no evidence of forebrain development. This forebrain gene expression in Foxa2 embryos begins in distal ectoderm rather than in anterior ectoderm (see also (Kinder et al., 2001a). We observed that the majority of Foxa2−/− embryos lack expression of AVE markers. Dufort and colleagues (1998) determined via chimera analysis that Foxa2 performs a critical role in extraembryonic tissues for primitive streak
elongation and rostral brain development. Our data reveal that Foxa2 is necessary for AVE development, as the AVE was abnormal in all mutants. In most, AVE markers were not expressed. In some, the markers were ectopically expressed in the distal portion of the conceptus rather than in an anterior location. This suggests that Foxa2 is required for the axial rotation of visceral endoderm toward the anterior (Rivera-Perez et al., 2003; Srinivas et al., 2004), and that Foxa2 plays an important role in promoting the unique gene expression profile of the AVE that presumably underlies this tissue’s function in promoting early head development.

We noted that a similar percentage of Foxa2 embryos exhibited distal expression of AVE markers as later showed forebrain gene expression, while the majority had neither AVE or forebrain gene expression. Based on our finding that the AVE can simultaneously promote forebrain gene expression and inhibit BMP signaling, this correlation leads us to suggest that the ectopic gene activities in the distal VE, such as Cerl and Lefty1 expression, contribute to forebrain establishment in these mutants.

The spatial relationship of distal VE and forebrain initiation in the Foxa2 mutants is somewhat similar to the scenario in the Cripto mutant, in which forebrain induction occurs in the distal epiblast rather than the anterior (Ding et al., 1998). In this case as well, markers of the anterior VE are found in the distal VE, suggesting a failure in the movement of the VE cells toward the anterior. These findings further suggest that the distal VE gene expression in Cripto mutants promotes forebrain gene expression in the distal ectoderm. However, the expression of forebrain genes is much weaker in the 27% Foxa2 mutants compared to that in Cripto mutants. Transient and/or weak forebrain gene expression in Foxa2 mutants might be explained by ectopic BMP signaling in the epiblast, whereas relatively strong Hesx1 expression in Cripto mutants may be explained by the restriction of posteriorizing signals in the proximal epiblast due to failure of embryonic mesoderm formation (Ding et al., 1998). Taken together, these findings suggest
that gene activities from the VE can promote forebrain initiation, and anterior movement of the VE cells is not necessarily a prerequisite for this function.

Our pSmad1/5/8 localization data confirmed ectopic BMP signaling activation in the anterior ectoderm and the visceral endoderm of Foxa2\(^{+/-}\) embryos during gastrulation and neurulation. Regional elevation of BMP signaling in the presumptive forebrain and primitive streak in Foxa2\(^{+/-}\) embryos is likely caused by BMP2, based on the expanded expression patterns that match pSmad1/5/8 protein distribution in the mutants. While AVE markers are not expressed in most mutants, it is intriguing to find that Bmp2 is expressed strongly in the VE of all mutants examined. It is possible that Bmp2 expression is not dependent on Foxa2 gene function in the in the VE, unlike other VE genes. Given that Bmp2 expression is not able to activate pSmad1/5/8 in the VE and ectoderm in wildtype but is able to do so in the mutants, this suggests that loss of normal AVE tissue, a potential source of BMP antagonism, favors the BMP signaling in the VE and may in turn strengthen BMP2 expression in a auto-regulatory fashion.

Our results suggest a model that over-activation of BMP signaling, due to lack of the organizer and the AVE, inhibits forebrain specification in Foxa2\(^{+/-}\) embryos (Figure 2.9). Given that AVE gene expression is absent in about 75% of Foxa2\(^{+/-}\) embryos, lack of normal AVE as a source of BMP antagonism can explain the ectopic pSmad1/5/8 localization in the VE and underlying epiblast. In the remaining quarter of Foxa2\(^{+/-}\) embryos, it is possible that distally localized VE gene activities sufficiently attenuate BMP signaling regionally, thus promoting some level of forebrain establishment (Figure 2.9).

However, maintenance signals such as BMP antagonism from the AME are absent in these mutants, so forebrain development is not sustained.

Our results not only support the double assurance model for forebrain formation proposed by Shawlot et al. (1999), but also add a molecular mechanism for the key signaling that occurs between tissues -- specifically BMP signaling and its antagonism.
**Figure 2.9: A Model for Forebrain Promotion by BMP Antagonists.** The AVE, the gastrula organizer (GO) and the AME promote forebrain formation by BMP antagonism. At E6.75, anti-BMP activities emitted from the AVE (orange) and the GO (green) restrict BMP signaling activation (blue dots) to proximal epiblast, allowing forebrain establishment in anterior ectoderm. At least until E8.25, specified forebrain (red dot) cell fate is maintained by AME (green), which also provides anti-BMP activities. In about 75% of *Foxa2*\(^{-}\) embryos, which lack anti-BMP activities of the AVE and the organizer, ectopic activated BMP signaling in the ectoderm (light blue) and the VE (purple) inhibits forebrain establishment. In remaining 25% of *Foxa2*\(^{-}\) embryos, distally localized VE (light orange), which antagonizes BMP signaling regionally, allows forebrain establishment to occur. However, forebrain maintenance fails (light red dot) due to the lack of the organizer and the AME.
Figure 2.9: A Model for Forebrain Promotion by BMP Antagonists
Our data further provide insight into the means of forebrain establishment in the absence of the gastrula organizer. Clearly, undiscovered signals are also involved. Their identities and relationships to BMP antagonism require further research.
Chapter 3:

Synergistic Interaction Between Nodal Signaling and BMP Antagonism During Mammalian Anterior Head Patterning

This work was done in collaboration with Ryan M. Anderson, who performed the morphological analyses (E9.5-E10.5) of Chordin+/−;Nodal+/− and Noggin+/−;Nodal+/− embryos.
Summary

Holoprosencephaly (HPE) is a common and often lethal syndrome of forebrain and craniofacial midline defects. The molecular and cellular causes of HPE are poorly understood; however, mutations in the Sonic Hedgehog (SHH) and Nodal signaling pathways have been associated with HPE both in humans and mice. In the mouse, genetic reduction of Bone Morphogenetic Proteins (BMP) signaling antagonists also results in HPE. Based on the inheritance patterns of human HPE, we hypothesize that HPE often results from compromises in multiple independent loci or on functional couple developmental pathways. We use mouse mutations to explore the effect of elevated BMP signaling combined with compromised Nodal signaling on early head development. We found that embryos with mutations in Nodal and the BMP antagonist Chordin exhibit HPE (23% penetrance), and those with mutations in the Nodal signaling effector Smad3 and another BMP antagonist Noggin also exhibit HPE (60% penetrance). This suggests that HPE can result from a combination of reduced Nodal signaling and BMP antagonism. Affected Chrd;Nodal and Nog;Smad3 embryos show loss of Foxa2 and Shh in the anterior midline, suggesting that they have defects in the anterior primitive streak (APS) and axial mesendoderm (AME). A deficient rostroventral foregut was revealed upon histological staining and loss of Hex expression. Furthermore, these compound mutant embryos displayed reduced Nodal signaling and ectopic BMP signaling activation in the APS and/or the AME, suggesting an imbalance between Nodal and BMP signaling in these tissues. These data, supported by additional molecular data, indicate that Nodal and BMP antagonism act synergistically to pattern the APS and its derivatives. The data also suggest that human birth defects of the forebrain and facial midline can result from patterning errors that occur during early gastrulation.
Introduction

Holoprosencephaly (HPE) is caused by defects in the anterior midline and is the most common anomaly of forebrain development in humans. As a result of genetic studies in humans, mutations in several genes were found to associate with the HPE phenotype, such as *SHH* and *NODAL* (Roessler and Muenke, 2001). However, the extreme variability in severity of HPE patients with single gene mutations cannot be explained by simple single gene haploinsufficiency. Therefore, a digenic HPE pathogenesis model was proposed in which HPE might be the result of multiple genetic mutations impacting common (sequential) or interacting (synergistic) developmental pathways during forebrain formation (Ming and Muenke, 2002).

The understanding of the cellular and molecular causes of HPE are largely comprised of functional studies of the tissues and genes involved in forebrain patterning in different animal models. In the mouse, forebrain initiation occurs at the anterior tip of the A-P axis during gastrulation and requires signals from the gastrula organizer, which is located at the anterior end of the primitive streak (APS) at the mid-streak (MS) gastrula stage (Kinder et al., 2001b). This group of cells exhibits classic “organizer” activity, i.e. the ability to induce a secondary axis following transplantation (reviewed by (Camus and Tam, 1999), giving rise to the anteriormost axial mesendoderm (AME). The AME itself is comprised of prechordal plate mesoderm (PCP) and part of the anterior definitive endoderm (ADE) (Camus et al., 2000; Lawson, 1999). The PCP and ADE migrate anteriorly to underlie the developing anterior neural plate, and are thought to promote and reinforce the initial anterior identity established by the anterior visceral endoderm (AVE) (reviewed by (Stern, 2001). Defects in these midline tissues, by either embryological or genetic manipulations, result in a failure of forebrain initiation and/or maintenance and lead to a spectrum of forebrain defects, including HPE.
Among the many genes and signaling pathways required in early mammalian forebrain establishment, the Transforming Growth Factor β (TGFβ) ligand Nodal plays a pivotal role in specifying the APS and its derivatives. Nodal acts via phosphorylation of its intracellular effectors Smad2 and Smad3, which activate transcription of target genes--including Nodal itself (reviewed by (Whitman, 2001). Genetic reduction of Nodal-Smad2/3 signaling results in anterior midline defects due to a failure of patterning the APS, anterior AME and its derivatives PCP and ADE (Dunn et al., 2004; Liu et al., 2004; Lowe et al., 2001; Vincent et al., 2003). Gradually decreasing Nodal-Smad2/3 signals by generating compound mutations in its pathway, such as Nodal\textsuperscript{+/-};Smad2\textsuperscript{+/-};Smad3\textsuperscript{-/-}, and ActRIIA\textsuperscript{-/-};Nodal\textsuperscript{+/-}, results in similar anterior midline defects (Dunn et al., 2004; Liu et al., 2004; Nomura and Li, 1998; Song et al., 1999), further suggesting that Nodal signaling acts in a dose-dependent manner. Moreover, Nodal also interacts synergistically with other genes, such as Foxa2 and Gdf1, in the APS during forebrain formation. An HPE phenotype is found in embryos carrying compound mutations of Nodal and Foxa2 (Varlet et al., 1997), as well as Nodal and Gdf1 (Andersson et al., 2006). These studies reveal that anterior malformations can arise from the co-occurrence of mutations in two different genes that are either both related to Nodal signaling or when at least one of them is Nodal. This suggests that Nodal interacts not only with genes within Nodal signaling pathways, but also with genes of other signaling pathways. Therefore, these compound mutation studies in mouse not only provide supporting evidence for the “digenic” pathogenesis model (Ming and Muenke, 2002), but also raise the possibility that other signaling pathways interact with Nodal during forebrain patterning, and thus also are responsible for causing some human HPE cases.

One of the many key molecular mechanisms mediating the inductive influence of various tissues on forebrain formation is the BMP signaling pathway, which also
possibly interacts with Nodal signaling. BMPs, specifically BMP2 and BMP4 (BMP2/4), activate downstream signaling via phosphorylation of its intracellular effectors Smad1, 5 and 8 (Smad1/5/8). Extracellularly, BMP2/4 ligands are antagonized by Chrd and Nog proteins, and this antagonism is a key mechanism involved in vertebrate neural patterning. In *Xenopus*, the Spemann organizer factors *Chrd* and *Nog* are required for neural induction, and each of them can mediate the induction of forebrain gene expression (De Robertis and Kuroda, 2004). In the mouse, BMP antagonism by *Chrd* and *Nog* is critical for forebrain initiation and maintenance. During gastrulation, *Chrd* and *Nog* are expressed in a similar expression pattern in the AME and later in the PCP, with the exception that *Chrd* is also expressed in the APS during early gastrulation (Bachiller et al., 2000). Prior to forebrain initiation, Chrd and Nog protein can promote forebrain character in naïve ectoderm; after forebrain specification, the AME where *Chrd* and *Nog* are expressed is able to maintain forebrain identity (Yang and Klingensmith, 2006). Genetically, the absence of both *Chrd* and *Nog* results in embryos with defects in all three axes, including forebrain truncation in many mutants (Bachiller et al., 2000). Reduced BMP antagonism (by decreasing the gene dosage of these BMP antagonists) results in variability of the severity of HPE due to defects in the PCP (Anderson et al., 2002). Interestingly, embryos carrying compound mutations in *Nog* and a Wnt antagonist *mDkk1* display a severe HPE phenotype, which is possibly due to mispatterning of the AME (del Barco Barrantes et al., 2003). This finding supports not only the dual inhibition model in frogs, which proposes that head induction requires both anti-Wnt and anti-BMP signals (Glinka et al., 1997), but also the digenic model that proposes inappropriate interaction between BMP and Wnt signaling will result in defects in the AME leading to HPE. Altogether, these studies not only suggest an essential role for BMP antagonism by *Chrd* and *Nog* in axial tissues during forebrain formation, but also provide another mouse model for digenic inheritance of HPE, even
though the role of CHRD and NOG in HPE have not been investigated in human genetic studies.

