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# Smad2 Signaling in Extraembryonic Tissues Determines Anterior-Posterior Polarity of the Early Mouse Embryo

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## Summary

**Smad proteins transmit TGFβ signals from the cell surface to the nucleus. Here we analyze Smad2 mutant embryos created using ES cell technology. Smad2 function is not required for mesoderm production per se, but, rather unexpectedly, in the absence of Smad2 the entire epiblast adopts a mesodermal fate giving rise to a normal yolk sac and fetal blood cells. In contrast, Smad2 mutants entirely lack tissues of the embryonic germ layers. Smad2 signals serve to restrict the site of primitive streak formation and establish anterior-posterior identity within the epiblast. Chimera experiments demonstrate these essential activities are contributed by the extraembryonic tissues. Thus, the extraembryonic tissues play critical roles in establishing the body plan during early mouse development.**

## Introduction

Pattern within the embryo is established by a developmentally regulated program of molecular and cellular compartmentalization. In lower vertebrates, molecules that specify axis determination are maternally supplied and locally sequestered within the egg cytoplasm (Gurdon, 1992). Subsequent cell cleavages divide the embryo into distinct regions that interact during establishment of the dorso-ventral axis. The key roles of growth factors, such as TGFβ, FGF, and Wnt family members and their specific antagonists, have been extensively characterized using *Xenopus* gain-of-function assays (Kessler and Melton, 1994; Heasman, 1997). By contrast, mechanisms responsible for patterning the early mammalian embryo are poorly understood. Moreover, a critical aspect of mammalian development is the formation of the trophectoderm and primitive endoderm cell lineages necessary for supporting growth of the embryo within the intrauterine environment. Their contributions during the formation of highly specialized extraembryonic structures such as the placenta and yolk sac are well described (Rossant, 1995). However, the molecular basis of their specification and their potential signaling activities remain to be determined. The overt anterior-posterior (A-P) axis of the embryo only becomes evident at the onset of gastrulation, coincident with the appearance of the primitive streak marking the site of mesoderm formation and thus the most posterior aspect of the embryo. Recent observations suggest that both the epiblast and overlying primitive endoderm acquire a molecular asymmetry many hours before primitive streak formation (Tam and Behringer, 1997; Thomas et al., 1998). However, nothing is known about signal(s) responsible for controlling these early patterning events.

Over recent years, many of the morphogens identified in *Xenopus* assays have been evaluated for their possible contributions to early mammalian development. Rather than reinforcing the notion that mechanisms of axis formation are conserved, for the most part these experiments have underscored the differences in the genetic programs between higher and lower vertebrates. Thus, for example, in contrast to the potent mesoderm inducers such as activin and Vg1, none of the mammalian TGFβ family members expressed during gastrulation appear responsible for mesoderm specification per se. However, studies of loss-of-function mutations have shown that individual TGFβ family members regulate different aspects of embryonic growth and tissue morphogenesis. For example, mouse embryos lacking Bmp4 activity generally fail to develop beyond the egg cylinder stage, but some mutants were recovered at the head fold stage and appear developmentally retarded, having disorganized posterior structures and a reduction in extraembryonic mesoderm (Winnier et al., 1995). On the other hand, Bmp2 mutants typically display defective heart morphogenesis and amniotic fold closure (Zhang and Bradley, 1996). Another TGFβ family member nodal is required for primitive streak formation and anterior patterning during gastrulation (Conlon et al., 1991, 1994; Zhou et al., 1993; Varlet et al., 1997). These previous experiments provide a partial view of TGFβ pathways controlling cell fate specification and mesodermal patterning in mammalian embryos.

Recent studies have described a family of intracellular effector molecules, the so-called Smad proteins, acting downstream of TGFβ receptors (Baker and Harland, 1997; Heldin et al., 1997; Massague et al., 1997). Upon ligand-induced activation at the cell surface, TGFβ/activin or BMP receptor kinases specifically phosphorylate either Smad2 or Smad1, respectively (Hoodless et al., 1996; Marcias-Silva et al., 1996; Kretzschmar et al., 1997). Consequently, these activated Smads form heteromeric complexes with Smad4, are transported to the nucleus, and gain the ability to function as transcriptional regulators via their interactions with DNA-binding proteins such as FAST-1 (Chen et al., 1996, 1997). Thus, TGFβ/activin or BMP receptors specifically activate Smad2 or Smad1, while their partner Smad4 is common to both signaling pathways (Baker and Harland, 1997; Heldin et al., 1997; Massague et al., 1997). The evidence to date suggesting Smad proteins influence cell fate decisions comes entirely from gain-of-function experiments testing their activities in *Xenopus* explant assays (Baker and Harland 1996; Graff et al., 1996; Thomsen, 1996). Nothing is known about possible functional roles

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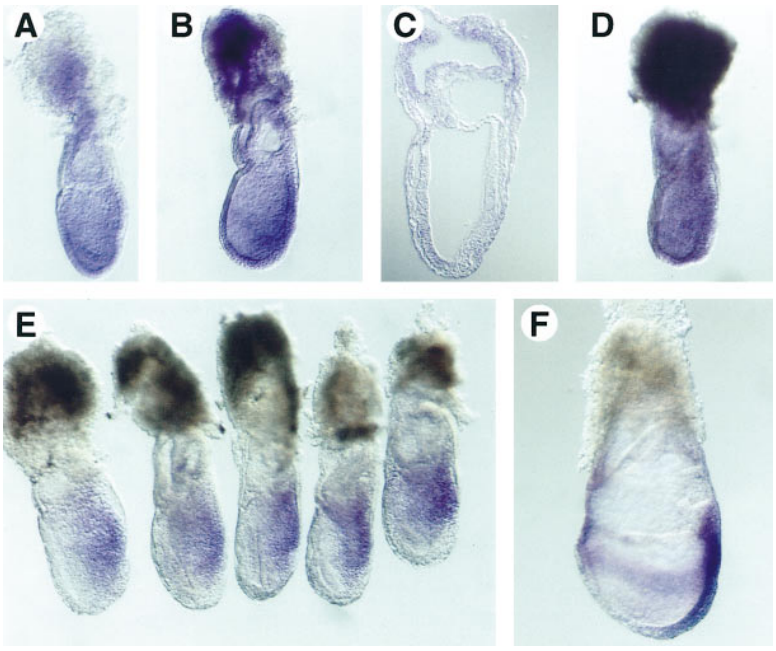


Figure 1. Distinct Smad1 and Smad2 Expression Patterns in Early Mouse Embryos

*Smad2* mRNA is ubiquitously expressed at pregastrulation (A) and late-streak (B) stages. (C) Sections through an amniotic fold stage embryo confirm that the signal is distributed throughout the embryonic and extraembryonic tissues. (D) *Smad4* mRNA is widely expressed in mid-streak stage embryos. (E) *Smad1* expression is initially detected at gastrulation in mesoderm cells migrating from the primitive streak. (F) At early head fold stages, *Smad1* is most strongly expressed in the primitive streak, lateral mesoderm, and the most anterior, precardiac mesoderm. All embryos are viewed laterally with anterior placed to the left.

contributed by these cytoplasmic effectors during early mammalian development. Moreover, recent reports describe additional Smad family members including Smad5 and Smad3, which are remarkably similar to Smad1 and Smad2, respectively (Liu et al., 1997; Nakao et al., 1997a; Suzuki et al., 1997). Inhibitory Smad's such as Smad6 and Smad7 have been shown to function as antagonists of TGFβ signaling (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997b). Thus, a high degree of fine tuning created by the specific molecular associations amongst diverse TGFβ receptor complexes and Smad family members serves to regulate their signaling properties.

