Targeted Mutagenesis of Smad1 Reveals an Essential Role in Chorioallantoic Fusion

Robert J. Lechleider,*1,2 Julie L. Ryan,* Lisa Garrett,† China Eng,‡ Chu-xia Deng,§ Anthony Wynshaw-Boris,†,3 and Anita B. Roberts‡

*Department of Pharmacology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799; †Laboratory of Genetic Disease Research, NHGRI, Building 49, Bethesda, Maryland 20892; ‡Laboratory of Cell Regulation and Carcinogenesis, NCI, 41 Library Drive, Bethesda, Maryland 20892; and §Genetics of Development and Disease Branch, NIDDK, Building 10, 9000 Rockville Pike, Bethesda, Maryland 20892

The Smad family of intracellular signaling intermediates transduce signals downstream from the transforming growth factor beta (TGF-β) family of receptor serine threonine kinases. The original member of this family, Smad1, has been shown to mediate signals from receptors for the bone morphogenetic proteins (BMPs), a large group of ligands in the TGF-β superfamily that mediate important developmental events. We have targeted the Smad1 gene in mice and created mutants null at this locus. Smad1 mutant mice die at approximately 9.5 days postcoitum due to defects in allantois formation. In Smad1 mutant mice, the allantois fails to fuse to the chorion, resulting in a lack of placenta and failure to establish a definitive embryonic circulation. Although vasculogenesis is initiated in the mutant allantois, the vessels formed are disorganized, and VCAM-1 protein, a marker for distal allantois development, is not expressed. Smad1 null fibroblasts are still able to respond to BMP2, however, suggesting that the defect observed in the developing extraembryonic tissue is caused by a very specific loss of transcriptional activity regulated by Smad1. Our data further demonstrate that although highly similar structurally, Smad proteins are not functionally homologous.

Key Words: TGF-β; Smads; allantois; BMP; targeted mutagenesis; VCAM-1; placenta.

INTRODUCTION

The TGF-β superfamily of intercellular signaling molecules is a large, conserved family that regulates important developmental events from early patterning to postnatal development (Hogan, 1996; Letterio and Roberts, 1997; Whitman, 1998). All TGF-β superfamily members studied to date signal through a highly conserved mechanism of transmembrane receptors and receptor-activated, translocatable transcription cofactors called Smads. Ligand binds to the type-II transmembrane serine-threonine kinase receptor which subsequently recruits and phosphorylates the type-I receptor kinase. Following type-I receptor activation by phosphorylation, receptor-activated Smads (R-Smads) act as direct substrates for the kinase activity of the type-I receptor. Phosphorylated R-Smads associate with the common Smad, Smad4, and this complex then translocates to the nucleus where it is involved in mediating transcriptional responses on target genes (Massague and Chen, 2000; Hill, 1999). Interactions between the R-Smads and type-I receptors are specific. Smad2 and Smad3 interact only with receptors for TGF-β or activin, while Smad1 and Smad5 interact with receptors for the BMP family (Massague, 1998). Smad activation has been shown to be essential for mediating growth inhibitory and certain transcriptional responses for all members of the TGF-β superfamily tested thus far, although accessory pathways likely play an important role in modulating this activity (Mulder, 2000; Miyazono, 2000).

The BMPs, the largest family within the TGF-β super-
family, regulate several key processes in development. For example, Bmp4 has been shown to be a potent inducer of ventral mesoderm during gastrulation in Xenopus laevis. In the mouse, Bmp4 is also essential for gastrulation (Winnier et al., 1995). Embryos with targeted disruption of both alleles of Bmp4 die between 6.5 and 9.5 days postcoitum (dpc), with the majority failing to progress beyond the egg cylinder stage. Interestingly, targeted mutagenesis of Bmp2, which is greater than 90% indentical to Bmp4, caused a defect in closure of the proamniotic canal and defects in heart formation, although embryos appeared to undergo gastrulation normally (Zhang and Bradley, 1996). Other BMPs appear to mediate effects later in development. Bmp7 (Op-I) is essential for eye and kidney development, while loss of Bmp5 does not lead to a discernible phenotype during development, but causes skeletal defects in postnatal life (Dudley et al., 1995; Karsenty et al., 1996; Storm et al., 1994). Similarly, mutations in both type-II and type-I receptors for BMPs cause severe developmental defects. Targeted deletion of Bmpr-II (Beppu et al., 2000) as well as Bmpr-IA, one of the two known type-I receptors for BMPs, leads to death at the egg cylinder stage with a failure to undergo gastrulation, as does mutation of Actr-1A, which has been shown to bind to and activate Smad1 (Goumans and Mummery, 2000).