Nodal and the anti-BMP activity by Chrd and/or Nog are similar in their spatiotemporal expression domains and some of their function that: (1) they are expressed in the APS and/or its derivatives; (2) they are required for normal AME and PCP patterning independently; and (3) the phenotype of compound mutations found in either signaling pathway supports the proposed digenic HPE model. Given these observations, we hypothesized that Nodal and BMP antagonism act synergistically during mammalian forebrain formation. Here we investigate the possible synergistic functions of Nodal signaling and BMP antagonism by examining the genetic interaction between Nodal, Smad3, Chrd and Nog genes in mutant mice. We found that Nodal signaling and BMP antagonism synergize in the APS and anterior AME during early forebrain patterning. Our results suggest mutual antagonism between Nodal and BMP signaling, and their fine balance is required for normal forebrain formation. The results of this study not only reveal novel functions for Nodal and BMP signaling during early forebrain patterning, but also provide a novel mechanism for digenic pathogenesis of human HPE.
Materials and methods

Embryo Collection

ChrdRV heterozygotes (Bachiller et al., 2000) or NogαE heterozygotes (McMahon et al., 1998) were bred to Nodal heterozygotes (Collignon et al., 1996) or Smad3 heterozygotes (Datto et al., 1999) to generate double heterozygous F1 progeny. Chrd+/−;Nodal+/− and Chrd+/−;Smad3+/− mice were generated by F1 intercrossing. All mice were maintained on a random outbred ICR (Harlan) background. Mutant embryos were generated by timed mating of 4 different stocks, Chrd−/−;Nodal+/−, Chrd−/−;Smad3+/−, Nog+/+;Nodal−/− and Nog−/−;Smad3+/−. Tg(Hex-eGFP)ARbe heterozygotes (or Hex-GFP) (Rodriguez et al., 2001) were bred to ChrdRV heterozygotes to generate double heterozygous F1 progeny. These were intercrossed to generate Chrd+/−;HexGFP mice, which were used to generate Chrd+/−;Nodal+/−;Hex-GFP embryos by crossing to Chrd+/−;Nodal−/− mice. Hex-GFP mice were introduced to Nog−/−;Smad3+/− mice and backcrossed to generate Nog−/−;Smad3+/−;Hex-GFP embryos. All embryos were genotyped by PCR as described previously (Bachiller et al., 2000; Collignon et al., 1996; Datto et al., 1999; McMahon et al., 1998) (Yang and Klingensmith, 2006). Embryos were staged as described (Downs and Davies, 1993).

Whole Mount In Situ Hybridization (WMISH) and Immunohistochemistry

Double- and single-colored WMISH was performed based on an established protocol (Davis et al., 2004). Whole-mount immunohistochemistry for anti-pSmad1/5/8 (Cell Signaling) was performed as described (Yang and Klingensmith, 2006).

Introduction of Expression Vectors Into the APS
Expression vectors were introduced into the APS as described (Yamamoto et al., 2004). E6.5 embryos were used and embryos were cultured for 14h at 37 °C in medium containing DMEM (Gibco) supplemented with 50% rat serum (Biomeda) with rotation. Embryos were then prepared for WMISH.

**Explant, Protein Treatment and Quantitative PCR (qPCR)**

APS explants from MS stage embryos were isolated (as shown in Figure 3.5A) by glass needles and cultured in 96-well plates at 37°C. Recombinant BMP2 and Nodal protein (R&D Systems) were used at 400ng/mL and 2µg/ml respectively. SB-431542 (Sigma) was used at 100µg/ml. SB-431542 or DMSO (Sigma) pretreatment length was 30 min, followed by 1.5 hr protein treatment. BSA (Sigma, 1µg/ml) and DMSO were used in control explants. qPCR was performed as described (Yang and Klingensmith, 2006).
Results

Genetic interaction between Nodal signaling and BMP antagonism

During mouse gastrulation, patterning of the APS is crucial for the function of the anterior AME, which is essential for normal forebrain formation. It has been shown that the graded Nodal signals within the epiblast govern the specification of the APS (Vincent et al., 2003) and that BMP antagonism by Chrd and Nog promotes the functions of AME and PCP, two derivatives of the APS (Anderson et al., 2002; Bachiller et al., 2000). To test our hypothesis that these signals function synergistically, we used a strategy to generate compound loss of function mutations of both components of Nodal signaling and BMP antagonists. Since the spatiotemporal expression of Nodal and Chrd partially overlaps in the APS (Bachiller et al., 2000; Collignon et al., 1996) (which gives rise to other key axial tissues related to forebrain formation), we first generated compound mutations of these genes.

Nodal\textsuperscript{-/-} mice die at gastrulation, where as Nodal\textsuperscript{+/-} mice appear phenotypically normal (Collignon et al., 1996; Lowe et al., 2001). While Chrd\textsuperscript{+/-} embryos display a DiGeorge syndrome phenotype in a hybrid background, they are viable and fertile in an outbred background (Bachiller et al., 2003). In either background, Chrd\textsuperscript{+/-} embryos do not show any indication of anterior truncation or midline deletions. Variable phenotypes were found in E9.5-E10.5 Chrd\textsuperscript{+/-};Nodal\textsuperscript{+/-} embryos (Chrd;Nodal), which were not present in Chrd\textsuperscript{+/-} single mutant littermates. We found that about 23% (19/83) of them showed morphological defects in anterior midline tissues, while the posterior development appeared normal. Among these embryos, 14 of them showed HPE in association with gross anterior truncation and fused first branchial arches (BA1; Figure 3.1C). Five of them possessed a cardiac looping phenotype, including reversed and ventral looping
Figure 3.1: Embryonic Manifestations of Holoprosencephaly (HPE). Lateral view of E9.5 embryos of different mutation classes. (A,B) Telencephalic vesicles (tv) and optic vesicles (op) are evident in WT and Chrd+/− embryos. (C) Chrd+/−;Nodal+/− embryos have a single holopsheric vesicle (hs) and fused branchial arch 1 (BA1). (D) Anterior structures are normal in Nog+/− embryos. (E) Nog+/−;Smad3+/− embryos lack a telecephalon (arrow) and normal BA1 morphology. (F) Severe Nog+/−;Smad3+/− embryos show forebrain truncation. Asterisks denote the midbrain/hindbrain boundary. Tv; telecephalic vesicles; op, optic vesicles; ot, otic vesicles; m/h, mid/hindbrain boundary; BA1, branchial arch 1.
Figure 3.1: Embryonic Manifestations of Holoprosencephaly (HPE)
(data not shown). This finding provides evidence of a genetic interaction between Nodal and BMP antagonism that is required for anterior patterning.

Chrd;Nodal embryos have a genetic composition of reduced Nodal and anti-BMP signals, which could be a possible molecular cause of the anterior malformations that were detected. If the HPE phenotype is indeed the result of the combination of reduced Nodal and anti-BMP signals, one will expect to detect similar anterior defects by analogous genetic manipulations, such as reducing Nodal signals by removing Nodal signaling effector Smad3, or similarly reducing anti-BMP signals by removing a second antagonist Nog. Based on this logic, we test our hypothesis by preparing a series of analogous compound mutants bearing mutations affecting the same pathways, including Nog;Nodal, Chrd;Smad3 and Nog;Smad3 (Table 1).

Table 1: Distribution of Anterior Defects Among Genotypic Classes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Chrd&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Nog&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Nog&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Nodal&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Smad3&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Sample #</td>
<td>36</td>
<td>68</td>
<td>39</td>
<td>18</td>
<td>44</td>
</tr>
<tr>
<td>Anterior defects</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

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<tr>
<th>Genotype</th>
<th>Chrd&lt;sup&gt;−/−&lt;/sup&gt;;Nodal&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Nog&lt;sup&gt;−/−&lt;/sup&gt;;Nodal&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Chrd&lt;sup&gt;−/−&lt;/sup&gt;;Smad3&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Chrd&lt;sup&gt;−/−&lt;/sup&gt;;Smad3&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Sample #</td>
<td>83</td>
<td>22</td>
<td>37</td>
<td>25</td>
</tr>
<tr>
<td>Anterior defects</td>
<td>19 (23%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Nog&lt;sup&gt;−/−&lt;/sup&gt;;Smad3&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Nog&lt;sup&gt;−/−&lt;/sup&gt;;Smad3&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Nog&lt;sup&gt;−/−&lt;/sup&gt;;Smad3&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Nog&lt;sup&gt;−/−&lt;/sup&gt;;Smad3&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Sample #</td>
<td>61</td>
<td>16</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>Anterior defects</td>
<td>3</td>
<td>2</td>
<td>17 (29%)</td>
<td>18 (60%)</td>
</tr>
</tbody>
</table>
All of these three classes of mutations showed a decrease in the genetic dosage of Nodal signals and anti-BMP signals; however, anterior malformations were only detected in the Nog;Smad3 class of mutations, and not in the other two (when we examined their morphological phenotype at E9.5-E10.5). This observation suggests that the spatiotemporal differences in the expression and function of these genes may possibly account for the phenotypic differences between these classes of mutations. For instance, Chrd and Nodal expression partially overlap in the APS, but Nog expression do not overlap with Nodal in the APS. Although Chrd and Nog expression in the AME overlap with that of Smad3, the fact that only Nog;Smad3 but not Chrd⁻/⁻;Smad3 compound mutants display phenotype suggests a distinct function and/or regulation of Chrd and Nog.

Smad3 is expressed ubiquitously in the early mouse embryo, particularly in the node and axial midline at E7.5 (Dunn et al., 2004). Although mutation of Smad3 does not cause any obvious defects in embryogenesis (Datto et al., 1999), it interacts synergistically with Smad2 during mesodermal and endodermal patterning (Dunn et al., 2004; Liu et al., 2004) and can functionally replace Smad2 (Dunn et al., 2005). Nog is expressed in the node and AME at E7.5 (Bachiller et al., 2000), and Nog⁻/⁻ mutants have abnormalities in the posterior spinal cord, somites and limbs, but not anteriorly in the head when they are in an outbred background (McMahon et al., 1998). It is likely that the anterior phenotype of Nog⁻/⁻ embryos is dependent on the genetic background (Stottmann et al., 2006); however, we did not see any anterior phenotype in embryos only carrying Nog mutation (n=39; Table 1). While Nog⁻/⁻ embryos were never found with anterior phenotype in previous reports or this study (n=68; Table 1), Nog⁻/⁻;Smad3⁻/⁻ embryos with HPE were only found occasionally (2/16, Figure 3.1E), while a higher penetrance of the HPE phenotype was revealed in Nog⁻/⁻;Smad3⁻/⁻ and Nog⁻/⁻;Smad3⁻/⁻ embryos (29% and 60% respectively, Figure 3.1F). This observation shows that the
penetrance of the HPE phenotype is associated with a decrease in $Nog$ and $Smad3$ alleles in a dosage-dependent manner. It further reiterates the genetic interaction between Nodal and BMP antagonism. In this study, we focus on $\text{Nog}^{+/}\text{Smad3}^{+/}$ embryos specifically, referred to from here on as $\text{Nog};\text{Smad3}$ embryos.

**Synergy between Nodal and BMP antagonism is required for the patterning the APS and anterior AME**

To determine the mechanisms underlying anterior malformations in $\text{Chrd};\text{Nodal}$ and $\text{Nog};\text{Smad3}$ embryos, we examined the expression of several key molecular markers that are required for normal anterior formation. In the mouse, the AVE promotes initial forebrain identity, which is followed by forebrain maintenance activities from the AME (Kimura et al., 2000; Yang and Klingensmith, 2006). To evaluate the cause(s) of anterior defects in these compound mutants, we first examined the expression of AVE genes $\text{Hex}$ and $\text{Cerl}$ at E6.5 by using a $\text{Hex-GFP}$ reporter (Rodriguez et al., 2001) or whole mount in situ hybridization (WMISH). All the $\text{Chrd};\text{Nodal}$ and $\text{Nog};\text{Smad3}$ compound mutant embryos examined show normal $\text{Hex}$ and $\text{Cerl}$ expression in the AVE (Figure 3.2E,F; n=4 and 3 for each gene), while we did detect abnormal expression of these genes in ADE cells in the same embryos (see below and Figure 3.2). These results suggest that the AVE functions and rotates normally in these embryos during early gastrulation.

We next considered the patterning of the APS in the epiblast. The APS gives rise to the anteriormost AME, which is comprised of the PCP and ADE (Camus et al., 2000). These tissues are thought to be sources of signals required for the maintenance of the forebrain identity (Rubenstein and Beachy, 1998; Stern, 2001), including anti-BMP signals (Yang and Klingensmith, 2006). The APS is first identifiable by the expression of the transcription factors $\text{Foxa2}$ and $\text{Gsc}$ at MS stage, and both genes function in the APS for normal A-P axis patterning (Ang and Rossant, 1994; Belo et al., 1998). We found
Figure 3.2: Loss of the APS and/or the Anterior AME in Mutant Embryos. (A) At MS stage, Foxa2 expression is detected in the APS in WT, Chrd\textsuperscript{+/−}, Nog\textsuperscript{+/−} and Nog\textsuperscript{−/−};Smad3\textsuperscript{+/−} embryos, but is significantly decreased in Chrd\textsuperscript{−/−};Nodal\textsuperscript{+/−} embryo (arrow). (B) Gsc expression is normal in WT and Nog\textsuperscript{−/−};Smad3\textsuperscript{+/−} embryos but decreased in Chrd\textsuperscript{−/−};Nodal\textsuperscript{+/−} embryos. (C) At bud stage, Foxa2 expression is detected in the extending AME in WT, Chrd\textsuperscript{+/−} and Nog\textsuperscript{−/−} embryos, but is lost in the anterior AME of Chrd\textsuperscript{−/−};Nodal\textsuperscript{+/−} and Nog\textsuperscript{−/−};Smad3\textsuperscript{+/−} embryos (arrowheads). Chrd\textsuperscript{−/−};Nodal\textsuperscript{+/−} embryos display a morphological constriction at the extraembryonic-embryonic junction (arrowhead). (D) Shh expression in the anterior AME is lost in Chrd\textsuperscript{−/−};Nodal\textsuperscript{+/−} and Nog\textsuperscript{−/−};Smad3\textsuperscript{+/−} embryo. (E) In LS stage WT embryos, Hex-GFP is found in the AVE (white bar) and in the ADE in the distal epiblast (arrows). In both Chrd\textsuperscript{−/−};Nodal\textsuperscript{+/−} and Nog\textsuperscript{−/−};Smad3\textsuperscript{+/−} embryos, expression of Hex-GFP in the AVE is normal; however, expression the ADE is significantly decreased. (F) Cerl expression is found in ADE cells normally located in the anterior-distal half of the epiblast (WT embryos, between arrowheads). However, in Chrd\textsuperscript{−/−};Nodal\textsuperscript{+/−} and Nog\textsuperscript{−/−};Smad3\textsuperscript{+/−} embryo, Cerl expression is significantly decreased and is located distally. (G) Hex expression is observed in the foregut endoderm pocket of WT, Chrd\textsuperscript{−/−} and Nog\textsuperscript{−/−} embryo, but is decreased or lost in those of Chrd\textsuperscript{−/−};Nodal\textsuperscript{+/−} and Nog\textsuperscript{−/−};Smad3\textsuperscript{+/−} embryo. (H) The PCP located in the ventral forebrain is normally marked by Shh expression in WT embryos. Shh is lost in the PCP of Chrd\textsuperscript{−/−};Nodal\textsuperscript{+/−} and Nog\textsuperscript{−/−};Smad3\textsuperscript{+/−} embryo but is retained in the rest of the midline. Fg, foregut pocket; h, heart.
**Figure 3.2: Loss of the APS and/or the Anterior AME in Mutant Embryos**
that *Chrd;Nodal* embryos showed decreased *Foxa2* (5/22) and *Gsc* expression (4/13) when compared to that of *Chrd* c littermates (n=9; Figure 3.2A,B). Interestingly, we did not detect any significant change in *Foxa2* or *Gsc* expression in *Nog* c or *Nog;Smad3* embryos (n=4 and 8, respectively). These data suggest that APS specification is defective in *Chrd;Nodal* but not in *Nog;Smad3* embryos. Therefore, even though both classes of mutants possess a similar rostral phenotypes at mid-gestation, the cellular causes of their phenotypes may be different, possibly due to differences in spatiotemporal gene functions between *Chrd*, *Nodal*, *Nog* and *Smad3*.