To learn more about the molecular signals that control patterning in the early mammalian embryo, we generated a loss-of-function mutation of the *Smad2* gene, which functions as an essential mediator of TGFβ and activin signaling. The present experiments demonstrate Smad2 signals are not essential for mesoderm induction per se. Rather, the Smad2 pathway determines anterior-posterior identity both within the epiblast and in the overlying visceral endoderm. Remarkably, in the absence of Smad2 the epiblast exclusively forms extraembryonic mesoderm and fails to give rise to the three primary germ layers, namely ectoderm, mesoderm, and the definitive endoderm of the embryo. Moreover, chimera experiments described in this report demonstrate that Smad2 signals responsible for early A-P axis formation are contributed by the extraembryonic tissues. These findings thus reveal a distinct feature of early mammalian development, namely that initial anterior-posterior identity is provided by a TGFβ-dependent signal originating in the extraembryonic cells.

## Results

### *Smad1*, *-2*, and *-4* Expression Patterns in Early Postimplantation Stage Mouse Embryos

At late gastrulation stages, mouse embryos broadly express *Smad2* transcripts (Baker and Harland, 1996). To learn more about possibly distinct expression patterns of individual Smad family members, we carried out whole-mount in situ hybridization experiments. *Smad2* and *Smad4* were both weakly expressed throughout the embryo at day 6.0, the earliest time point analyzed (Figure 1). Following the onset of gastrulation, as expected, *Smad2* transcripts were uniformly expressed throughout all tissue layers (Figure 1C). In contrast, *Smad1* mRNA was not detectable prior to gastrulation. Interestingly, *Smad1* expression initially appears in the mesodermal cell populations emerging on both sides of the primitive streak (Figure 1E). One day later, at the early head fold stage, *Smad1* is expressed at highest levels in the primitive streak, the lateral mesoderm, and the most anterior precardiac mesoderm (Figure 1F). *Smad1*, *-2*, and *-4* are expressed throughout all the tissues of the embryo at later stages (day 8.5, 6–8 somites).

### Loss of Smad2 Activity Results in Early Embryonic Lethality

To investigate whether Smad2 signaling is essential for early mammalian development, we created a loss-of-function mutation via homologous recombination in ES cells. A positive/negative targeting vector containing 6 kb of 5′ and 5 kb of 3′ genomic homology was designed to replace the first coding exon with the MC1*neo* cassette (Figure 2). Six out of eight targeted ES clones gave rise to male chimeras that transmitted the mutation to their offspring. Smad2$^{+/-}$ heterozygotes were phenotypically normal, but we failed to detect any live-born homozygous progeny from intercross matings (>300 offspring screened). To examine the timing of developmental arrest, embryos from Smad2$^{+/-}$ intercross matings were genotyped by PCR. At 8.5 and 9.5 days of development, we recovered Mendelian ratios of homozygous embryos. However, Smad2 embryos are resorbed shortly thereafter, since we failed to identify any homozygous mutant embryos at day 12.5 pc. The homozygous mutant embryos derived from all six ES cell

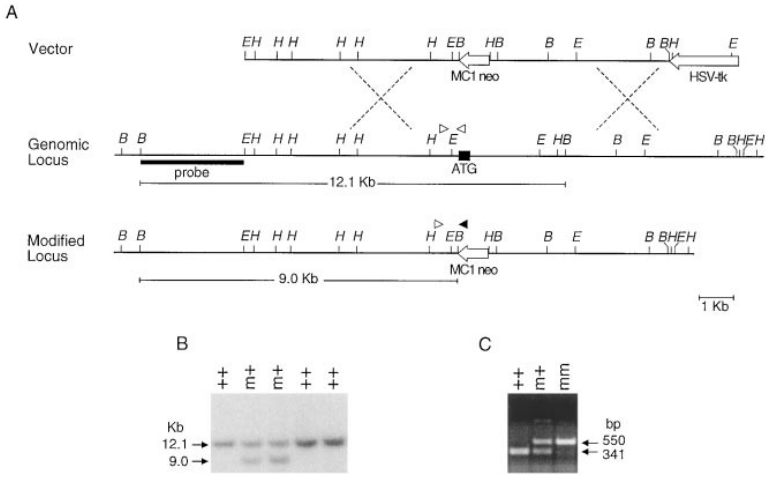


Figure 2. Generation of a Null Allele at the Smad2 Locus

(A) Schematic representation of the wild-type and mutant alleles and the targeting vector. E, EcoRI; H, HinDIII; B, BamHI.

(B) Southern blot analysis of Smad2$^{+/-}$ intercross progeny. Genomic DNA digested with BamHI was hybridized with the 5′ flanking probe. Positions of the 12.1 kb wild-type and 9.0 kb mutant fragments are indicated.

(C) PCR genotyping of 8.5 dpc embryos from heterozygous intercrosses. The three primers used amplify a 341 bp fragment specific for wild type and a 550 fragment derived from the mutant allele.

clones exhibited identical tissue disturbances described below, irrespective of whether the mutation was analyzed on an inbred or outbred background. This allele of Smad2 has been designated Smad2$^{Robm1}$.

At 8.5 days of development, as shown in Figure 3, the extraembryonic tissues including the ectoplacental cone, Reichert's membrane, and visceral yolk sac (VYS) appear to develop normally in homozygous Smad2 mutants. In striking contrast, any structures resembling an embryo proper are completely absent (Figure 3A). The VYS is initially formed during gastrulation when the extraembryonic mesoderm migrates around the exocoelom and comes to underlie the visceral endoderm, a derivative of the primitive endoderm. The visceral endoderm initially covers both the extraembryonic and embryonic ectoderm, but during gastrulation endoderm overlying the epiblast is displaced proximally by definitive endoderm emerging from the midline (Lawson et al., 1987; Thomas and Beddington, 1996). Thickened regions of the yolk sac mesoderm termed blood islands

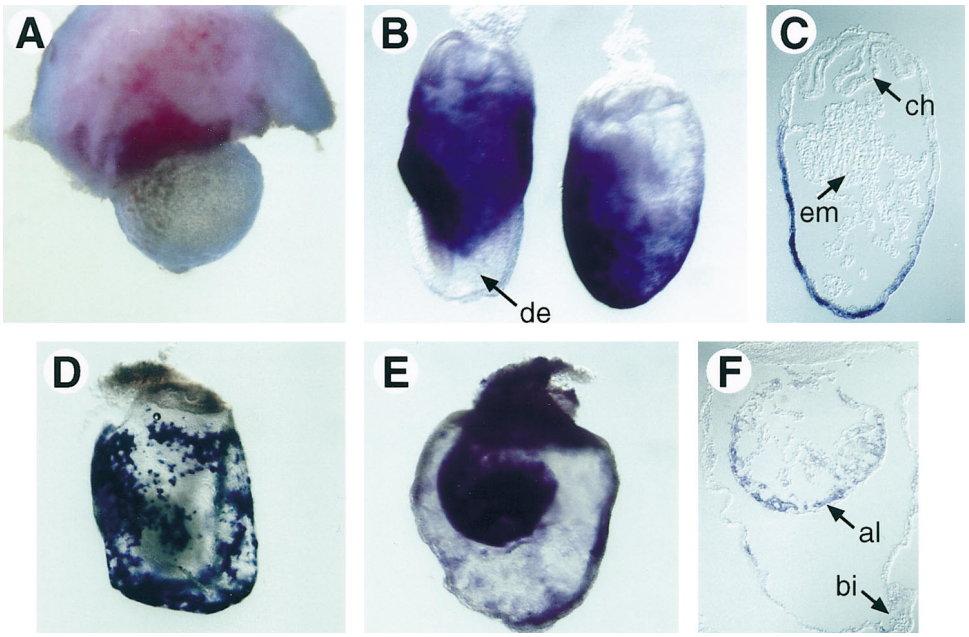


Figure 3. Normal Development of Extraembryonic Tissues in Smad2 Mutant Embryos

(A) A Smad2-deficient embryo at 8.5 dpc. Reichert's membrane has been removed. Despite the normal appearance of the VYS, no embryo proper is present. (B) Whole-mount in situ hybridization analysis examining AFP expression in wild-type (left) and Smad2-deficient (right) embryos at head fold stages. AFP expression is normally confined to the visceral endoderm layer of the VYS and absent from the definitive endoderm overlying the distal embryonic region (lateral view, anterior to left). In contrast, AFP is strongly expressed in distal regions of Smad2-deficient embryos. Sections taken from this mutant embryo (C) show signal localized to the outer layer of the VYS. The embryo appears to lack an embryonic component. Cells of a loose mesenchymal appearance and a morphologically normal chorion are present. (D) Expression of epsilon fetal β-globin localized to the blood islands in Smad2-deficient embryos. (E) *Flk1* expression restricted to the endothelial component of the VYS. Section taken from this Smad2 mutant (F) showing a strong signal localized to mesodermal cells resembling normal allantois tissue. de, definitive endoderm; ch, chorion; em, extraembryonic mesoderm; al, allantois-like tissue; bi, blood island.

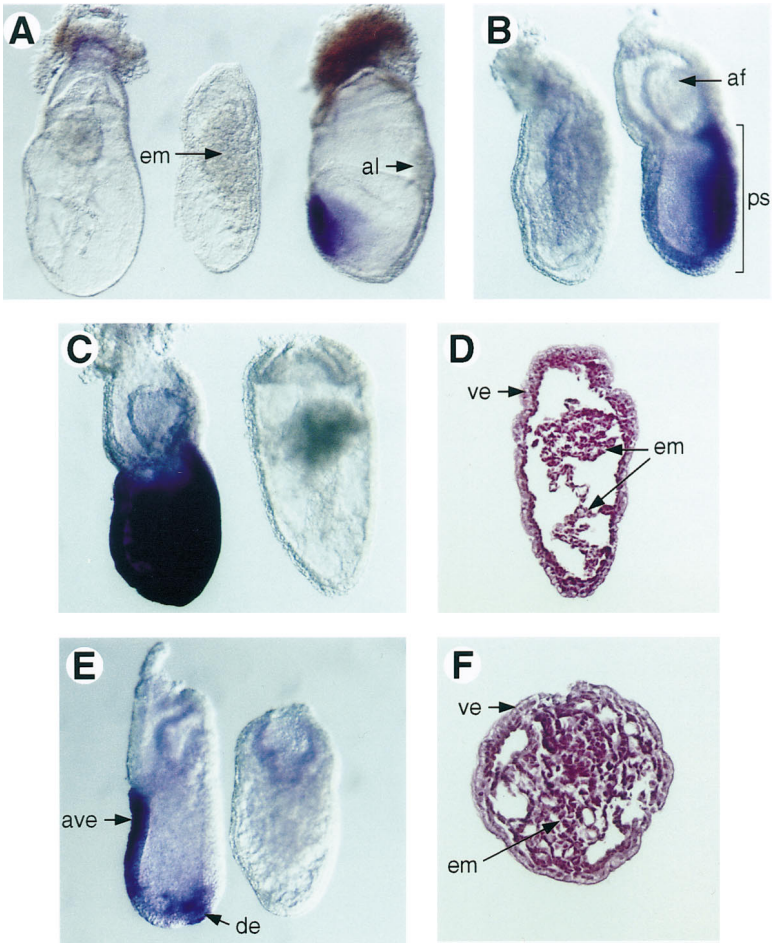


Figure 4. Absence of Epiblast Derivatives at Late-Streak to Early Head Fold Stages

(A) *Otx2* expression normally restricted to the anterior regions at head fold stages (right) is absent in Smad2 mutants (center and left). (B) *Fgf8* mRNA abundantly expressed in the posterior epiblast and nascent mesoderm at the late-streak stages (right) is absent in a Smad2 mutant littermate (left). (C) *Lim1* normally expressed in embryonic mesodermal populations at the late-streak stage (left) is not detectable in Smad2 mutants at similar developmental ages (right). (E) *Cer-l* expression (left), which normally marks the anterior visceral endoderm and cells of the definitive endoderm emigrating from the mid-line, is absent in a Smad2 mutant (right). (D and F) Sagittal and transverse sections of the Smad2 mutants shown in (C) and (E), respectively, show the lack of an obvious primitive streak. Neither embryo contains ectodermal-like cells nor has an amnion formed, but the internal population is comprised exclusively of a loose mesenchymal cell type. ve, visceral endoderm; em, extraembryonic mesoderm-like cells; de, definitive endoderm; ave, anterior visceral endoderm; af, amniotic fold.

give rise to fetal blood cells. To further describe the extraembryonic derivatives in Smad2-deficient embryos, we assessed expression of a panel of molecular markers (Figure 3). As expected at the head fold stage, wild-type embryos strongly express AFP localized to the VYS endoderm (Figure 3B). Without exception, Smad2 mutant embryos at day 8 of development show ectopic expression of AFP in VYS endoderm surrounding the distal portion of the embryo (Figures 3B and 3C). As judged by morphological criteria, the yolk sac blood vessels appear to form normally. As a marker for hematopoiesis, we tested for expression of the epsilon form of fetal β-globin. Whole-mount analysis confirmed abundant fetal erythroid cells localized to blood islands in Smad2-deficient embryos (Figure 3D). We also examined expression of *Flk1*, a receptor tyrosine kinase selectively expressed in the endothelial cells of the blood islands (Yamaguchi et al., 1993) and known to be required for embryonic vasculogenesis (Shalaby et al., 1997). As shown in Figure 3E, Smad2 mutant embryos showed normal patterns of *Flk1* expression. The allantois, a derivative of the extraembryonic mesoderm, normally gives rise to umbilical blood vessels that connect the fetus to the chorio-allantoic placenta. Interestingly, we also observed balled masses of *Flk1*-positive cells bearing a strong morphological resemblance to allantoic tissue (Figures 3E and 3F). Thus, Smad2 function is not required for the formation of extraembryonic tissues at early postimplantation stages of mouse development.