Next, we examined the development of the anterior AME that is derived from the APS at E7.25-E7.5. *Foxa2*, which patterns and is expressed in the extending AME, is diminished in the anterior AME in *Chrd;Nodal* embryos (5/20). *Nog;Smad3* embryos also have a similar expression pattern with only distal *Foxa2* (5/10; Figure 3.2C). *Shh* is expressed and required in the AME for ventral forebrain development (Chiang et al., 1996) and is significantly decreased anteriorly in both mutant classes (5/12 and 2/3; Figure 3.2D). Later at neurulation, the anteriormost AME forms the PCP, which underlies the forebrain region and secretes inductive cues, including *Shh* (Chiang et al., 1996). *Shh* expression in the PCP is greatly reduced in both *Chrd;Nodal* (2/5) and *Nog;Smad3* (4/10) mutant embryos (Figure 3.2H). Together, these results suggest that the anterior AME and the PCP are abnormally patterned in embryos of both mutant classes. The other derivative of the APS is the ADE. The ADE emerges from either side of the midline to displace VE anteriorly and is marked by the expression of *Hex* and *Cerl*. Expression levels of these diagnostic markers are decreased though not completely lost in the distal epiblast of both classes of mutants (n= 4 and 3; Figure 3.2E,F), allowing us to conclude that ADE specification is significantly impaired. At E8.25, *Hex*, which normally marks the invaginating foregut pocket derived from the ADE, is lost in both
mutant classes (2/5 and 2/4; Figure 3.2G), further supporting the finding that the ADE is not normally patterned.

Taken together, these results show that \textit{Chrd;Nodal} and \textit{Nog;Smad3} mutants a loss of anterior AME specification compromises the subsequent patterning of the PCP and ADE. For \textit{Chrd;Nodal} embryos, the loss of anterior AME at E7.5 is likely the result of abnormal APS patterning at E6.5; however, for \textit{Nog;Smad3} embryos, the loss of anterior AME is the earliest defect detected. Therefore, it is possible that the interaction between \textit{Chrd} and \textit{Nodal} occurs in the APS and/or the AME, whereas the interaction between \textit{Nog} and \textit{Smad3} occurs in the anterior AME. These results demonstrate that Nodal signaling and BMP antagonism cooperatively specify the APS and the anterior AME. They further indicate that Nodal signals and BMP antagonists interact synergistically during anterior patterning, and suggest that disruption of this interaction leads to anterior malformation.

**Disruption of the synergistic Nodal and anti-BMP interaction leads to the alteration of Nodal and BMP signaling levels**

Our genetic analyses in \textit{Chrd;Nodal} and \textit{Nog;Smad3} embryos suggest that a synergistic interaction between Nodal and anti-BMP activities is crucial for APS patterning. This finding suggests that Nodal signals assist \textit{Chrd} and/or \textit{Nog} in inhibiting BMP activation in wildtype embryos. Similarly, \textit{Chrd} and/or \textit{Nog} may assist \textit{Nodal} in promoting Nodal signaling activation. To assess these hypotheses, we sought to examine the spatiotemporal alteration of Nodal and BMP signaling levels in response to the genetic disruption of Nodal and BMP pathway members in these mutants. To visualize Nodal signaling levels, we examined \textit{Nodal} expression by WMISH since \textit{Nodal} is a downstream positive target gene for Nodal signaling through a positive feedback mechanism (Whitman, 2001). We first compared Nodal expression in WT and \textit{Nodal}^{+/-} embryos in
the APS and detected no differences, suggesting that the lack of only one Nodal gene copy does not affect Nodal expression. While Nodal expression remained similar in Chrd\(^+/\) embryos, a fairly high percentage of Chrd;Nodal embryos showed significantly diminished Nodal mRNA levels in the APS (11/11, Figure 3.3B2,B3) and in the node (6/9, Figure 3.3C2,C3). This result suggests that Nodal signaling is decreased in Chrd;Nodal embryos, and in particular the decreased signaling in the APS is likely to cause defects in its patterning (Vincent et al., 2003). In Nog;Smad3 embryos, while we did not detect any change in Nodal expression in the APS (n=3), we found decreased levels of Nodal expression in the node of some embryos (2/9; Figure 3.3C5). Therefore, it is possible that that Nodal signal level is downregulated in some Nog;Smad3 mutants.

Immunohistochemistry with an antibody specific to phosphorylated Smad1/5/8 (pSmad1/5/8), which labels activated intracellular BMP2/4 signaling effectors, revealed BMP signaling activities in the mouse gastrula (Yang and Klingensmith, 2006). We used this approach to determine the spatiotemporal BMP signaling activities in our mutants. In E7.0 WT embryos, pSmad1/5/8 was detected in the extraembryonic region, resembling BMP4 expression (Figure 3.3A1) (Yang and Klingensmith, 2006); however, its distribution pattern in the epiblast was very dynamic. At MS-LS, pSmad1/5/8 was found in the posterior epiblast in the elongating primitive streak, but this gradually clears and becomes restricted to the proximal one-third of the epiblast at around 0B (Figure 3.3A1). In MS-LS Chrd\(^+/\) and Chrd;Nodal embryos, pSmad1/5/8 was detected in the primitive streak and displayed no significant differences as compared to WT (data not shown). However, we found that ectopic pSmad1/5/8 became prominent in the APS located at the distal posterior epiblast in 0B stage Chrd;Nodal embryos (7/18; Figure 3.3A3), while Chrd\(^+/\) embryos showed a similar pattern to WT embryos (n=16; Figure 3.3A2). Interestingly, this ectopic staining is likely to be very transient because we did not see any embryos displaying a similar pattern at slightly later stages, i.e. EB to
Figure 3.3: Alteration of BMP and Nodal Signaling Levels in Mutant Embryos. (A) At 0B stage, pSmad1/5/8 protein is found in the extraembryonic tissues and the junction but is absent in the distal epiblast of WT (A1), Chrd<sup>−/−</sup> (A2) and Nog<sup>−/−</sup> embryos (A4). Ectopic pSmad1/5/8 is detected in a group of cells located in the APS region of the posterior-distal epiblast of Chrd<sup>−/−</sup> embryo (arrow in A3). It is also ectopically distributed all through the epiblast in Nog<sup>−/−</sup>;Smad3<sup>+/−</sup> embryos (arrowheads in A5). (B) At MS-LS stage, Nodal expression in the APS is significantly decreased in Chrd<sup>−/−</sup>;Nodal<sup>+/−</sup> embryo (B3), while the expression is normal in other mutant embryos. (C) At EB stage, Nodal is expressed in the node located at the distal tip of the epiblast in WT embryos (C1). The expression is normal in Chrd<sup>−/−</sup> and Nog<sup>−/−</sup> embryos (C2, C4), while it is lost or decreased in Chrd<sup>−/−</sup>;Nodal<sup>+/−</sup> (C3) and Nog<sup>−/−</sup>;Smad3<sup>+/−</sup> (C5) embryo.
Figure 3.3: Alteration of BMP and Nodal Signaling Levels in Mutant Embryos
HF (n=23, data not shown). Similarly, we examined the pSmad1/5/8 staining patterns in WT, Nog−/− and Nog;Smad3 embryos. Nog−/− embryos did not have any significantly different pSmad1/5/8 staining during gastrulation (Figure 3.3A4), though they did at the neurulation stage in the neural tubes (Y.P.Y and J.K., unpublished). However, a few Nog;Smad3 embryos displayed ectopic pSmad1/5/8, with staining throughout the distal epiblast (3/13; Figure 3.3B5), suggesting ectopic activation of BMP signaling. These data suggest that both Chrd;Nodal and Nog;Smad3 classes of double mutants have ectopic BMP activation as demonstrated by ectopic Smad1/5/8 phosphorylation.

Taken together, the findings above support our hypothesis that Nodal and anti-BMP cooperatively promote Nodal signaling while inhibiting BMP activation. Furthermore, this interaction appears to maintain a fine balance between mutually antagonistic BMP and Nodal levels, and it is required for normal APS and anterior AME patterning. Genetic lesions which disrupt this balance lead to the downregulation of Nodal levels and the ectopic activation of BMP levels within or around the APS and AME. The alteration of levels of either signaling patterning potentially disrupts the function of the APS and AME, thus resulting in anterior defects.

**Nodal antagonizes the inhibitory effect of BMP on APS gene expression**

Our molecular analyses in mutant embryos suggest that the defects in the APS and anterior AME could be caused by abnormal Nodal and BMP signaling levels, thereby reflecting an inappropriate BMP-Nodal interaction. Specification of the APS can be severely compromised by decreased Nodal expression (Vincent et al., 2003), it remains unclear whether its specification is also affected by ectopic BMP signaling levels, which are possibly regulated by Nodal signaling. Previous studies suggest that inhibition of BMP signaling is required from the AVE, AME and PCP for normal forebrain initiation and specification (Anderson et al., 2002; Bachiller et al., 2000; Yang
and Klingensmith, 2006). However, whether it is also required in the APS was not directly assessed.

To address this question, we utilized a previously developed embryo culture assay (Yamamoto et al., 2004) which allows for the introduction of ectopic gene expression vectors into the primitive streak area via microinjection. E6.5 WT embryos were injected between the mesoderm and endoderm layers in the primitive streak region with liposomes containing a DNA construct of the gene of interest, and were cultured for 14 hours in rotating culture (Figure 3.4A). These embryos were subjected to two-colored WMISH specific for both the ectopic gene and the endogenous APS marker Foxa2. An eGFP construct was first injected as a control, and the expression of eGFP and Foxa2 expression in the APS was confirmed. The transfected eGFP cells migrated during streak elongation, and by the end the culture period the embryos were divided into 2 major groups: those with eGFP cells localized in the distal epiblast and those with eGFP cells localized in the proximal epiblast (Figure 3.4A). Embryos without ectopic expression, or with ectopic expression only in the extraembryonic portion, were excluded from the data pool. Most of the embryos with ectopic eGFP expression showed normal Foxa2 expression (Figure 3.4B1, 2), suggesting that the injection and culturing did not affect endogenous Foxa2 expression in these embryos. Using this assay, we tested the effect of ectopic Bmp2 on Foxa2 expression. In embryos injected with the Bmp2 vector construct, we found that Foxa2 expression was lost or significantly decreased when the ectopic Bmp2 was transfected in the distal epiblast (Figure 3.4C1). Foxa2 expression remained unchanged if ectopic Bmp2 was localized more proximally in the epiblast (Figure 3.4C2). Similar results were found for Gsc expression (data not shown). These results suggest that ectopic Bmp2 exerts its inhibitory effect on Foxa2 expression at a close range. It further suggests that the ectopic BMP signaling in the distal epiblast of Chrd;Nodal and Nog;Smad3 likely inhibits normal APS gene expression.
Our genetic studies suggest an antagonistic relationship between Nodal and BMP signaling. If this hypothesis is correct, we expect that Nodal can relieve the inhibitory effect caused by ectopic BMPs. To test this, we co-injected Bmp2 and Nodal vectors into the primitive streak and then examined Foxa2 expression. All the embryos with ectopic Bmp2/Nodal expression in the proximal epiblast had normal Foxa2 expression (n=14, Figure 3.4D2). Ectopic Bmp2/Nodal expression in the distal epiblast did not inhibit Foxa2 expression (Figure 3.4D1) as compared those with Bmp2 injection alone (16/20 vs 10/31). Similar results were observed for Gsc expression in the APS (Figure 3.4F). These results suggest that Nodal rescues the loss of Foxa2 expression caused by ectopic Bmp2
Figure 3.4: The Effect of Ectopic Bmp2 and Nodal on Expression of the APS Genes Foxa2 and Gsc. (A) A diagram of the experimental strategy. An effector gene was introduced into APS cells of E6.5 WT embryos. The embryos were cultured for 14hr, after which the embryos were divided into 2 groups (distal, proximal) depending on the location of ectopic gene expression. The ectopic gene expression and endogenous APS gene Foxa2 expression were visualized by 2-color WMISH. (B) Ectopic eGFP expression (green arrowheads) does not affect Foxa2 whether it is located in the distal (B1) or proximal epiblast (B2). (C) Ectopic Bmp2 in the distal epiblast inhibits Foxa2 expression (C1), whereas that in the proximal epiblast does not affect Foxa2 (C2). (D) Co-expression of Bmp2 and Nodal does not affect Foxa2 expression whether it is located distally (D1) or proximally (D2). (E-F) Summary of the effects on Foxa2 and Gsc expression of ectopic expression of the indicated genes. The numbers of embryos with normal (red) or no expression (blue) are indicated.
Figure 3.4: The Effect of Ectopic Bmp2 and Nodal on Expression of the APS Genes

*Foxa2 and Gsc*
(Figure 3.4E,F). It further supports our hypothesis that BMP and Nodal signaling act antagonistically, where BMP is inhibitory to expression of the APS genes Foxa2 and Gsc, while Nodal relieves this inhibitory effect.