### Selective Induction of Extraembryonic Mesoderm in Smad2 Mutant Embryos

Cell marking and grafting experiments demonstrate that all three germ layers, namely ectoderm, mesoderm, and definitive endoderm, are derived from the epiblast during the process of gastrulation (Hogan et al., 1994). Since development of the embryo proper appears severely compromised by the loss of Smad2 activities, it was important to examine the specific tissue disturbances underlying this profound defect. We assessed germ layer formation and patterning by examining expression of a panel of genes showing regionalized expression in the embryo during late-streak to early head fold stages. As markers for definitive ectoderm, we analyzed expression of *Otx2* and *Hesx1*. *Otx2*, a homeobox-containing transcription factor, is initially expressed prior to gastrulation throughout the epiblast and gradually becomes restricted to the anterior third of the embryo by the head fold stage (Ang et al., 1994). *Hesx1* is expressed in the anterior visceral endoderm beginning at pregastrulation stages onward and in the rostral neurectoderm, eventually becoming confined to the prosencephalic neurectoderm and rostral foregut by late-streak stages (Hermesz et al., 1996; Thomas and Beddington, 1996). *Fgf8*

is specifically expressed in the posterior region of the epiblast prior to streak formation (Crossley and Martin, 1995). During gastrulation, *Fgf8* marks the posterior ectoderm cells just prior to their ingression into the primitive streak. The transcription factor *Lim1*, strongly expressed in mesoderm, is confined to the embryonic region from early streak stages onward (Barnes et al., 1994; Shawlot and Behringer, 1995). At early allantoic bud stages, *Lim1* is also expressed in the cells of the node, the mouse organizer tissue. *Cerberus-like* (*cer-l*), a homolog of the secreted molecule Cerberus implicated in head formation in *Xenopus* (Bouwmeester et al., 1996), provides a convenient marker for anterior visceral endoderm and definitive embryonic endoderm (Belo et al., 1997; Thomas et al., 1997). Remarkably, we found no evidence for expression of any of these marker genes in Smad2 mutant embryos (Figure 4). The absence of node tissue and axial mesoderm derivatives was confirmed using a probe for *HNF3*β (data not shown). Thus, at the equivalent of mid- to late-streak stages, none of the normal descendent tissues of the anterior or distal regions of the epiblast are present in Smad2 mutants.

On the other hand, histological data (Figures 4D and 4F) suggested that tissues comprised of a loose mesenchymal cell type, morphologically reminiscent of mesoderm, occupy the embryonic region. Moreover, we observe extraembryonic mesoderm derivatives including fetal blood cells at day 8.5 pc. Fate maps have shown that different mesodermal derivatives arise from different positions along the primitive streak (Hogan et al., 1994). During the first day of gastrulation, extraembryonic mesoderm is produced from the posterior end of the streak. To examine mesoderm induction and patterning in Smad2 mutants, we assessed expression of Brachyury (T) at day 7.5 of development. T is transiently expressed by nascent mesoderm in the primitive streak throughout gastrulation, and as mesoderm differentiates to form embryonic and extraembryonic derivatives, T expression is down-regulated (Wilkinson et al., 1990). None of the mutant embryos (>20 examined) contain T-expressing cells at late streak through to the equivalent of head fold stage (Figure 5B), allowing us to conclude that production of mesoderm has ceased in these embryos. Next we examined *Bmp4* expression patterns. As expected, *Bmp4* is strongly expressed in both posterior and extraembryonic mesoderm arising from the proximal epiblast (Winnier et al., 1995; Figures 5C and 5D). Interestingly, in both wild-type and Smad2 mutant embryos, we also found abundant *Bmp4* transcripts localized to the chorion (Figures 5C–5E). In many mutant embryos the chorion appears highly folded and misplaced, being situated laterally and distally, in contrast to the strictly proximal position occupied by this tissue in normal embryos (Figure 5E). However, at slightly later stages the chorion assumes a more normal location due to expansion of the VYS (Figure 5D). The loosely packed mesodermal population attached to the surface of the visceral endoderm also weakly expresses *Bmp4*. In normal embryos the extraembryonic ectoderm actively inhibits expression of AFP, thus restricting its expression to visceral endoderm contacting the extraembryonic mesoderm (Dziadek, 1978). Interestingly, in Smad2 mutant embryos we similarly observe AFP expression only adjacent to this *Bmp4*-positive mesodermal cell population (Figure 5F). Thus, extraembryonic tissue interactions appear largely unperturbed in Smad2-deficient embryos.

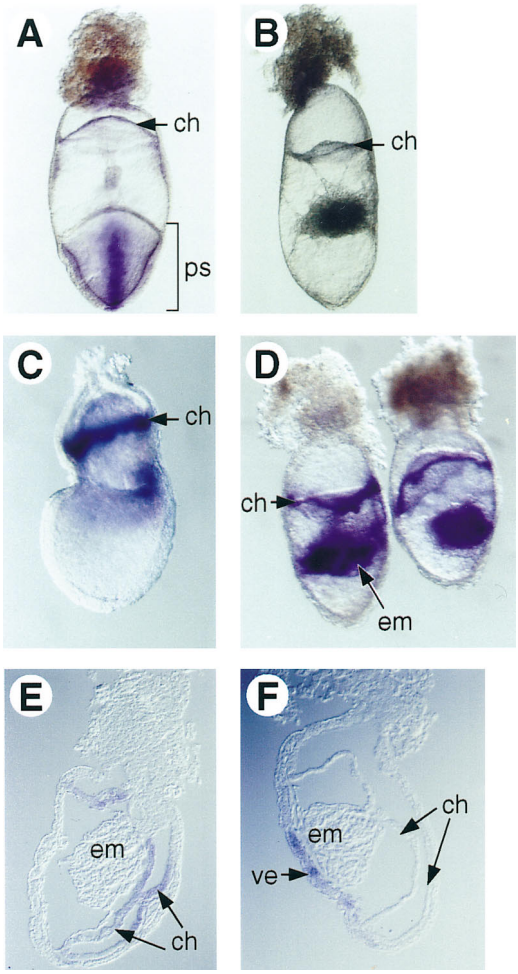


Figure 5. Selective Development of Mesodermal Derivatives at Late-Streak to Early Head Fold Stages

(A) T expression normally marks nascent mesoderm forming in the primitive streak (posterior view). No T-positive cells are detected in Smad2 mutant embryos at similar developmental stages (B). The apparent signal in the balled mass of cells in the embryo shown is due to tissue thickness. Sectioning of this and other mutant embryos failed to identify T expressing cells. (C) *Bmp4* expression localized to posterior embryonic mesoderm, extra embryonic mesoderm, allantoic bud, and the periphery of the chorion at early head fold stages. (D) Smad2 mutants at the equivalent stage express *Bmp4* in the chorion and more distal mesodermal cells. (E) Sagittal section through a Smad2 mutant shows *Bmp4* expression localized to chorion tissue. (F) Mid-sagittal section through a Smad2 mutant embryo at the equivalent of late-streak stages shows the onset of AFP expression confined to visceral endoderm overlying extraembryonic mesoderm. ch, chorion; ps, primitive streak; em, extraembryonic mesoderm; ve, visceral endoderm.

### Tissue Disturbances during Gastrulation and Streak Formation

Results above strongly suggest that Smad2 mutants lack definitive tissues of the embryo, but extraembryonic components derived from the epiblast appear relatively

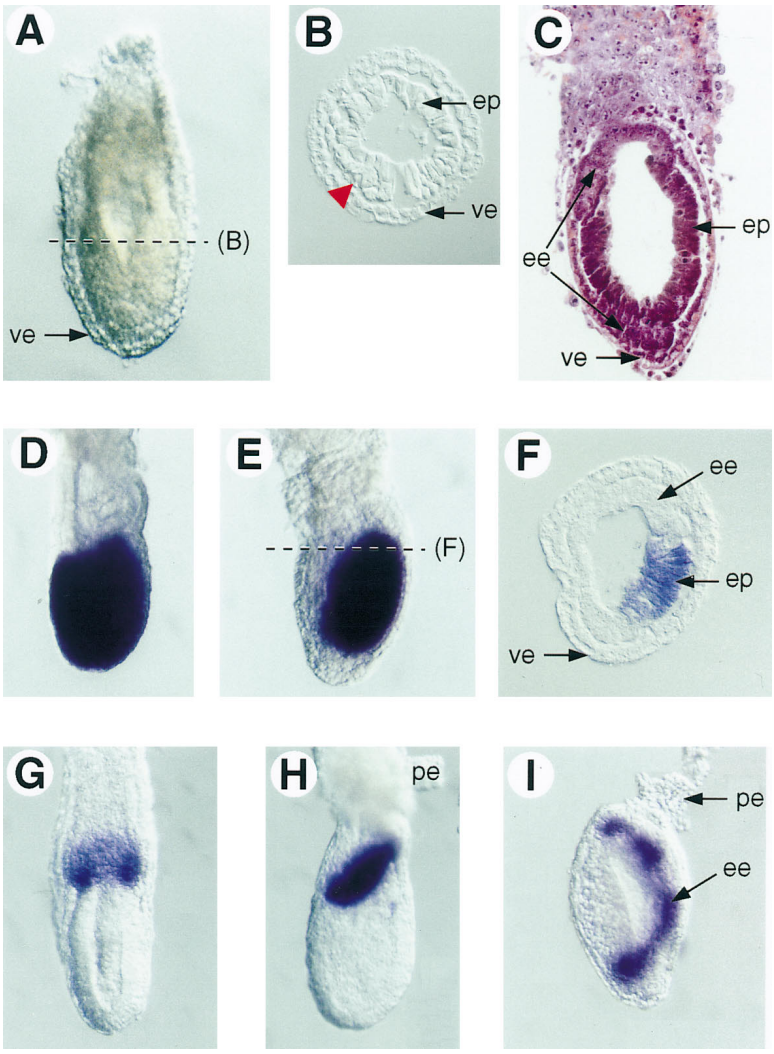


Figure 6. Loss of Normal Tissue Boundaries and Gene Expression Domains at Gastrulation

At the equivalent of early streak stages (A), no primitive streak is evident in Smad2 mutant embryos. The proamniotic cavity has been lost in the distal region, and the visceral endoderm appears thickened over the entire surface of the epiblast. In transverse section (B), the mutant retains a near perfect radial symmetry but shows columnar cells of the epiblast mixed with loose mesodermal-like cells (red arrow). (C) Sagittal section of a second representative Smad2 mutant embryo showing disturbance of the normal proximal and distal position of the extraembryonic ectoderm (ee) and epiblast (ep), respectively. *Oct4* expression normally present throughout the epiblast prior to and during early gastrulation (D) is abnormally placed in Smad2 mutant embryos (E and F). Posterior (G) and lateral views (H) of *Bmp4* expression restricted to the distal cells of the extraembryonic ectoderm at the onset of gastrulation. This population becomes displaced during formation of the amniotic fold. (I) In Smad2 mutant embryos, *Bmp4* marks extraembryonic ectoderm lying lateral to its normal position. ee, extraembryonic ectoderm; ve, visceral endoderm; ep, epiblast; pe, parietal endoderm.

unaffected by the loss of Smad2 signals. To describe the underlying cellular and molecular disturbances leading to this distinctive phenotype, we next examined Smad2 mutants at early gastrulation stages. As shown in Figure 6, Smad2 mutant embryos initially form a proamniotic cavity that in many cases is not retained. These embryos lack a distinct primitive streak, and the epiblast fails to maintain a uniform columnar appearance (Figures 6A and 6B). Moreover, the spatial relationship between the epiblast and the extraembryonic ectoderm is severely disturbed (Figure 6C). Normally, the extraembryonic ectoderm directly overlies the epiblast and is displaced proximally by newly formed mesoderm migrating from the posterior end of the streak. In contrast, many Smad2$^{-/-}$ embryos fail to demonstrate this proximal-distal polarity.

To further characterize the embryonic epiblast tissue in Smad2 mutant embryos, we analyzed *Oct4* expression. *Oct4*, a member of the POU family of transcription factors, is strongly expressed by epiblast cells of pregastrulation stage embryos and is down-regulated as this pluripotential cell population differentiates, eventually becoming confined exclusively to germ cells (Rosner et al., 1990). As shown in Figure 6, the *Oct4* expression domain in many Smad2 mutant embryos is displaced laterally. Sectioning–confirmed *Oct4* expression is confined to cells of a columnar and thus epiblast morphology (Figure 6F). By the equivalent of mid-streak stages, all Smad2 mutants completely lack *Oct4* expression, indicating that pluripotential epiblast cells are prematurely lost. The overall size and total cell numbers are unperturbed in mutant embryos, suggesting that the absence of *Oct4* expression is not the result of a proliferation defect. Smad2 mutant embryos also exhibit abnormal *Fgf8* expression patterns (Figure 7B). In normal embryos, the extraembryonic ectoderm lying just proximal to and abutting the rim of the epiblast strongly expresses *Bmp4* transcripts (Figure 6G) and, coincident with the onset of gastrulation, this population is displaced proximally by posterior mesoderm migrating from the streak (Figure 6H). At the equivalent stages, Smad2 mutants display similar *Bmp4* expression patterns (Figure 6I).

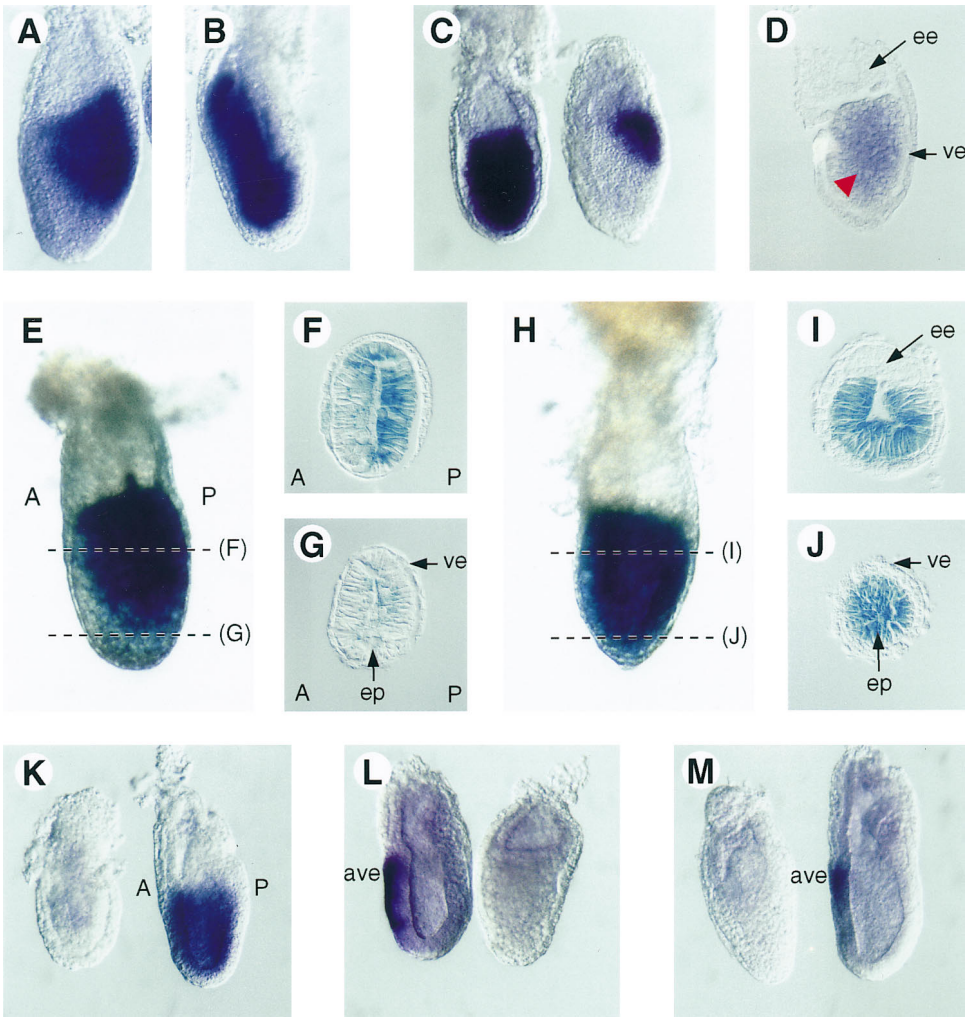


Figure 7. Smad2 Function Required for Molecular Patterning of the Epiblast prior to Streak Formation