**BMP inhibits while Nodal promotes APS gene expression**

Our in vivo transfection experiments showed that ectopic Bmp2 inhibits expression of the APS genes Foxa2 and Gsc and this inhibition can be rescued by Nodal expression. To overcome the limitations of this experimental design and to further understand the mechanisms behind these signaling interactions on APS patterning, we devised an APS explant system to facilitate our investigations. We isolated APS fragments from MS stage embryos with glass needles; these explants were first pre-cultured, with either Nodal inhibitor SB-431542 or DMSO as control, and this was followed by BMP2 or Nodal protein treatment (Figure 3.5A). SB-431542 is a potent and specific inhibitor of Nodal receptors but does not have an effect on BMP signaling levels. The purpose of using this drug is to block Nodal signaling at the receptor level without affecting BMP signaling function in order to address the possible mechanisms of the BMP-Nodal interaction (see next section for details). After culturing, explants were prepared for RT-qPCR (qPCR). First, the efficiencies of BMP2 and Nodal protein treatments were validated by examining their target genes Msx2 and Nodal respectively. An increase in Msx2 mRNA levels was detected after BMP2 treatment (Figure 3.5E), and an increase in Nodal mRNA was seen after treatment with Nodal protein (Figure 3.5D). In the same batch of explants, the mRNA levels of the APS genes Foxa2 and Gsc was significantly decreased by BMP2 treatment; in contrast, their mRNA level were increased by Nodal protein treatment (Figure 3.5A2,3). These findings are not only consistent with those from in vivo transfections, but they also provide further support that Nodal is able to promote expression of APS genes.
Figure 3.5: Mutual Antagonistic BMP and Nodal Signals Interact Extracellularly. (A) A diagram of the APS explant assay. APS fragments were separated from E6.5 embryos and then cultured. These explants were pre-treated with DMSO or Nodal inhibitor SB-431542, followed by protein treatment and qPCR. (B-D) Charts of relative mRNA levels of Foxa2, Gsc, Nodal and Msx2 as detected by qPCR in the explants after drug and protein treatment. Protein treatment sets include BSA, BMP2 (BMP), Nodal (NOD), and BMP2-Nodal co-treatment (B/N).
Figure 3.5: Mutual Antagonistic BMP and Nodal Signals Interact Extracellularly
Mutual antagonism between BMP and Nodal signaling

It is intriguing that Chrd;Nodal embryos show ectopic BMP and decreased Nodal in the APS. This result suggests that an imbalance between BMP and Nodal occurs in the APS when the synergy with BMP antagonists is absent. This molecular phenotype also strongly suggests the possibility that BMP and Nodal signaling are antagonizing each other. To further investigate the possible molecular mechanisms behind this interaction and to test for BMP-Nodal mutual antagonism, we used our APS explant system to assess the alteration of BMP and Nodal signaling in response to a combination of protein treatments by examining the mRNA levels of their target genes.

As mentioned above, treatment with BMP2 or Nodal protein can effectively induce expression of their target genes Msx2 and Nodal respectively (Figure 3.5E,D). To investigate the possible interactions between BMP and Nodal signaling, we treated APS explants with BMP and Nodal proteins at the same time and then examined the expression levels of their target genes. Interestingly, while Nodal mRNA was significantly increased with Nodal protein treatment, we found that it was decreased in the explants treated with both BMP2 and Nodal proteins (Figure 3.5D). This result suggests that BMP2 protein treatment is able to antagonize Nodal transcription by inhibiting Nodal protein. Conversely, we also detected significant reduction of Msx2 expression levels after combined BMP2/Nodal treatments, as compared to those treated only with BMP2 protein (Figure 3.5E), suggesting that Nodal is also able to suppress the activation of BMP signaling induced by BMP2 protein. Together, these data suggest a mechanism of mutual antagonism between BMP and Nodal signaling, which is supported by previous studies in frogs (Yeo and Whitman, 2001). This possible mechanism is also supported by our observation that the compound mutants display ectopic BMP signaling that is associated with decreased Nodal signaling.
BMP and Nodal signaling may interact extracellularly

Since the genetics and embryonic data support a possible mechanism of mutual antagonism between BMP and Nodal signaling, we next investigated at which cellular levels these two signaling cascades interact. We made use of Nodal inhibitor SB-431542 (SB), which in cell culture studies is known to inhibit activation of TGFβ signaling by blocking the phosphorylation ability of Activin type I receptors Alk4, 5 and 7, without affecting BMP signaling activation (Inman et al., 2002). In addition, SB is able to block both endogenous and exogenous signaling activation via Smad2 phosphorylation in frog and fish embryos which further supports its specificity (Ho et al., 2006). By using this drug, we examined whether the mutual antagonistic interaction of BMP and Nodal protein is dependent on function of Nodal receptors, which can be blocked by SB. We tested the efficiency and the specificity of this SB inhibitor in our explant system. In control explants, Nodal protein was able to induce an increase in Nodal expression of about 1.5 fold; however, in explants pretreated with SB, Nodal was unable to induce expression of Nodal mRNA. In addition, these explants displayed a decrease in endogenous Nodal levels (Figure 3.5D). In contrast, SB treatment did not affect the induction of Msx2 by BMP2 (Figure 3.5E). These results are consistent with the previous findings that SB is able to inhibit both exogenous and endogenous Nodal signaling.

Since SB inhibits Nodal signaling effectively via disabling the type I receptor required for Nodal signaling activity, we next tested whether BMP and Nodal are able to still interact when the Nodal receptor is disabled and thus unable to activate downstream signaling events. If Nodal protein cannot inhibit induction of Msx2 by BMP2 protein after SB treatment, this would suggest that Nodal signaling via its receptor is required to inhibit BMP signaling. If Nodal protein does inhibit Msx2 expression, this would suggest that Nodal inhibits BMP signaling extracellularly and is
not dependent on the function of Nodal receptors. To test these possibilities, SB-
pretreated APS explants from E6.75 mouse embryos were co-treated with BMP and
Nodal proteins together. We found that Msx2 expression was significantly reduced in
explants after SB/BMP/Nodal treatment as compared to those treated only with BMP
(Figure 3.5E).

This result demonstrates that Nodal protein is able to antagonize BMP signaling,
even though Nodal signaling cannot be transduced downstream due to dysfunctional
receptors. Although we cannot exclude other possible means of interaction, these data
suggest that the BMP/Nodal mutual antagonism (which does not require the function of
Nodal receptors) may happen extracellularly. The possible molecular mechanism
underlying their extracellular interaction is currently under investigation, and is
discussed in Chapter 5.
Discussion

Dose-dependent BMP signals and TGFβ/Activin/Nodal signals play essential roles during early A-P axis patterning of the vertebrate embryo (reviewed by (De Robertis and Kuroda, 2004; Whitman, 2001). In the mouse, anterior malformations were observed in embryos with either decreased BMP antagonism (Anderson et al., 2002; Bachiller et al., 2000) or decreased Nodal-Smad2/3 signaling (Dunn et al., 2004; Liu et al., 2004; Vincent et al., 2003). These mutant embryos display defects in the anterior AME. Thus, the patterning of the anterior AME, is tightly associated with the dosage of both BMP antagonism and Nodal signals. Given that Nodal and BMP antagonists Chrd and Nog are expressed and function in the mouse gastrula organizer and its derivatives, an interesting question is whether BMP and Nodal signaling interact.

A couple of studies in Xenopus suggest an interaction between BMP and Nodal signaling. One nodal family member Xnr3 lacks mesodermal-inducing activity via activating Smad2/3, but promotes neural induction by antagonizing BMP4 (Hansen et al., 1997; Haramoto et al., 2004). Nodal can antagonize the endogenous activation of Smad1 by BMP7 signals, and BMP7 can antagonize the activation of Smad2 by Nodal (Yeo and Whitman, 2001). Based on these findings, the antagonistic activities between Nodal and BMP signaling are at least in part due to the formation of signaling-inactive Nodal (or Xnr3) -BMP heterodimers (Haramoto et al., 2004; Yeo and Whitman, 2001). While these studies suggest a mutual antagonistic function between BMP and Nodal in amphibian neural induction, this type of interaction in mammals had not yet been demonstrated. The present work reveals the in vivo significance of BMP-Nodal interactions during mouse gastrulation. It demonstrates that: (1) synergistic interaction between BMP antagonism and Nodal signaling occurs and is required during anterior formation; (2) mutual antagonism between BMP and Nodal signaling patterns the APS
and anterior AME; (3) BMP antagonists Chrd and Nog reveal a spatiotemporally unique functions during their interaction with Nodal signaling; and (4) mouse Nodal and BMP most likely interact extracellularly.

It has previously been shown that Nodal-Smad2 signals are required in the VE to establish the AVE and initiate anterior specification (Brennan et al., 2001); however, attenuated Nodal-Smad2/3 signals, such as in embryos carrying a Nodal hypomorph mutation (Vincent et al., 2003) and Smad2;Smad3 compound mutations (Dunn et al., 2004), do not affect AVE patterning. Embryos with decreased BMP antagonism by Chrd and Nog do not display defects in AVE specification (Bachiller et al., 2000), even though the AVE is a possible source of these BMP antagonists (Yang and Klingensmith, 2006). Similarly, in Chrd;Nodal and Nog;Smad3 embryos, the AVE forms normally and the anterior cell fate is imposed in the epiblast. Instead, a lack of normal production of the anterior AME, which emerges from the APS, provides compelling evidence that BMP antagonism and Nodal signaling synergize during APS patterning. This further suggests that a fine balance between mutually antagonistic BMP and Nodal signaling is maintained in the APS and the anterior AME during their specification.

In the absence of a balance between BMP and Nodal signaling, head formation is severely compromised. In Chrd;Nodal embryos, an imbalance between these signals caused ectopic pSmad1/5/8 expression and diminished Nodal expression in the APS. This alteration also suggests that BMP signaling and Nodal signaling are the downstream targets of this interaction, and that this interaction occurs in the APS. We propose that the alteration in both signaling levels contributes to the defects in the APS and its derivatives (based on the following reasons). The Chrd;Nodal phenotype resembles the Nodal hypomorph in many aspects, including the loss of APS specification, defects in the anterior AME, and an ultimate phenotype of anterior
malformation (Vincent et al., 2003). Therefore, a decrease of Nodal expression in the APS can be destructive to the development of the APS and anterior AME.

However, whether ectopic BMP activation in the APS would affect anterior patterning had not previously been determined. In embryos carrying mutations in BMP antagonists, such as Chrd;Nog and Twsg1, loss of the anterior structures as well as the PCP and ADE suggests that BMP antagonism is required in the anterior AME for head formation (Anderson et al., 2002; Bachiller et al., 2000) (Petryk et al., 2004). This requirement has obviously occurred in the extending AME at E7.5, but whether it occurs earlier during APS formation is not clear. BMP signaling may possibly be required to confine the specification domain of the APS, because ectopic endoderm cells marked by the APS gene Foxa2 were found in E6.5 Bmpr1a conditional mutants (Davis et al., 2004). The data herein demonstrate that BMP signaling negatively regulates the expression of Foxa2 in the APS, which can be overcome by Nodal expression. Consistent with these findings, Bmpr1a mutant embryos may have ectopic Nodal signaling, because Foxa2 is speculated to be a Nodal target gene (Liu et al., 2004; Vincent et al., 2003). Altogether, these data suggest that the BMP signaling levels in the APS are tightly regulated and may directly affect Nodal signaling due to the nature of BMP-Nodal mutual antagonism. It will also be interesting to examine Nodal signaling levels, by using pSmad2/3 or other reporter assays in embryos with mutations in BMP antagonists to further understand their interaction.

Among all classes of mutations we examined, including Chrd;Nodal, Nog;Nod, Chrd;Smad3, and Nog;Smad3, we only observed anterior malformation in two mutations: Chrd;Nod and Nog;Smad3. While the anterior malformation found in these embryos provides evidence of BMP-Nodal signaling interaction, the fact that only two mutant classes show a synergistic phenotype further supports that the BMP antagonists Chrd and Nog have unique functions during early gastrulation. Chrd and Nog mRNA emerge in
different domains and at different times during early gastrulation. *Chrd* is expressed at E6.5 in the APS and later in the midline, while *Nog* is expressed in the midline from E7.25 but not earlier (Bachiller et al., 2000). The difference in endogenous *Chrd* and *Nog* expression may explain the different phenotype we observed in *Chrd;Nodal* and *Nog;Nodal* mutants. *Nodal* is expressed in the APS at E6.0-E6.5 (Collignon et al., 1996), where early *Chrd* expression is found at the same stage (Bachiller et al., 2000; Klingensmith et al., 1999). In *Chrd;Nodal* mutants, ectopic pSmad1/5/8 and decreased *Nodal* expression in the APS vicinity (which leads to the mispatterning of the APS) matches the expression domains of *Chrd* and *Nodal*. These data demonstrate that *Chrd* and *Nodal* synergize in the APS, which is a novel function for *Chrd* has been uncovered. In contrast, *Nog* expression is found in the node, while *Nodal* expression is found around the node, where *Nodal* is not required for anterior patterning (Brennan et al., 2001). Therefore, in *Nog;Nodal* mutants, there may be changes in BMP or Nodal signaling levels occur but it appears that there is no combinational effect in the spatiotemporal domain required for anterior patterning.

Surprisingly, between the two analogous *Nog;Smad3* and *Chrd;Smad3* mutation classes, anterior malformation only occurs in *Nog;Smad3*. In the Nodal signaling pathway, *Smad2* and *Smad3* are independently regulated but co-expressed. *Smad3* expression was found throughout gastrulation, with strong expression in the AME at mid-gastrulation (Dunn et al., 2004). Although *Smad3* mutants do not possess any embryonic phenotype (Datto et al., 1999), one would expect to see compromised Nodal signaling (though at a tolerable level for development) in *Smad3* mutant embryos, especially in the midline. Interestingly, *Nog* is expressed in the AME, where its expression partially overlaps with that of *Smad3*. Despite the fact that *Nog* does not play an essential role in the AME (McMahon et al., 1998), several studies have shown that *Nog* does cooperate with other genes during AME and PCP patterning, including
Chrd (Anderson et al., 2002; Bachiller et al., 2000), mDkk1 (del Barco Barrantes et al., 2003), and Smad3 (this study). The interaction between Nog and Smad3 provides an additional line of evidence that the balance between BMP and Nodal signaling is crucial during AME patterning in a dosage-dependent manner (Table 1). While Nog and Smad3 together act to maintain the BMP-Nodal balance in the anterior AME, we found no evidence that they interact in the APS. A similar scenario was also found with Nog-mDkk1 interaction, which occurs in the AME but not the APS (del Barco Barrantes et al., 2003). This is possibly because Nog is not expressed or does not function in the APS. Chrd expression and/or function overlaps with that of Smad3 and mDkk1 in the AME.