*Fgf8* transcripts normally confined to the proximal-posterior region at pregastrulation stages (A) are transiently expressed throughout the epiblast of Smad2 mutant embryos (B). Similarly, T is expressed throughout the epiblast of a Smad2 mutant embryo (left) versus normal littermate (right) at the onset of gastrulation (C). (D) Sagittal section through the mutant embryo in (C) shows T mRNA confined to the epiblast derivatives (red arrow). Nodal.LacZ expression in normal (E–G) and Smad2 mutant embryos (H–J) prior to gastrulation. (K) *Otx2* expression in a Smad2 mutant (left) and normal littermate (right) at gastrulation. (L) *Cer-l* expression normally detected in the AVE (left) is absent in Smad2 mutant embryos (right). (M) *Hesx1* expression in a Smad2 mutant (left) and normal littermate (right) at mid-streak stages of development. ee, extraembryonic ectoderm; ve, visceral endoderm; ep, epiblast; ave, anterior visceral endoderm; A, anterior; P, posterior.

### Smad2 Mutants Fail to Establish an A-P Axis

Recent data suggests that the epiblast is molecularly patterned many hours before the initiation of primitive streak formation. For example, nodal transcripts initially detected throughout the epiblast gradually become restricted proximally and are subsequently localized to the most posterior region of the embryo prior to overt streak formation (Varlet et al., 1997; Figure 7E). Similarly, T transcripts initially appear radially throughout the proximal epiblast, and expression is sharply restricted to the primitive streak during the onset of gastrulation (Thomas and Beddington, 1996). Additionally, *Fgf8* mRNA is selectively expressed on the posterior side of the epiblast prior to gastrulation (Crossley and Martin, 1995). Thus, it was of interest to examine whether Smad2-deficient embryos maintain correct positional information at the egg cylinder stage of development. As a marker for nascent mesoderm, we analyzed T expression. As expected, in normal embryos T transcripts appear asymmetrically distributed at very early gastrulation stages. Surprisingly, in contrast, we observe T mRNA throughout the epiblast of Smad2-deficient embryos (Figures 7C and 7D). This expression is highly transient, because at later stages, as shown above, Smad2 mutants entirely lack T transcripts.

Highly dynamic nodal expression patterns at early stages of mouse development were previously described using a nodal.LacZ reporter allele (Collignon et al., 1996). It was also important to test nodal.LacZ expression in the context of the Smad2 mutant background. As expected, in normal embryos we observe

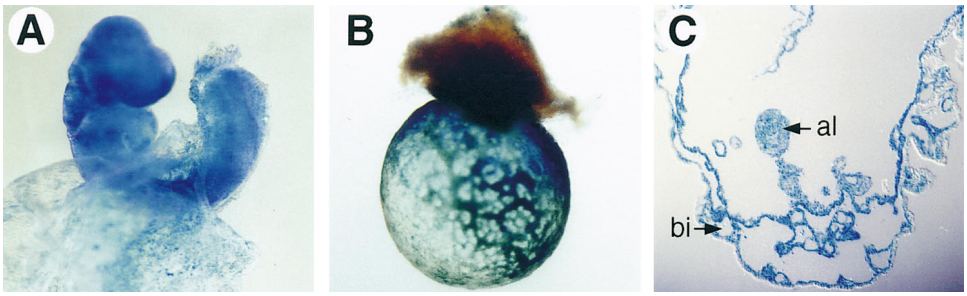


Figure 8. Extensive Colonization by Wild-Type ES Cells Fails to Rescue Smad2 Mutant Embryos

(A) Chimeric embryo (9.5 dpc) resulting from extensive ES cell colonization of a wild-type or Smad2$^{+/-}$ blastocyst. (B and C) In the context of a Smad2 mutant blastocyst, wild-type LacZ$^{+}$ ES cells give rise to exclusively extraembryonic mesoderm derivatives. bi, blood islands; al, allantois tissue.

that nodal.LacZ expression is rapidly lost from the distal region (Figures 7E–7G). In striking contrast, staining appears throughout the epiblast in Smad2 mutants (Figures 7H–7J). Moreover, widespread nodal expression patterns are maintained at slightly later stages when nodal transcripts normally become strictly confined to the prospective posterior region of the epiblast. Additionally, *Fgf8* expression domains were markedly expanded in Smad2 mutants, showing a detectable hybridization signal (Figure 7B). Thus, we conclude that in the absence of Smad2 function the entire epiblast becomes proximal in character.

Mouse embryos have been shown to display morphological asymmetry at pre-streak stages. Thus, the visceral endoderm overlying the proximal region of the embryo appears distinctly thickened in comparison to that located at the distal tip of the egg cylinder. Moreover, the prospective anterior visceral endoderm (AVE) selectively expresses anterior markers such as VE-1 (Rosenquist and Martin, 1995), *Hesx1*, *gsc*, *Lim1*, and *Cer-l* at pre-streak stages (Thomas and Beddington, 1996; Thomas et al., 1997). Recent evidence suggests this extraembryonic cell population provides anterior patterning signals during gastrulation (Popperl et al., 1997; Thomas et al., 1997). Interestingly, the visceral endoderm of Smad2-deficient embryos is uniformly thickened over the entire surface of the epiblast (Figure 6A). Moreover, as judged by the absence of *Cer-l*, *Hesx1*, and *Lim1* expression (Figures 7L and 7M; data not shown), Smad2 mutants appear to lack a distinct AVE region. Similarly, there was no evidence for nodal expression in the visceral endoderm at these stages (Figures 7I and 7J). Smad2-deficient embryos also fail to express *Otx2* at early gastrulation stages (Figure 7K). These results demonstrate that Smad2 expression is required for molecular patterning of the visceral endoderm lineage.

### Essential Smad2 Signals Are Provided by Extraembryonic Tissues

ES cells, introduced into blastocysts, predominantly colonize the embryonic epiblast (Beddington and Robertson, 1989). Thus, in chimeric embryos ES cell derivatives are confined to the embryonic portion, while the extraembryonic tissues are of host origin. This pronounced developmental bias was previously exploited to distinguish nodal activities contributed by the embryonic ectoderm for primitive streak formation and those provided by the primitive endoderm required for anterior patterning (Varlet et al., 1997). Similarly here, wild-type ES cells carrying a ubiquitously expressed *LacZ* gene (Varlet et al., 1997) were injected into host blastocysts from Smad2$^{+/-}$ intercrosses. Thus, one-quarter of the resulting chimeric embryos are comprised of Smad2-deficient primitive endoderm and extraembryonic ectoderm. Approximately 70% of the chimeric embryos recovered at day 8.5 and stained for LacZ expression (33/48) were morphologically normal and demonstrate widespread ES cell contributions to epiblast derivatives (Figure 8A). In striking contrast, the remaining 30% (15/48) closely resembled Smad2$^{-/-}$ embryos (Figure 8B). Interestingly, the LacZ-positive wild-type cells in these chimeric embryos all become fated to form posterior mesoderm. As shown in histological section, LacZ$^{+}$ cells give rise solely to extraembryonic mesoderm, fetal blood cells, and tissue strongly resembling the allantois (Figure 8C). Retrospective genotyping confirmed these chimeric embryos are derived from the injection of Smad2$^{-/-}$ blastocysts. Thus, massive colonization of the epiblast by wild-type cells fails to rescue the developmental block imposed by Smad2-deficient extraembryonic tissues.