Unexpectedly, we have so far found no evidence that Chrd interacts with Smad3 genetically; and whether Chrd interacts with mDkk1 remains to be studied. The phenotypic difference between Nog;Smad3 and Chrd;Smad3 embryos, or even possibly between Nog;mDkk1 and Chrd;mDkk1, may be due to unknown and distinct regulatory mechanisms of Chrd and Nog that require further investigation.

Altogether, our data lead us to propose that the crosstalk between BMP and Nodal signaling occurs via the synergistic functions of: (1) Chrd and Nodal in the APS at E6.5 (Figure 3.6A1), and (2) Nog and Smad3 in the anterior AME at E7.0 (Figure 3.6A2). These two distinct spatiotemporal regulations may result in similar signaling alterations, i.e. increased BMP activation combined with decreased Nodal activity. In Chrd;Nodal embryos, APS specification is deficient due to an excess of BMP signaling and reduced Nodal signaling (Figure 3.6B1), and it is this APS which gives rise to a deficient AME that especially lacks its most anterior region (Figure 3.6B2). In Nog;Smad3 embryos, even though the APS may have moderately reduced Nodal signal levels because the embryos lack Smad3 alleles, APS patterning appears normal, possibly because Chrd and Nodal are still expressed in the APS (Figure 3.6C1). However the AME, emerging from the compromised APS, lacks both Nog and Smad3 functions and can no longer provides the
Figure 3.6: Model for Interaction of Nodal and Anti-BMPs in Early Forebrain Patterning.  

(A) Synergistic interaction of Chrd and Nodal in the APS (green/red) at E6.5 (A1) and Nog and Smad3 in the anterior AME (green) at E7.5 (A2) restricts BMP signaling activation (blue) and promotes Nodal signaling, allowing specification of the APS and AME. (B) In Chrd<sup>+/−</sup>;Nodal<sup>+/−</sup> embryos, the APS is defective due to excess BMP activation and reduced Nodal signaling (B1) and therefore gives rise to a deficient AME (B2). (C) In Nog<sup>+/−</sup>;Smad3<sup>+/−</sup> embryos, the APS is possibly compromised due to moderately reduced Nodal signals but its patterning appears normal. However, the AME emerging from the compromised APS is defective due to a lack of collaborative Nog and Smad3 functions and this results in ectopic BMP activation and decreased Nodal signaling.
Figure 3.6: Model for Interaction of Nodal and Anti-BMPs in Early Forebrain Patterning
cues required to antagonize BMPs (Figure 3.6C2). In embryos of both classes of mutation, failure in maintaining balanced BMP-Nodal levels causes a destruction of the boundaries between the mutually antagonistic signals in the APS and/or the anterior AME, which thus leads to the gross anterior truncation.

In human genetics studies, which are supported by findings in mouse models, HPE has been shown to be likely caused by the digenic inheritance pattern, in which multiple mutations in the same developmental pathway affect phenotype severity (Ming and Muenke, 2002). The present work not only establishes the significance of BMP-Nodal interaction during mammalian anterior development, but it also demonstrates that HPE can arise from the co-occurrence of mutations in Chrd and Nodal, as well as Nog and Smad3. Given the finding that mutations in Nodal signaling components are associated with human HPE (Roessler and Muenke, 2001), it is highly possible that these cases also involve mutations in BMP antagonists. Whether mutations in human CHRD or NOG, in combination with mutations in Nodal signaling components such as SMAD3, are responsible for some cases of human HPE requires further research.
Chapter 4:

Spatiotemporal Pattern of BMP Signaling Activation During Early Mouse Embryogenesis
Summary

BMPs, a family of secreted proteins related to the cytokine TGFβ, are involved in multiple processes during development. Their signal transduction acts via phosphorylation and activation of Smad1/5/8 proteins. Here we used an antibody specific for the activated form of Smad1/5/8 to examine endogenous BMP signaling activities during early mouse embryogenesis in either wildtype embryos or embryos carrying mutations for the BMP antagonists 

Chordin (Chrd) and Noggin (Nog). We found that BMP signaling is generally activated in the extraembryonic portion of the mouse egg cylinder; however, it is ectopically activated in the primitive streak of Chrd⁻/⁻;Nog⁻/⁻ embryos, which may be related to their forebrain truncation phenotype. BMP signaling is active in the dorsal neural fold (where neural crest cells originate), the ventral foregut endoderm, in somites and in the presomitic mesoderm. Increased BMP activation was detected in these tissues in Chrd⁻/⁻;Nog⁻/⁻ embryos, suggesting that BMP signaling is normally antagonized in these tissues. Asymmetric BMP activation was also revealed in the lateral plate mesoderm suggesting a role during left-right axis formation. Our data suggest that redundant functions of BMP antagonists Chrd and Nog restrict BMP signaling from the APS, the node, and the anterior endoderm during gastrulation. They also confine BMP activation in the dorsal neural tube posterior to the optic vesicles during neurulation. We discuss the implication of phospho-Smad1/5/8 distribution during these stages in both wildtype and mutant embryos during early mouse development.
Materials and methods

Mice

Wildtype embryos were obtained from timed matings of ICR mice (Harland). Embryos with double mutations carried ChrdRV (Bachiller et al., 2000) and Nog9E (McMahon et al., 1998) alleles were obtained from timed matings of Chrd−/−;Nog+/−; Nog−/+;Nog−/− mice generated as described previously (Anderson et al., 2002). Chrd−/−;Nog+/− mice were maintained in an outbred (ICR) background. All mutant embryos were genotyped by PCR as described previously (Bachiller et al., 2000; McMahon et al., 1998), and staged (Downs and Davies, 1993) as described.

Western Blots

Western immunoblots were performed as described (Yeo and Whitman, 2001). Tissue from one E8.5 mouse embryo was used per lane. Antibodies used were rabbit-α-pSmad1/5/8 (Cell Signaling; 1:1000) and rabbit-α-pSmad2 (Cell Signaling; 1:1000).

In Situ Hybridization, Immunohistochemistry and Histological Sectioning

Whole-mount in situ hybridization (WMISH) was performed using an established protocol (Belo et al., 1997). Whole mount immunohistochemistry for α-pSmad1/5/8 (Cell Signaling) was performed as described previously (Yang and Klingensmith, 2006). Stained embryos were cryosectioned and counter stained with histo-green based on standard protocols (Hogan, 1994).
Results and Discussion

Endogenous pSmad1/5/8 during mouse primitive streak formation

Smad1/5/8 are the intracellular effectors that are activated by the Type I receptors upon binding of BMP ligands. The distribution of activated Smad1/5/8--phospho-Smad1/5/8 (pSmad1/5/8) is by far the most reliable readout of BMP signaling activity, which cannot be represented or simply predicted by known target genes (e.g. Foxa2\textsuperscript{\textdagger}; Yang and Klingensmith, 2006). Therefore, the understanding of endogenous pSmad1/5/8 expression pattern in WT and mutant embryos is the best approach towards the understanding of the function and the regulation of BMP signaling activity.

We sought to examine the endogenous pattern of BMP signaling activation during early mouse embryogenesis. We used an anti-phospho-Smad1/5/8 antibody (anti-pSmad1/5/8), which detects endogenous levels of phosphorylation of the BMP signaling effectors Smad1/5/8. By western blot analysis, only a single band is detected in mouse embryonic lysates when using anti-pSmad1/5/8 (Figure 4.1). Anti-pSmad1/5/8 antibodies were used to detect BMP signaling activation in the embryos by whole-mount immunohistochemistry. From about early-streak (ES) stage, prior to and at the beginning of primitive streak formation, pSmad1/5/8 is detected in the extra-embryonic portion of the egg cylinder and in a few epiblast cells close to the junction (Figure 4.2A). Similarly at mid-streak (MS) stage, pSmad1/5/8 is detected strongly in extraembryonic tissues and weakly in some cells in the elongating primitive streak in the posterior epiblast (Figure 4.2B, arrow). At late-streak (LS) stage, when the primitive streak is halfway up the posterior epiblast (Figure 4.2C, arrowhead), pSmad1/5/8 is also detected within the elongating streak, although the signal appears much weaker.
Figure 4.1: Anti-pSmad1/5/8 Immunoblotting in Mouse Lysates.

AntipSmad1/5/8 antibodies specifically distinguish a single band of activated Smad1/5/8 in E8.5 mouse embryo lysates. pSmad1/5/8 is approximately 60kDa.
Figure 4.1: Anti-pSmad1/5/8 Immunoblotting in Mouse Lysates
Figure 4.2: Endogenous pSmad1/5/8 Pattern During Early and Late Mouse Gastrulation. (A, B) At ES to MS stage, pSmad1/5/8 is detected strongly in the extraembryonic portion of the egg cylinder. Arrows denote the distal limit of expression. (C, D) From LS to 0B stage, pSmad1/5/8 is detected in the more posterior part of the elongating primitive streak (black arrows) but not the anterior tip of primitive streak (red arrowheads). (E-F') From EB to LB, endogenous pSmad1/5/8 is detected in the extraembryonic endoderm, posterior endoderm close to the streak, and the allantois, but is excluded from the anterior neuroectoderm and the distal epiblast. (G-H') Later at headfold stages, pSmad1/5/8 is similar to that in EB stages, but is highly distributed throughout the invaginating foregut pocket (arrow in H'). Ne, neuroectoderm; al, allantois; n, node.
Figure 4.2: Endogenous pSmad1/5/8 Pattern During Early and Late Mouse Gastrulation
towards the anterior streak (Figure 4.2C, arrow). In the no-bud (0B) stage epiblast (Figure 4.2D, arrowhead), Smad1/5/8 activation is gradually extinguished at the anterior tip of the primitive streak, and is more strongly detected at the posterior streak, which is located closer to the extraembryonic-embryonic junction (Figure 4.2D, arrow).

The dynamic pattern of pSmad1/5/8 distribution (generally intense in the extraembryonic tissues, and in embryonic tissues restricted to the posterior streak while low or undetected in the anterior streak) suggests that BMP activation is tightly regulated during early gastrulation. This prompted us to compare the expression of possible BMP ligands (Bmp2, Bmp4, Bmp7) at these stages. During ES-MS stage, the pSmad1/5/8 pattern is similar to Bmp4 expression, which is detected throughout the extraembryonic tissues but not detected in the embryonic tissues (Figure 4.3A1). At MS-0B, phosphorylation of Smad1/5/8 is not simply predicted by Bmp4 expression, or by Bmp2 or Bmp7. Bmp4 mRNA is strongly expressed in the extraembryonic tissues next to the junction; therefore, it is possible that BMP4 protein travels to the epiblast region and activates Smad1/5/8 in the primitive streak. Bmp2 is weakly expressed in the nascent primitive streak in the posterior epiblast at ES-MS (Figure 4.3B1, arrow) but is strongly expressed in the anterior primitive streak (APS) region at LS stage (Figure 4.3B2). Bmp7 is also expressed in the primitive streak (Figure 4.3C1). Based on this observation, it is also likely that Bmp2 and Bmp7 activate Smad1/5/8 in the posterior primitive streak.

Even though Bmp2 and Bmp7 expression is prominent in the APS, they are not able to activate Smad1/5/8 in the same tissue because pSmad1/5/8 is gradually cleared out of the APS at the same stage. This observation further raises the possibility of additional regulatory mechanisms for BMP signaling activation. Both Nodal and the BMP antagonist Chrd are expressed in the APS and they synergize in the APS to antagonize BMP activation (Chapter 3). The expression and function of these genes, as well as other organizer factors, might account for the limitation of pSmad1/5/8
Figure 4.3: Expression of BMP Ligands and Downstream Target Genes in Gastrulating Embryos. (A1-A3) *Bmp4* is expressed in the extraembryonic tissues but not detected in the epiblast, with expanded expression around the extraembryonic-embryonic junction at slightly later stages in A2, A3. (B1-B5) *Bmp2* expression is first detected in the AVE (arrowhead in B1) and the primitive streak region (arrow in B1), and later can be found in the APS (arrow in B2). *Bmp2* is detected in the anterior part of elongation streak (arrow in B3), and later in the node (B4, B5). (C1-C3) *Bmp7* is detected in the APS and the node (arrow in C1 and C2 respectively) at E6.5 and early bud stages, and in the headfold region but not in the node at headfold stage (C3). (D1-D3) *Msx2* expression resembles that of *Bmp4*, and is detected mostly in the extraembryonic tissues and the junction. (E1, E2) *Bambi* is found around the junction especially in the anterior region of headfold close to the junction border (arrow).
Figure 4.3: Expression of BMP Ligands and Downstream Target Genes in Gastrulating Embryos
distribution in the APS. BMP inactivation in the APS has been implicated to be essential for normal forebrain ectoderm specification and maintenance (Foxa2 mutants; Yang and Klingensmith, 2006), and the patterning of the APS and its derivatives (such as in Chrd;Nodal and Nog;Smad3 mutants; Chapter 3).

Interestingly, pSmad1/5/8 expression is detected in the extraembryonic endoderm; however, it is not detected in the anterior visceral endoderm (AVE) even though Bmp2 is (Figure 4.3B1, arrowhead). Therefore, the expression of BMP ligands does not necessarily activate the downstream signaling cascade since BMP signaling is tightly regulated by extracellular inhibitors. In addition, given that Bmp2 in the AVE is not able to activate Smad1/5/8 phosphorylation, it suggests and supports our previous finding that the AVE is a possible source of anti-BMP signals (Yang and Klingensmith, 2006).

**Endogenous pSmad1/5/8 during headfold formation**

At early-bud (EB) to late-bud (LB) stage, pSmad1/5/8 is strongly detected throughout the extraembryonic endoderm and allantois (Figure 4.2E’,F’, arrows). In the epiblast, all three layers are almost free of Smad1/5/8 activation, except for the cells next to the extraembryonic-embryonic junction, which display a weak pSmad1/5/8 signal (Figure 4.2E’). From early-head-fold (EHF) to late-head-fold (LHF) stage, pSmad1/5/8 is still detected in the extraembryonic endoderm and allantois; however, not in the epiblast (Figure 4.2G,H). The pattern at these stages resembles Bmp2 and Bmp4 expression (Figure 4.3A2, B3-B5), as well as the BMP target genes Msx2 (Figure 4.3D2,D3). Interestingly, before and during foregut invagination, pSmad1/5/8 is found in the endoderm overlying the head fold area at EHF stage (Figure 4.2G’, arrow); later it is found in the endoderm overlying the opening of the foregut endoderm pocket right next to the anterior tip of the axial mesendoderm (AME) (Figure 4.2H’, arrow). This
pattern is similar to that of Bmp2, which is found anteriorly at the junction (Figure 4.3B5, arrowhead). However, in these tissues, even when Smad1/5/8 is activated, we did not see expression of Msx2 (Figure 4.3D3) or Msx1 (data not shown), two major downstream target genes of BMP signaling (Balemans and Van Hul, 2002). Instead, Bambi, which is a membrane-bound BMP inhibitor that is induced by BMP itself (Onichtchouk et al., 1999), reveals a similar expression pattern at the head fold area (Figure 4.3E1, arrow). This observation suggests a complexity of BMP signaling activation even via the same pSmad1/5/effectors 8, where different target genes are activated in different sub-domains of the gastrulating embryo. Similarly, in EB stage Foxa2−/− embryos, ectopic pSmad1/5/8 is detected in the primitive streak and visceral endoderm (VE) in EB stage embryos, but none of the known BMP target genes (Msx1, Msx2, Smad6, Bambi, Dlx5) show ectopic expression in the corresponding area (Yang and Klingensmith, 2006). In addition, E8.25 Foxa2+/− embryos also have ectopic pSmad1/5/8 in the head mesenchyme and in the heart tube; however, among the known BMP target genes only Msx2 is expressed ectopically in the head but not in the heart (Yang and Klingensmith, 2006). Therefore, it is possible that these genes differ in their competence to respond to pSmad1/5/8 in different domains. In addition, it is also possible that other target genes and/or other regulatory signaling mechanisms are involved that require further identification and investigation.

Intriguingly, pSmad1/5/8 was never detected in the mouse node or axial mesendoderm in the WT embryos, a result different from the finding that pSmad1/5/8 is weakly detected in Hensen's node in chick (Faure et al., 2002). Mouse Bmp2 and Bmp7 are expressed in the node (Figure 4.3B4,B5, 3C2) but are not able to activate Smad1/5/8 in this area. It is possibly due to the expression of BMP antagonists Chrd and Nog in the axial tissues (Bachiller et al., 2000). However, in the chick, BMP4 is weakly expressed in Hensen's node and is possibly the ligand that activates
Smad1/5/8—even though Nog is also expressed in the same domain (Faure et al., 2002). Nonetheless, in both organisms, pSmad1/5/8 is rapidly cleared out from the node and APS during neural induction, suggesting an anti-BMP requirement in these tissues for normal neural plate patterning, especially of the forebrain ectoderm, which is more sensitive to BMP signaling levels (Yang and Klingensmith, 2006).

**Endogenous pSmad1/5/8 in early somite stages**

From neurulation to early somitogenesis (0-7 somites), pSmad1/5/8 is detected in the neural fold, along the A-P axis (Figure 4.4A, black arrow), but is not very prominent in the surface ectoderm (Figure 4.4A, blue arrow). Slightly later, at about the 10-somite stage, pSmad1/5/8 is still detected in the neural fold and the surface ectoderm next to it (Figure 4.4C, blue arrow). Specifically at this stage, pSmad1/5/8 expression is also increased in the rhombomere (R) 3 and R5 (Figure 4.4C, arrowhead). This pattern resembles that of BMP4 expression, which promotes apoptosis of neural crest cells (NCCs) in the same region (Farlie et al., 1999). Anteriorly, pSmad1/5/8 is found in the anterior neural ridge (Figure 4.4B, arrow), but gradually clears out at about 10-somite stage.

A boundary of pSmad1/5/8 distribution is detected close to the optic vesicles (Figure 4.4D, arrow), and no Smad1/5/8 activation is detected in the surface ectoderm or neural fold rostral to this boundary (Figure 4.4D). This observation suggests a down-regulation of BMP signaling during anterior structure formation. It is also possibly related to anti-BMP signals expressed in the forebrain region, such as Six3 (Gestri et al., 2005). In cross sections of these embryos, pSmad1/5/8 is detected in the dorsal neural tube (or neural ridge) and in some migrating neural crest cells (NCCs, Figure 4.4E), which is consistent with the roles of BMP signaling in D-V patterning of the neural tube and neural crest specification. Smad1/5/8 is also activated in the ventral foregut endoderm
Figure 4.4: Endogenous pSmad1/5/8 Pattern During Early Somite Stages of Mouse Embryos. (A) At the 5-somite stage, pSmad1/5/8 is detected in the dorsal neural fold anteriorly (black arrow), but is not detectable in the surface ectoderm next to the neural fold (blue arrow). Expression is strong in the tail bud and allantois. (B) Anteriorly, pSmad1/5/8 is detected in the anterior neural ridge (arrow) and the opening of the foregut pocket (arrowheads). (C) At around the 7-somite stage, the anterior limit of pSmad1/5/8 distribution is at the optic vesicles (black arrow). At this stage, it is found strongly in the surface ectoderm (blue arrow), especially in R3 and R5 (arrowheads). (D) Higher magnification of (C) showing that the optic vesicles and the forebrain is pSmad1/5/8 negative. Staining is found in the splanchnic mesoderm (red arrowheads). (E) Anterior section showing that Smad1/5/8 is activated in the neural fold, ventral foregut endoderm, NCCs, surface ectoderm, and both myocardium and endocardium in the heart. (F-H) pSmad1/5/8 is also detected in the dorsal neural tube, dorsal aorta, somites. Caudally it is found in the presomitic mesoderm. OV, optic vesicles; NF, neural fold; VFE, ventral foregut endoderm; NCCs, neural crest cells; SE, surface ectoderm; Myo, myocardium; Endo, endocardium; DA, dorsal aorta; NT, neural tube; Som, somites; PSM, presomitic mesoderm.
Figure 4.4: Endogenous pSmad1/5/8 Pattern During Early Somite Stages of Mouse Embryos
(VFE, Figure 4.4E), suggesting a role for BMP signaling in the GI system and also in early heart development, which requires signals from the VFE (Rossi et al., 2001). In the heart, pSmad1/5/8 is detected in both myocardium and endocardium, but the expression is not ubiquitous (Figure 4.4E). Further analysis of BMP signaling activation in sub-domains of the heart in wildtype or related mutant embryos will be crucial in understanding its function during heart development.

Caudally, pSmad1/5/8 is detected strongly in the tail bud, the allantois, and in the somites, where it is stronger in the segmented somites (Figure 4.4C). In sections, pSmad1/5/8 expression is intense in the presomitic mesoderm (PSM) and the ventral neural tube right next to it (Figure 4.4H). However, we did not detect any molecular-clock like pattern similar to that seen with ERK activation (Corson et al., 2003). Given the fact that embryos with decreased BMP signaling (as in BmprIa deficient embryos), as well as increased BMP signaling (as in Nog-/- embryos), display defects in the patterning of somites (Davis et al., 2004; Stottmann et al., 2006), BMP signaling activation in the PSM is possibly regulating the patterning of somites.

Asymmetrical Smad1/5/8 activation in the lateral plate mesoderm

While present in the segmented paraxial mesoderm, pSmad1/5/8 is also detected in the lateral plate mesoderm (LPM), although it is weakly expressed if not absent in the intermediate mesoderm in between, except for the most caudal part (Figure 4.5C, arrowhead). In the chick, asymmetric Shh on the left side of Hensen’s node induces the perinodal domain of Nodal expression, and endogenous BMP signaling in the left LPM induces Cfc expression to promote Nodal signaling, which presumably diffuses from the perinodal domain (Piedra and Ros, 2002). In addition, enhanced Bmp4 expression, as well as Smad1/5/8 phosphorylation, is found on the right side of the perinodal area (Faure et al., 2002). This phenotype is consistent with the finding that
Figure 4.5: Asymmetrical pSmad1/5/8 Distribution in the LPM. (A, A’) At the 3-5-somite stage, pSmad1/5/8 displays enhanced distribution in the right LPM (arrowheads) and is weakly detected at the intermediate mesoderm region (arrows). (B-C) Later at 5-7 or 7-10-somite stages, asymmetrical distribution is no longer detectable in the LPM. Intermediate mesoderm region remains weakly stained for pSmad1/5/8 (arrowhead in C).
Figure 4.5: Asymmetrical pSmad1/5/8 Distribution in the LPM
BMP acts on the right side of the node to antagonize Shh expression and establish the L-R axis (Monsoro-Burq and Le Douarin, 2000). At slightly later stages, while Nodal expression is established in the left LPM only, several BMPs are expressed symmetrically in the LPM. Consistently, pSmad1/5/8 is also symmetrically distributed (Faure et al., 2001). Unlike in the chick, mouse pSmad1/5/8 is not detected in the node or the perinodal area. However, it is distributed asymmetrically in the LPM across the L-R axis. In 3-5 somite stage mouse embryos, pSmad1/5/8 is more enhanced in the right LPM as compared to the left (Figure 4.5A,A’ arrowheads), but it is still detected in the left LPM (Figure 4.5A,A’, arrows). Asymmetrical BMP activation is not detectable at slightly later stages; it is the same in both sides of the LPM at 5-7-somite and 7-10-somite stage (Figure 4.5B,C). This observation that BMP signaling is asymmetrically activated in the right LPM across the L-R axis is further supported by the preliminary finding that expression of the BMP antagonist Nog is enhanced in the left LPM (N. Mine, J.K. unpublished). The timing of asymmetrical BMP activation matches the timing when Nodal and Pitx2 are expressed on the left side, which is crucial for L-R axis establishment. Given that BMP can antagonize activation of Nodal signaling in other tissues (Chapter 3), BMP signaling activation may possibly antagonizing Nodal signaling in the right LPM during L-R axis determination. Therefore, an interaction between BMP and Nodal might occur in the LPM. Future analyses of mutations with L-R axis defects are required for a better understanding of these interactions (collaboration with T. Sitzman and N. Mine).

Chrd+/Nog+/ embryos reveal ectopic pSmad1/5/8 in the APS

Embryos with mutations in the BMP antagonists Chrd and Nog possess severe defects in patterning of all three axes and many of them also show forebrain truncation (Bachiller et al., 2000). However, the spatiotemporal alteration of BMP signaling level,
and how ectopic BMP signals cause a wide spectrum of defects has not yet been
determined in Chrd<sup>+</sup>;Nog<sup>−/−</sup> embryos. Therefore, we used pSmad1/5/8 antibodies to
examine the BMP signaling level in Chrd<sup>+</sup>;Nog<sup>−/−</sup> mutants in order to understand how
BMP over-activation result in defects in the mutants.

We first examined pSmad1/5/8 in streak stage WT, Chrd<sup>+</sup> and Chrd<sup>+</sup>;Nog<sup>−/−</sup>
embryos because the earliest detectable Chrd expression by WMISH is at LS stage, when
Nog expression is not yet detected. We did not find any difference at this or any other
stages between WT and Chrd<sup>+</sup> embryos, which were obtained from an outbred genetic
background. In LS control (WT or Chrd<sup>+</sup>) embryos, pSmad1/5/8 is detected in the
posterior elongating primitive streak (Figure 4.6A1, arrow) but not in the APS located
more distally in the epiblast (Figure 4.6A1, asterisk). However, ectopic pSmad1/5/8 is
detected throughout the primitive streak so that the posterior half of the epiblast is
pSmad1/5/8-positive in Chrd<sup>+</sup>;Nog<sup>−/−</sup> embryos (Figure 4.6A2, arrow). This phenotype
suggests that BMP signaling is ectopically activated in the APS of Chrd<sup>+</sup>;Nog<sup>−/−</sup> embryos
but not Chrd<sup>+</sup> embryos. At 0B-EB stage, pSmad1/5/8 is cleared from the epiblast and
is restricted to the extraembryonic tissues and next to the junction in the control embryos
(Figure 4.6B1, arrow). However, in mutant embryos, ectopic pSmad1/5/8 is detected in
the distal epiblast, where the APS is located (Figure 4.6B2).

Two interesting questions are raised from the observation that Chrd<sup>+</sup>;Nog<sup>−/−</sup>
embryos possess ectopic activation of BMP signaling. First, ectopic pSmad1/5/8 is
detected in the APS at LS stage, when Nog expression by WMISH is not yet detected
(Bachiller et al., 2000). This observation suggests an unknown Nog function, whereby
Nog synergizes with Chrd to antagonize BMP activation at early gastrulation. Further
analysis, possibly by qPCR, is required to determine the expression and function of Nog
during early gastrulation. Interestingly, Nog is expressed in the extraembryonic parietal
endoderm (data not shown), which is located outside and adjacent to the epiblast.
Figure 4.6: Ectopic pSmad1/5/8 Is Found in the APS and Anterior Endoderm in Chrd<sup>+/−</sup>;Nog<sup>+/−</sup> Embryos. (A, B) At LS stage, pSmad1/5/8 is found in the posterior part of the primitive streak in Chrd<sup>+/−</sup> embryos (arrow in A1) but ectopically in the anterior streak located distally in Chrd<sup>+/−</sup>;Nog<sup>+/−</sup> embryo (arrowhead and asterisk in A2). At 0B stage, pSmad1/5/8 is restricted to the proximal part of the Chrd<sup>+/−</sup> embryo; however, ectopic pSmad1/5/8 is found in the primitive streak located at the distal epiblast of Chrd<sup>+/−</sup>;Nog<sup>+/−</sup> (arrow). Arrowhead denotes the constriction at extraembryonic-embryonic junction. (C) In Chrd<sup>+/−</sup>;Hex-GFP embryo, similar to wildtype embryo, pSmad1/5/8 (red) is located in the extraembryonic endoderm, slightly extended in the posterior endoderm (C1), and does not overlap with Hex-GFP expression in the anterior endoderm (green, C2, C4). (D) In Chrd<sup>+/−</sup>;Nog<sup>+/−</sup>;Hex-GFP embryo, extended pSmad1/5/8 (red) is found in the anterior endoderm (arrow, D1), and partially overlaps with Hex-GFP expression in the anterior endoderm (green, D2, D4). Arrowheads denote the extraembryonic-embryonic junction.
Figure 4.6: Ectopic pSmad1/5/8 Is Found in the APS and Anterior Endoderm in $Chrd^{-/-}\;Nog^{-/-}$ Embryos
Whether or not Nog functions via the parietal endoderm to prevent BMP signaling in the APS or anterior ectoderm requires further investigation with chimeric embryos. Similarly, ectopic BMP activation is detected in Chrd$^+/−;Nodal^{+/−}$ embryos in the APS as a result of an imbalance between Nodal and BMP signaling (Chapter 3). Therefore, it is also possible that ectopic BMP activation in Chrd$^+/−;Nog^{−/−}$ APS may have an inhibitory effect on Nodal signaling levels, which were previously reported to govern APS specification during gastrulation (Vincent et al., 2003). Whether the abnormal patterning in the APS and its derivatives that causes future forebrain truncation in Chrd$^+/−;Nog^{−/−}$ embryos is solely the result of ectopic BMP signaling, or the indirect effect on Nodal signaling level, or a combination of both, requires further investigation.