## Discussion

The site of primitive streak formation at the proximal region of the epiblast marks the prospective posterior side of the mouse embryo. According to current views, the epithelial sheet delaminates in response to local cues initiating mesoderm production. On the other hand, recent experiments suggest that specification of the A-P axis is determined many hours before the onset of gastrulation. Thus, a graded pattern of gene expression develops along the proximal-distal axis of the embryo. For example, nodal expression, initially widespread, becomes restricted proximally and is later excluded from the prospective anterior portion of the epiblast (Varlet et al., 1997). Similarly, T transcripts are restricted to the proximal region of the epiblast (Thomas and Beddington, 1996). Coincident expression of both nodal and T transcripts thus marks prospective mesoderm prior

to streak formation (Lawson et al., 1991). The present results demonstrate that Smad2 activity confers this molecular asymmetry to the epiblast. Thus, Smad2 mutant embryos fail to exhibit a distinctive proximal-distal polarity. Rather, nodal as well as T and *Fgf8* transcripts all appear uniformly expressed throughout the epiblast at a time normally coincident with streak formation. These aberrant gene expression patterns cause profound cellular disturbances. Thus, the anterior region of the epiblast normally fated to become neurectoderm instead gives rise to posterior mesoderm. These findings demonstrate Smad2 function is essential for the establishment of anterior-posterior identity, and these events are independent of mesoderm specification per se. Rather, the Smad2 pathway restricts the site of primitive streak formation and is essential to maintain the prospective anterior region fated to give rise to anterior mesoderm, surface ectoderm, and neural tissues. Remarkably, in the absence of this pathway the entire epiblast adopts a posterior mesodermal fate and extensively proliferates, giving rise to yolk sac mesoderm and fetal blood cells.

The loss of Smad2 activity also disturbs development of the primitive endoderm lineage. Thus, recent experiments demonstrate a discrete patch of AVE specifically expressing many of the same genes, such as *Hesx1*, *Cer-l*, and *Lim1*, found later in the node-derived axial mesendoderm hours before primitive streak formation (Thomas et al., 1997). Functional data suggests the AVE confers anterior pattern to the epiblast (Thomas and Beddington, 1996). In contrast, the visceral endoderm of egg cylinder stage Smad2 mutant embryos shows no evidence for any morphological or molecular asymmetry. Instead, the endoderm tissue appears uniformly thickened over the entire surface of the epiblast and entirely lacks AVE marker transcripts. Does the loss of proximal-distal polarity in the epiblast simply reflect the absence of molecular patterning of the primitive endoderm? Consistent with this possibility, analysis of chimeric embryos reveals that essential Smad2 signals are provided by the extraembryonic tissues. We recently used a similar experimental strategy to describe distinct nodal activities contributed by the epiblast and primitive endoderm derivatives. Thus, chimeric embryos generated by introducing wild-type ES cells into nodal-deficient host blastocysts proceed beyond gastrulation and instead display anterior patterning defects (Varlet et al., 1997). In contrast here, extensive colonization by wild-type ES cells fails to rescue developmental defects of Smad2-deficient embryos. Rather, wild-type ES cells injected into Smad2 mutant host blastocysts exclusively give rise to extraembryonic mesoderm and tissue strongly resembling the allantois. These findings strengthen the idea that Smad2-mediated signals from the primitive endoderm are necessary for patterning the overlying epiblast.

It is also possible that essential patterning function(s) are contributed by the extraembryonic ectoderm. Considering their well-known developmental fates (Beddington and Robertson, 1989), descendants of Smad2 host blastocysts and not wild-type ES cell derivatives probably gave rise to this tissue. Moreover, distinct tissue boundaries normally observed at the junction between epiblast and extraembryonic ectoderm appear severely disturbed in Smad2 mutant embryos. It is obviously important to learn more about this inability to maintain proximal-distal polarity at the egg cylinder stage and, in particular, whether abnormalities are predominantly caused by defective Smad2 activities in the primitive endoderm, extraembryonic ectoderm, or both tissues. Perhaps the initiation of primitive streak formation at the proximal rim of the epiblast reflects broadly received inductive signals from the extraembryonic ectoderm to elicit nascent mesoderm, acting in opposition to those from the AVE responsible for the formation of anterior structures. Interestingly, recent lineage analysis strongly suggests a small patch of visceral endoderm at the distal tip of the egg cylinder gives rise to the AVE (Thomas et al., 1998). Thus, the A-P axis appears to derive from this earlier proximal-distal asymmetry. Considering that Smad2 mutant embryos display subtle changes affecting tissue architecture of the visceral endoderm and lack anterior pattern, we suggest that the loss of signals from this cell lineage is sufficient to disrupt the earliest steps of A-P specification.

During *Xenopus* gastrulation, ventral and dorsal signals act combinatorially to pattern mesoderm formed in the marginal zone (Harland, 1994; Graff, 1997; Heasman, 1997). Recent experiments demonstrate Smad1 functions downstream of Bmp4 to elicit the formation of ventral mesodermal derivatives such as blood (Graff et al., 1996; Thomsen, 1996), whereas Smad2 induces dorsal mesoderm such as the notochord and can mimic the organizer, causing axial duplication (Baker and Harland, 1996; Graff et al., 1996). The present experiments demonstrate that extraembryonic mesoderm derivatives such as fetal blood cells develop normally in Smad2 mutant embryos in the absence of expression of genes such as *HNF3*β and *Lim1*, which identify anterior axial cell types (Ang et al., 1993; Shawlot and Behringer, 1995). The Smad2 phenotype thus shows striking parallels to defects exhibited by UV-treated *Xenopus* embryos. This treatment selectively destroys dorsal/neural-inducing signals without affecting production of ventral cell types (Heasman, 1997). In mouse, the Smad2 pathway is initially activated in extraembryonic tissue to specify anterior cell types. Overexpression of Smad2 in frog similarly elicits formation of dorsal/anterior tissue, although it is unclear when and where the endogenous pathway might normally act in establishing anterior pattern.

In gastrulation stage *Xenopus* embryos, *Bmp4* transcripts are present in the marginal zone between the animal and vegetal hemispheres (Heasman, 1997). Similarly, prior to the onset of mouse gastrulation *Bmp4* expression is strictly localized to the distal portion of the extraembryonic ectoderm immediately adjacent to the epiblast. These observations suggest that nascent mesoderm emerging from posterior regions of the primitive streak and migrating across the proximal boundary of the epiblast is efficiently exposed to abundant Bmp4 signals. Interestingly, *Smad1* transcripts initially appear coincident with the onset of gastrulation, and indeed at this stage *Smad1* is predominantly expressed in mesodermal cell populations emerging from the streak. At mid-streak stages, posterior mesoderm also strongly expresses *Bmp4* (Winnier et al., 1995). Thus, the extraembryonic mesoderm fated to give rise to cell types

such as blood appears well equipped for efficient delivery of Bmp signals via paracrine pathways. Thus, we conclude that the strongly ventralized phenotype observed here for Smad2-deficient mouse embryos probably reflects their selective responses to Smad1 signals.