**Chrd$^+/−;Nog^{+/−}$ embryos reveal ectopic pSmad1/5/8 in the ADE at E7.5**

During gastrulation, the definitive endoderm (DE) emerging from the APS normally displaces the VE, which moves into the extraembryonic visceral yolk sac (Thomas and Beddington, 1996). Failure of this process often causes improper DE specification, as well as later defects in anterior gut tube formation. To further understand the effect of ectopic BMP signaling on anterior definitive endoderm (ADE) specification, we introduced Tg(Hex-eGFP)ARbe (or Hex-GFP, Rodriguez et al., 2001), a reporter of Hex expression, to Chrd$^+/−;Nog^{+/−}$ embryos and examined the pattern of pSmad1/5/8 in these embryos. By immunofluorescent staining and optical sectioning, we found that Smad1/5/8 phosphorylation is restricted in the extraembryonic endodermal cells (Figure 4.6C1), a pattern consistent with the results obtained by immunohistochemistry (Figure 4.2E'). In addition, pSmad1/5/8 protein is almost absent in Hex-GFP labeled ADE cells (Figure 4.2C2). However, in Chrd$^+/−;Nog^{+/−}$ embryos at the same stage, the pSmad1/5/8 signal extends more distally towards the epiblast region and partially overlaps with Hex-GFP cells (Figure 4.6D1, arrow), and also is weakly
detected in the node. This observation suggests that BMP signaling is ectopically activated in the anterior endoderm region due to a lack of BMP antagonism by Chrd and Nog.

Interestingly, in control embryos, Hex-GFP positive cells are found continuously in the embryonic endoderm (Figure 4.6C2, arrow). These cells are ADE cells, which replace AVE cells at this stage, while the AVE cells have migrated to the extraembryonic yolk sac and no longer express Hex. In Chrd<sup>+/+</sup>;Nog<sup>+/+</sup> embryos, the endoderm displays continuous expression of Hex-GFP positive cells distally in about two-third of the endoderm (Figure 4.6D2, asterisk) but sporadic expression in cells proximally (Figure 4.6D2, arrow). It is possible that these cells sporadically expressing Hex-GFP are AVE cells that have failed to move properly into the extraembryonic tissues. This hypothesis is supported by the morphological constriction found at the extraembryonic-embryonic junction of Chrd<sup>+/+</sup>;Nog<sup>+/+</sup> embryos (Figure 4.6D2, arrowhead). This constriction is often found in other mutants with axial rotation defects (e.g. Foxa2 mutants, Ang and Rossant, 1994; Dufort et al., 1998). These data suggest that the ADE is not normally specified or localized in Chrd<sup>+/+</sup>;Nog<sup>+/+</sup> embryos. This is likely due to ectopic pSmad1/5/8 in the APS at earlier stages and in the anterior endoderm, possibly activated by Bmp4 expressed in the adjacent extraembryonic tissues. The defective ADE in Chrd<sup>+/+</sup>;Nog<sup>+/+</sup> embryos may also affect the process of recruiting embryonic lineage cells into the future gut tube. Whether extraembryonic lineage cells are abnormally contributing to the gut tube (and thus affect patterning of the anterior gut, craniofacial tissues and the heart) of Chrd<sup>+/+</sup>;Nog<sup>+/+</sup> embryos requires further analyses.

**Ectopic pSmad1/5/8 in Chrd<sup>+/+</sup>;Nog<sup>+/+</sup> in E8.5 embryos**

At E8.5, Chrd<sup>+/+</sup>;Nog<sup>+/+</sup> embryos reveal a more complicated pattern of ectopic Smad1/5/8 phosphorylation throughout the body. In WT or Chrd<sup>+/+</sup> control embryos, pSmad1/5/8 is
detected in the dorsal-most part of the ectoderm of the neural tube and in the migrating NCCs (Figure 4.7C1). It is not detectable in the rostral-most part of the neural tube (where the optic vesicles are located) or in the head mesenchyme (Figure 4.7C1). However, in Chrd<sup>⁄⁺</sup>;Nog<sup>⁄⁻</sup> embryos, pSmad1/5/8 is detected in a more expanded area of the dorsal neuroectoderm (Figure 4.7C2, bracket) and in a significantly increased number of NCCs next to the neural fold (Figure 4.7C2). In Chrd<sup>⁺⁺</sup>;Nog<sup>⁻⁻</sup> embryos, because of the holoprosencephaly (HPE), the optic vesicles are not invaginating normally (Figure 4.7D1, asterisk). We found ectopic pSmad1/5/8 in the neuroectoderm and the adjacent surface ectoderm at rostral end of the embryo (Figure 4.7D1, arrowhead) which is normally pSmad1/5/8 negative. Scattered pSmad1/5/8 is also detected ectopically in the head mesenchyme in Chrd<sup>⁄⁺</sup>;Nog<sup>⁄⁻</sup> embryos (Figure 4.7D2). Ectopic BMP signaling is able to promote apoptosis and also affect the patterning of NCCs (Anderson et al., 2006) and the forebrain (Anderson et al., 2002). This expanded BMP activation on the dorsal side of the neural tube suggests that the BMP-Shh gradient along the D-V axis is abnormal. It also suggests that the boundary between neuroectoderm and surface ectoderm is not normally established.

More posteriorly, pSmad1/5/8 is weakly detected in the first branchial arches (BA1) in control embryos (Figure 4.7C2); however, it is ectopically distributed inside the BA1 of Chrd<sup>⁺⁺</sup>;Nog<sup>⁻⁻</sup> embryos (Figure 4.7D2). Chrd<sup>⁺⁺</sup>;Nog<sup>⁻⁻</sup> embryos often show reversed heart looping or an unlooped heart tube (Bachiller et al., 2000; Figure 4.7D3). Strong pSmad1/5/8 expressing cells are found in both myocardium and endocardium compared to control embryos (Figure 4.7C3, D3). In addition, the expression of pSmad1/5/8 in the ventral foregut endoderm is increased in the mutants (Figure 4.7C,D). Since BMP4 has been implicated in branchial-arch artery and outflow-tract development (Liu et al., 2004), the defects in heart development in Chrd<sup>⁺⁺</sup>;Nog<sup>⁻⁻</sup> embryos other than heart tube looping (Bachiller et al., 2000), require further analysis.
Figure 4.7: Ectopic pSmad1/5/8 Distribution in Chrd<sup>+/−</sup>;Nog<sup>+/−</sup> Embryos at around 9-Somite Stage. (A, B) Whole-mount immunohistochemistry staining against pSmad1/5/8 in wildtype and Chrd<sup>+/−</sup>;Nog<sup>+/−</sup> embryos. Chrd<sup>+/−</sup>;Nog<sup>+/−</sup> embryo displaying overall increased levels of pSmad1/5/8, including in the neural tube, heart, somites and the tail bud region. (C, D) Sections of wildtype and Chrd<sup>+/−</sup>;Nog<sup>+/−</sup> embryo shown in (A). (C1, C2, D1 and D2) In Chrd<sup>+/−</sup>;Nog<sup>+/−</sup> embryos, pSmad1/5/8 is expanded in the dorsal neural tube (nt, brackets), ectopically in NCCs (arrows), in the surface ectoderm (SE), and in the BA1 (D2). WT embryo shows no staining in the optic vesicles rostrally (ov, C1), but mutant show ectopic spots at rostral end (arrowheads, D1). (C3, C4, D3 and D4) At a more posterior axial level, a significant increase in pSmad1/5/8 is found in the dorsal neural tube, which is covered by a sheet of pSmad1/5/8 positive epithelium (arrow in D4), in the VFE (D3) and the heart (ht) of the mutants, including myocardium and endocardium (D3, D4) compared to that in the WT embryos (C3, C4). Asterisks denote increased level of pSmad1/5/8 staining. Nt, neural tube; NCCs, neural crest cells; ov, optic vesicles; SE, surface ectoderm; BA1, brachial arches 1; VFE, ventral foregut endoderm; ht, heart.
Figure 4.7: Ectopic pSmad1/5/8 Distribution in Chrd$^{+/+}$;Nog$^{+/+}$ Embryos at around 9-Somite Stage
Caudally, the morphological phenotype of Chrd\textsuperscript{+/};Nog\textsuperscript{+/} embryos is similar to that of Nog\textsuperscript{+/}, with a kinked neural tube and somites at E8.5 (Stottmann et al., 2006). In Chrd\textsuperscript{+/};Nog\textsuperscript{+/} embryos, pSmad1/5/8 is significantly expanded at the tail bud in presomitic mesoderm and is increased in the somites (Figure 4.7B, arrow). The ectopic BMP activation in these tissues may be responsible for the sclerotome defects. Ectopic pSmad1/5/8 is also detected in the dorsal neural tube and the covering sheet of surface ectoderm (Figure 4.7D4, arrow).

In summary, this atlas of pSmad1/5/8 domains provides an overview of BMP signaling activation regions during early mouse embryogenesis. Even though there is a complex network of BMP ligands and antagonists, the endogenous intracellular activation provides a direct examination of the signaling activities that cannot be predicted by the network. It is interesting that in embryos lacking Chrd/Nog function pSmad1/5/8 is not detected ubiquitously in the gastrula but ectopically in the APS and the anterior endoderm. Later, pSmad1/5/8 is expanded in the dorsal neural tube. This expression pattern provides a spatiotemporal function of Chrd and Nog in inhibiting BMP signaling activation. It is very likely that BMP2 (rather than BMP4) may be the possible ligand causing ectopic pSmad1/5/8 in the APS in the mutants, which function is not previously appreciated. Asymmetric activation of BMP signaling in the LPM, in an opposite direction to Nodal signaling, in short period of time raises possibilities for BMP-Nodal interaction during L-R axis patterning. These findings in spatiotemporal distribution and magnitude (as well as the alteration) of BMP signaling activation in WT and mutant embryos highlights further insight into how BMP signaling is functioning, regulated and responding in vivo.
Chapter 5:

Conclusions—

Summary and Future Directions
Summary

This dissertation has investigated cellular and molecular mechanisms governing mammalian forebrain patterning. A combination of embryological manipulations and genetic techniques were employed to elucidate (1) the timing of and tissue requirement for forebrain initiation; (2) the roles of BMP antagonism in promoting forebrain cell fates; (3) the significance and mechanism of the BMP-Nodal signaling interaction during anterior primitive streak (APS) and axial mesendoderm (AME) specification; and (4) the spatiotemporal regulation of BMP signaling activation during early mouse gastrulation. The key points resulting from these investigations are summarized below.

BMP antagonism from exogenous tissues is required during early forebrain patterning events

Previous studies have shown that forebrain initiation occurs during gastrulation, prior to or around the time forebrain genes are expressed. However, the timing, tissues and identities of the molecular signals required for this process are poorly understood. The study herein has demonstrated that, as a result of tissue interactions, mouse forebrain ectoderm is specified to express Six3 and Hesx1 at 0B stage, several hours before the time at which they are expressed. During specification, the initial forebrain character is inhibited by BMP signals but promoted by Chrd and Nog. This activity of Chrd and Nog, which is similar to that of the anterior visceral endoderm (AVE), can inhibit posteriorizing signals in naïve ectoderm. This suggests that BMP antagonism in the AVE is a likely molecular mechanism for promoting forebrain establishment. In this way, the AVE may inhibit endogenous BMP signaling in the ectoderm in order to promote forebrain gene expression and inhibit a few posterior genes. After forebrain character is initiated, the forebrain ectoderm requires signals to maintain its character
until at least somite stage. Interestingly, \textit{Six3} and \textit{Hesx1} expressions have differential dependencies on tissue interaction, which is provided by the anterior mesoderm and endoderm. During maintenance, BMP not only inhibits forebrain gene expression, but also has posteriorizing activities (in a gene-dependent manner). The requirement for BMP antagonism from the AVE and AME during early forebrain patterning is confirmed by the regional elevation of BMP signaling in the presumptive forebrain ectoderm, primitive streak and visceral endoderm (VE) of \textit{Foxa2}\textsuperscript{−/−} embryos. These data provide new insights into the molecular basis for initiating and maintaining forebrain cell fate.

**Nodal signals and BMP antagonism cooperate during forebrain induction**

The graded Nodal signals in mouse epiblast govern the cell fate of the APS, which gives rise to the forebrain (Vincent et al., 2003). Independently, BMP antagonism by \textit{Chrd} and \textit{Nog} promotes the function of rostral organizing centers for forebrain development (Anderson et al., 2002). This current study has linked these two signaling pathways by genetic studies and provides further evidence, as well as the functional relevance, for their interaction. Two analogous mutation classes, \textit{Chrd}\textsuperscript{−/−};\textit{Nodal}\textsuperscript{+/−} and \textit{Nog}\textsuperscript{−/−};\textit{Smad3}\textsuperscript{+/−/−}, display an anterior malformation phenotype with considerable penetrance at mid-gestation, demonstrating the genetic interactions between \textit{Chrd} and \textit{Nodal} and between \textit{Nog} and \textit{Smad3}. These interactions take place in the APS and/or the AME, and failure of this interaction causes ectopic BMP signaling and decreased \textit{Nodal} expression.

It is likely that a balance between BMP and Nodal signaling must be maintained by the synergistic effects of Nodal signaling (via \textit{Nodal} and \textit{Smad3}) and BMP antagonism (via \textit{Chrd} and \textit{Nog}) in the APS and the AME. This idea is supported by our molecular studies showing that BMP and Nodal signaling are mutually antagonistic, and that their interaction possibly occurs extracellularly. Therefore, the synergistic interaction
between Nodal and anti-BMPs is likely aimed at building a buffer zone between BMP and Nodal signals. Disruption of this interaction results in inappropriate BMP-Nodal levels in the APS and its derivatives, leading to anterior defect such as holoprosencephaly (HPE). These results provide deeper understanding about the complexity of regulation which exists between two signaling pathways in tissues required for forebrain patterning. In addition, these results present a novel genetic model and molecular mechanism which support the hypothesis of digenic HPE pathogenesis, and which will be helpful for our future understanding of the genetic etiology of human HPE.