Specific TGFβ ligands potentially activate cognate cell surface receptor kinases and Smad proteins that are widely expressed by responding tissues. This simple model predicts mutations disrupting expression of individual TGFβ family members, their receptors, or Smad proteins responsible for signaling to the nucleus could in principle cause similar developmental defects. However, mutant mice lacking candidate ligands upstream of Smad2, namely TGFβ1–3, activin, and nodal, all display different characteristics. Indeed, TGFβ1–3 (Shull et al., 1992; Kulkarni et al., 1993; Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997) and activin (Matzuk et al., 1995a, 1995b) deficient mice are normal at birth. Nodal (Conlon et al., 1994) and Smad2 mutant embryos exhibit quite distinctive phenotypes. It is of course possible that incomplete penetrance of these loss-of-function mutations, in comparison with the Smad2 defects, reflects overlapping activities contributed by closely related TGFβ family members. Consistent with this way of thinking, biochemical experiments extensively document promiscuous interactions amongst TGFβ ligands and cell surface receptor kinases (Massague, 1996). Analysis of appropriate compound mutant strains may therefore reveal tissue abnormalities more closely resembling the Smad2 phenotype. To date, Vg1, another potential ligand responsible for induction of dorsal mesoderm in *Xenopus* (Thomsen and Melton, 1993; Kessler and Melton, 1995), has no mammalian counterpart. Thus, it is also possible that Smad2 acts downstream of another as yet undiscovered TGFβ family member(s), perhaps the elusive Vg1 homolog.

The specific structural characteristics of Smad family members responsible for regulating molecular associations with each other and the many other critical components of the signaling pathway such as TGFβ receptor complexes, the extensive cytoplasmic phosphorylation machinery, and assemblies of transcription factors controlling downstream gene expression are highly conserved (Baker and Harland, 1997; Heldin et al., 1997; Massague et al., 1997). On the other hand, a strong argument can be made that Smad effector molecules may also be somewhat divergent to accomodate specific developmental programs and tissue interactions responsible for mesoderm induction and patterning in different organisms. The present experiments strongly suggest that critical aspects of Smad2 function are shared between frogs and mammals. It will be interesting to learn more about the specific molecular and cellular targets of this pathway.

## Experimental Procedures

### Generation of Smad2 Mutant Mice

A full-length mouse Smad2 cDNA isolated from a 16.5 day mouse embryo cDNA library (Novagen) was used to screen a 129/SvJ genomic library (Stratagene). We identified a 2.4 kb EcoRI fragment containing the first exon (290 bp). To construct a positive/negative targeting vector, the 5′ 6.0 kb EcoRI fragment and a 5.0 kb HindIII fragment lying 3′ to the first exon were ligated to the MC1*neo*pA cassette. The HSV-*tk* cassette was introduced 3′ to the HindIII fragment as shown in Figure 2. The resulting vector was linearized with SacII and electroporated into CCE ES cells. Drug-resistant colonies picked into 96-well colonies were screened by Southern blot analysis as described (Ramirez-Solis et al., 1993). Briefly, DNA samples digested with BamHI were probed with a 3.0 BamHI/EcoRI genomic fragment external to the vector. As shown in Figure 2, this probe detects a 12.1 kb and a 9.0 kb product corresponding to the wild-type and mutant alleles, respectively. Approximately one in five drug-resistant ES cells had undergone the predicted recombination event at the Smad2 locus. The fidelity of the 3′ side of the targeting event was verified using additional digests and a *neo*-specific probe. Correctly targeted clones were injected into C57Bl/6J host blastocysts to generate germ line chimeras using standard procedures (Bradley, 1987).

### Genotyping Procedures

$F_1$ progeny were genotyped by Southern blotting as described above. Subsequent offspring and embryos were genotyped by PCR using a common primer located 85 bp 5′ of exon 1 (5′-AGTTAATTGC CCAGAGCGTTGACA-3′) in conjunction with a *neo*-specific primer (5′-AGCACGTACTCGGATGGAAGC-3′) and a third primer corresponding to exon 1 sequences (5′-GCGGAGTGAATGGCAAGATGG-3′) designed to amplify 550 bp and 341 bp products specific for the targeted and wild-type alleles, respectively. The PCR conditions were 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 μM each primer, and 0.5 U Amplitaq. Following initial denaturation at 94°C for 3 min, reactions were conducted for 30 cycles at 93°C for 45 s, 60°C for 60 s, and 72°C for 60 s. The resulting products were separated on 2% agarose gels and visualized with ethidium bromide. For genotyping of early stage embryos, the entire embryo (day 6.5 and 7.5) or visceral yolk sac (day 8.5) was lysed in 50 μl buffer (50 mM KCl, 10 mM Tris-HCl [pH8.3], 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20) containing 2.0 mg/ml proteinase K, incubated for 12 hr at 55°C, and boiled for 5 min. A 1 μl aliquot was subjected to PCR amplification using the conditions above.

### Histology and In Situ Hybridization

Embryos were dissected free of maternal tissues, the Reichert's membrane reflected, and fixed overnight in 4% paraformaldehyde in PBS at 4°C. For conventional histology, tissue was dehydrated through an ethanol series, cleared in xylene, embedded in paraffin wax, sectioned at 6 μm, and stained with H and E using standard procedures. Whole-mount in situ hybridization analysis using digoxigenin-labeled probes was performed as described (Wilkinson, 1992). Wild-type and mutant embryos were processed in the same tube. Riboprobes for whole-mount analysis were as described: *Hesx1* (Thomas and Beddington, 1996), *Cer-l* (Thomas et al., 1997), T (Wilkinson et al., 1990), *Otx2* (Ang et al., 1994), *Oct4* (Rosner et al., 1990), *Lim1* (Barnes et al., 1994), *Fgf8* (Crossley and Martin, 1995), *Bmp4* (Jones et al., 1991), AFP (300 bp fragment of AFP, kind gift of R. Beddington and R. Arkell), *Flk1* (Yamaguchi et al., 1993), epsilon β-globin (Baron and Farrington, 1994), and *HNF3*β (Sasaki and Hogan, 1994). A minimum of eight mutant embryos were assessed for each developmental time point. Probes specific for *Smad1*, *-2*, and *-4* were synthesized from full-length cDNAs (P. H. and J. W., unpublished data). Embryos were post-fixed in 4% paraformaldehyde, photographed, dehydrated and cleared, embedded in paraffin wax, and sectioned at 8 μm. Sections were dewaxed using standard procedures, mounted in Cytoseal, and photographed using Nomarski optics.

### Analysis of Nodal.LacZ Expression

Heterozygous $Smad2^{+/-}$ mice were crossed to animals carrying the nodal.LacZ allele (Collignon et al., 1996), and the resulting progeny were genotyped for the Smad2 (see above) and nodal.LacZ mutant alleles (Collignon et al., 1996). $Smad2^{+/-}$/nodal.$LacZ^{+/-}$ and $Smad2^{+/-}$ animals were intercrossed, and embryos recovered between day 6.5 and 7.5 pc were stained for β-galactosidase activity and processed as described (Collignon et al., 1996).

### Generation and Analysis of Chimeric Embryos

Blastocysts recovered from Smad2$^{+/-}$ intercross matings were injected with 10–15 R26.1 ES cells as described (Varlet et al., 1997). Following transfer into pseudopregnant foster females, the manipulated embryos recovered at 8.5–9.5 days of development were fixed and processed for β-galactosidase staining. The genotypes of host blastocysts were determined retrospectively by PCR genotyping of a portion of the extraembryonic endoderm, as described (Varlet et al., 1997).

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