**Redundant BMP antagonists Chrd and Nog reveal distinct functions during early forebrain patterning**

Previous studies have shown that mouse Chrd and Nog have redundant functions and are required for patterning of all three axes (Bachiller et al., 2000). Intriguingly, even though their functions are redundant, Chrd and Nog expression are distinct during early gastrulation. Only Chrd is expressed in the APS, although its function in the APS is not clear. Four classes of mutations were generated to test the synergistic interaction between Nodal signals and BMP antagonism: Chrd<sup>−/−</sup>;Nodal<sup>+/+</sup>, Chrd<sup>−/−</sup>;Smad3<sup>−/−</sup>, Nog<sup>−/−</sup>;Nodal<sup>+/+</sup> and Nog<sup>−/−</sup>;Smad3<sup>+/−/−</sup>. Unexpectedly, only Chrd<sup>−/−</sup>;Nodal<sup>+/−</sup> and Nog<sup>−/−</sup>;Smad3<sup>+/−/−</sup> displayed phenotypes, which were similar morphologically (anterior malformation) but were different molecularly (in the APS and the AME respectively). It is apparent that Chrd and Nodal cooperate to pattern the APS; however, Nog does not possess this function. This observation suggests that one distinct function for Chrd in the APS is to synergize with Nodal signaling. Interestingly, Chrd and Nog are both expressed in the AME and are required redundantly there. However, it appears that only Nog interacts with Smad3 in the AME. This observation again suggests a
difference in their functions; however, the mechanism by which this is accomplished requires further investigation.

**Endogenous BMP signaling activity**

The distribution of the activated BMP signaling effectors pSmad1/5/8 is by far the most reliable spatiotemporal readout for BMP signaling activities during embryonic development. For example, ectopic pSmad1/5/8 is found in the primitive streak of Foxa2−/− embryos, although no known target genes have been found ectopically expressed (Yang and Klingensmith, 2006). This current study presents an atlas of the timing, location and distribution of pSmad1/5/8 during early mouse gastrulation and neurulation (E6.5-E8.5). The expression domain of pSmad1/5/8 cannot be simply predicted by the expression of Bmp genes. The primitive streak lacks pSmad1/5/8 activity, although Bmp2 and Bmp7 are expressed there. Ectopic pSmad1/5/8 is found in the primitive streak and anterior endoderm of Chrd−/−;Nog−/− embryos, suggesting their function in the surrounding tissues during gastrulation (which has not been fully studied yet). Since Bmp2 and Bmp7 are expressed in these tissues, it is likely that BMP2 and/or BMP7 are the ligands which cause this ectopic signaling activation. In addition, pSmad1/5/8 is found in several important tissues, including the foregut endoderm, some sub-domains of the heart, the outflow tract, neural crest cells, and in the left lateral plate mesoderm (LPM) asymmetrically. Overall, our study presents an overview of BMP signaling activation, providing insights into the regulation of BMP signaling in vivo and pinpointing regions where downstream target genes might be sought.
Future Directions

Function of Chrd and Nog in extraembryonic tissues

The function of Chrd and Nog in extraembryonic tissues has not yet been assessed, partially because neither is expressed in the AVE (as assayed by WMISH). However, our study shows that BMP antagonism from the AVE is required for forebrain initiation (Chapter 2). Since Chrd/Nog protein can directly trigger forebrain gene expression in the naïve ectoderm, which is similar to the function of the AVE, it is possible that they promote forebrain initiation via the adjacent AVE. In addition, Nog expression is detected in a discrete cluster of cells in the parietal endoderm, which is the external layer of extraembryonic tissue adjacent to the VE. These observations suggest that at least Nog may function via extraembryonic tissues influencing the AVE. Detailed analysis of Chrd and Nog expression by WMISH, or qPCR of microdissected extraembryonic tissue, will be helpful in determining their expression in extraembryonic tissues. Functional analyses (by generating tetraploid chimeric embryos comprised of WT epiblasts and mutant extraembryonic tissues) will help to elucidate the functions of Chrd and Nog in extraembryonic tissues.

Roles of Foxa2 and BMP antagonism in axis rotation

A key and early step of axis rotation is the rotation of the distal visceral endoderm (DVE) to the anterior epiblast and becomes the AVE. Currently it was understood as a process involves inhibition of involves inhibition of Nodal signaling (via Cerl and Lefty1) and of Wnt signaling (via mDkk1) in the AVE (Yamamoto et al., 2004; Kimura-Yoshida et al., 2005). It is therefore likely that Nodal and Wnt, normally found in the posterior-proximal epiblast, act as repulsive signals for the DVE migration. Interestingly, in Foxa2−/− embryos, the rotation of DVE cells is failed. These cells retain
some of the molecular function, such as expression of Cerl and Lefty1. However, they possess ectopic BMP signaling activation showing by pSmad1/5/8 expression. An interesting question raised here is whether BMP is also acting as a repulsive signal for the DVE migration. This question can be tested in embryo culture to examine the effect of BMP2 (using protein-coated beads) on the DVE rotation. Alternatively, whether Chrd/Nog in the VE cells will derive the direction of DVE rotation can be tested by ectopic Chrd/Nog expression in the VE cells via liposome transfection (as set up in Yamamoto et al., 2004). In addition, while Foxa2 plays a crucial role in AVE specification, its relationship with BMP signaling is not clear. Transcriptional regulation of Foxa2 by BMPs and antagonists can be assessed in explant or cell culture systems to further address this question.

The molecular mechanisms of BMP-Nodal interaction

One very interesting but also very difficult issue is raised by this study about our understanding of the molecular mechanism(s) underlying antagonistic BMP-Nodal interaction during forebrain patterning. This study raises the possibility that BMP and Nodal interaction occurs extracellularly. One plausible way to account for our observations, without conflicting with their mutually antagonistic character, can be found in the formation of BMP and Nodal molecular complex which fails to signal. For example, they may form heteromeric complexes. It was demonstrated that Xenopus BMP7 proprotein is able to heterodimerize with mouse Nodal proprotein when they are co-expressed (Yeo and Whitman, 2001). However, it is still unclear: (1) whether mouse BMPs and Nodal actually form protein complexes; (2) whether they do so as proproteins or mature proteins; (3) if they form complexes only when co-expressed in the same cells or instead when they are secreted into the extracellular space; and (4) whether their binding is dependent on the pro-domain of the proprotein or instead only
dependent on the mature protein region. These possibilities are currently under investigation utilizing co-immunoprecipitation (co-IP) of tagged BMP2 and Nodal proteins in COS cells and HEK293 cells. Two versions of these tagged proteins were prepared: one which only allows detection of the proprotein form and one which can be detected in either form (Figure 5.1A).

Two scenarios are possible regarding the potential BMP-Nodal heteromeric complex formation. First, BMP and Nodal proprotein may heterodimerize within the cell (perhaps via formation of disulfide bonds in the endoplasmic reticulum), followed by secretion of the BMP-Nodal proprotein heterodimers into the extracellular space where key proprotein convertases are located. Indeed, results from co-IP suggest that BMP-Nodal proprotein can heterodimerize within cells when they are co-expressed (Figure 5.1B). This data is consistent with the previous finding in Xenopus showing the similar heterodimerization (Yeo and Whitman, 2001). Alternatively, BMP and Nodal may form a heteromeric complex extracellularly, though not necessarily heterodimeric complexes. Preliminary data suggest that BMP and Nodal proproteins do form complexes extracellularly. Two groups of cells were independently transfected with Nodal-Flag or BMP-Myc, and then plated together at 24 hr post-transfection. Tagged BMP and Nodal proproteins were immunoprecipitated together in the conditioned medium (Figure 5.1C), suggesting that they can form complexes in the extracellular space. Whether mature BMP and Nodal proteins can form complexes extracellularly, which suggests the prodomain is not necessary for this interaction, requires further investigation.

Previous studies suggest that that both BMP4 and Nodal are secreted and travel in the extracellular space in the form of proproteins, which are more stable than the mature proteins (Constam and Robertson, 1999). Proproteins are more stable, which increases their signaling range by extending paracrine signaling (Le Good et al., 2005), but they also can initiate signaling activities via the same receptor complex.
Figure 5.1: BMP and Nodal Can Form Heteromeric Complexes Intracellularly and Extracellularly. (A) A diagram showing the protein tagging strategy. A Flag or a Myc epitope (green) is inserted in the pro-domain region (purple) to tag the proprotein, or in the mature domain (magenta) to tag both the mature protein and proprotein. (B) Nodal-Flag proprotein forms a heteromeric complex with BMP2-Myc proprotein within the cells. BMP2-Myc and Nodal-Flag were co-expressed in COS cells, which were then used for co-IP. Proproteins were immunoprecipitated by anti-Myc antibody and analyzed by anti-Flag immunoblotting, or vice versa. (C) Nodal-Flag and BMP2-Myc can form heteromeric complexes extracellularly. Nodal-Flag or BMP2-Myc constructs were independently transfected in two groups of COS cells. These two groups were plated together 24 hr post-transfection. Extracellular Nodal-Flag and BMP2-Myc proteins were collected from the conditioned medium and were immunoprecipitated by anti-Myc antibody and analyzed by anti-Flag immunoblotting. IP, immunoprecipitate; IB, immunoblotting; CM, conditioned medium.
Figure 5.1: BMP and Nodal Can From Heteromeric Complexes Intracellularly and Extracellularly
Therefore, in addition to the mature ligand, the extracellular proprotein is an obviously a likely determinant in the spatiotemporal activity of both BMP and Nodal signaling. Even though BMP-Nodal proproteins may form heteromeric complexes extracellularly, it is still unclear which domains are required for this interaction. The fact that this proprotein complex can form extracellularly suggests that this process may not necessarily depend on disulfide bond formation between the mature protein domains. Whether or not this type of BMP-Nodal interaction occurs in a pro-domain-dependent manner should be examined using truncated BMP and Nodal proteins with various domains.

The most important question here is whether the BMP-Nodal complex occurs in vivo. Although suitable antibodies are not available, co-IP of endogenous BMP and Nodal protein from the APS of mouse embryos would help clarify this question. So far, the evidence suggests it is likely that BMP and Nodal can interact extracellularly (based on their expression in mouse embryos). During early gastrulation (E6.5-E6.75), *Nodal* expression is detected in the APS. *Bmp2* and *Bmp7* expression are detected in the posterior-distal part of the epiblast, which appear to be in the same vicinity as *Nodal* expression (though they may not be necessarily overlap). *Nodal* expression does not overlap much with *Bmp4* (Figure 5.2). Nonetheless, since the proteins from these mRNA are able to travel into broader domains, it is likely that Nodal does encounter BMP2 and BMP7 extracellularly. Whether they are truly co-expressed would require close examination by fluorescent in situ with multiple probes.

Together, these data provide compelling support for the understanding the BMP-Nodal interaction gained from Chapter 3. However, it is unclear whether BMP and Nodal signaling interact via a competition for the Co-Smad Smad4, which is shared between these pathways. This hypothesis could be tested by studying the phenotype of compound mutations *Chrd;Smad4* or *Nog;Smad4*. 

Figure 5.2: Comparison of the Spatiotemporal Expression of Nodal and Bmp During Early Gastrulation. (A) At E6.5, Nodal is expressed in the primitive streak, including the APS (bracket, A1). Bmp2 is also detected in the APS region (bracket, A2), but Bmp4 is only detected in the extraembryonic region. (B) At E6.75, Nodal expression is found in the extending streak (B1). At the same stage, Bmp2 and Bmp7 are expressed in the APS (B2, B3), where Nodal expression is also detected (arrows). The arrowheads denote the junction between embryonic and extraembryonic tissues.
Figure 5.2: Comparison of the Spatiotemporal Expression of Nodal and Bmp During Early Gastrulation
**BMP-Nodal interaction in other developmental contexts**

Synergistic interaction of Nodal and anti-BMP signals happens not only during forebrain development. One possible role for their interaction is during Left-Right (L-R) axis establishment since both Nodal and BMP antagonism are involved in L-R patterning. Firstly, Nodal signaling functions during determination of the L-R axis, and \( Chrd^{\pm};Nog^{\pm} \) mutant embryos possess a L-R axial phenotype. This suggests that BMP antagonism in the midline is crucial for L-R determination (Bachiller et al., 2000).

Secondly, in \( Chrd^{\pm};Nodal^{\pm} \) and \( Nog^{\pm};Smad3^{\pm} \) mutants, abnormal heart tube looping, reversed or straight heart tubes, are often detected. Thirdly (and surprisingly), endogenous pSmad1/5/8 is found asymmetrically along the L-R axis, with stronger expression in the right LPM, but only at the time when Nodal is expressed asymmetrically in the left LPM (Chapter 4). Although normally Nodal expression is only detected in the left LPM, the fact that in some mutant embryos (e.g. \( Smad1;Smad5 \) compound mutants) Nodal is expressed in both side of the LPM supports the antagonistic BMP-Nodal interaction in the LPM. Altogether, these data suggest the possibility that Nodal inhibits BMP in the left LPM while Nodal is inhibited by BMPs in the right LPM. In addition this process may also involve the asymmetric expression of Nog. Analysis of compound mutants by morphology and molecular markers, along with ectopic BMP/Nodal expression in the LPM will further elucidate the underlying mechanisms (currently under investigation, collaboration with T.J.S. and N.M.).

**BMP-Nodal interaction genes in the genetic etiology of human HPE**

The data from our study of BMP-Nodal interaction supports the digenic model for HPE pathogenesis. One obvious question which arises is whether \( Chrd, Nog \) and \( Smad3 \) mutations are involved in the genetic etiology of human HPE. In collaboration
with human genetics researchers, genomic libraries derived from HPE patients could be assessed for mutations in these candidate genes from Nodal or BMP signaling pathways.
References


Biography

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Publications:


Yang Y-P, Anderson RM, Klingensmith J. BMP antagonism and Nodal signaling act synergistically in mammalian rostral patterning. \textit{(Manuscript in prep)}

Yang Y-P, Anderson RM, Klingensmith J. Spatiotemporal regulation of BMP signaling activation during early mouse embryogenesis. \textit{(Manuscript in prep)}