Three Smad proteins have been shown to signal from receptors of the BMP family. Smad1 was the first mammalian Smad gene to be described and is structurally highly similar to Drosophila mad. Smad5 and Smad8 are two highly similarly vertebrate isoforms that have not been as well studied at a biochemical level (Hill, 1999). Smad1 is phosphorylated and activated by the BMP receptors BMP PR-IA and BMP PR-IB, the related activin receptor Actr-I (Macias-Silva et al., 1998), and also Actr1 (Chen and Massague, 1999), an orphan receptor for which ligand specificity remains uncertain. In developmental systems, Smad1 and Smad5 can induce ventral mesoderm, similar to ectopic expression of BMP receptors, and can block the dorsalizing effects of activin (Lagna and Hemmati-Brivanlou, 1999). Dominant inhibitory forms of Smad1 can block the ventralizing effects of constitutively active BMP receptors, and overexpression of wild-type Smad1 can overcome loss of signaling induced by dominant inhibitory forms of BMP receptors (Graff et al., 1996). Taken together, these studies demonstrate that Smad1 can mediate the principle effects of BMP receptors in inducing ventral mesoderm formation in Xenopus.

Studies of the roles of Smad1, Smad5, and Smad8 in murine development suggest a more complicated picture that has emerged from Xenopus. Expression patterns of Smad1 suggest a role throughout development (Dick et al., 1998), although expression at around the time of gastrulation has not been examined. Targeted disruption of Smad5 revealed a role for this gene in vascular development and patterning, yet mutant embryos underwent normal gastrulation (Yang et al., 1999; Chang et al., 1999). Since Smad1 and Smad5 share approximately 92% amino acid identity and appear to be functionally homologous in vitro, we sought to further examine the role of Smad1 in mammalian physiology by targeted disruption of Smad1 in embryonic stem (ES) cells and generation of mutant mice. Our results indicate that, like Smad5 mutant mice, Smad1 null mice proceed through gastrulation normally, but die in midgestation due to defects in allantois development and chorioallantoic placenta formation. Together, these data indicate both redundant and specific roles for these two highly related proteins that signal from BMP receptors.

MATERIALS AND METHODS

Construction of the Smad1 targeting vector and generation of Smad1 null mice. A mouse 129SvEv genomic library was screened with a Smad1 probe and a 16-kb NotI fragment containing exon 3 (Huang et al., 2000) was isolated. A targeting vector containing approximately 8.5 kb on the 3' arm and 4.4 kb in the 5' arm was constructed in pPNT. The vector was electroporated into TC1 129Sv/Ev ES cells (Deng et al., 1996), which were selected in G418 and FIAU. Two correctly targeted clones as identified by Southern blot analysis were injected into C57/B16 blastocysts and chimeric mice generated for both clones. Chimeric males were mated into 129Sv/Ev or NIH Black Swiss females and the F1 and subsequent generations intercrossed. There were no differences observed between the two clones used for this analysis.

Genotype analysis. Southern blot analysis was performed as described on genomic DNA isolated from E9.5 embryos by using the Trizol reagent (Gibco/Life Technologies) or from tail clips. PCR genotyping of yolk sac DNA was performed by using the primers 5'-gaagcacagcgagtacaatcct-3' and 5'-ccgcttgaccttcctttc-3' for the wild-type allele and 5'-gaagcacagcgagtacaatcct-3' and 5'-ccgcttgaccttcctttc-3' for the mutant, yielding products of 1.1 kb and 700 bp, respectively. PCR conditions were: 94°C for 4 min then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension of 72°C for 7 min.

Western blot and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. Protein extracts from 9.5-dpc embryos were separated by SDS-PAGE, transferred to PVDF membrane, and probed with a mouse monoclonal antibody to Smad1 raised against the full-length GST-fusion protein (Santa Cruz Biotechnologies). Genotypes were confirmed by Southern blotting of the genomic DNA isolated from the same embryos. The Smad1 antibody was evaluated for specificity by lack of cross-reactivity with other Smad proteins, and blocking with GST–Smad1 fusion protein but not with GST alone (data not shown). The truncated Smad1 expression construct was designed to express amino acids 1–212 of Smad1 in the pCMV2-FLAG vector. Lysates of COS1 cells transiently transfected with either the full-length or truncated Smad1 expression construct are included as controls. RNA and DNA for genotyping were extracted from 9.5-dpc embryos by using Trizol (Life Technologies). cDNA was prepared by using a commercial kit (Life Technologies). Primers were derived from the M.H1 domain or spanning the linker and MH2 domain. β-Actin primers served as a control.

Histological analysis. Embryos were dissected free from the uterus and the yolk sac reserved for genotyping. Embryos were fixed in cold 4% paraformaldehyde and embedded in paraffin for sectioning (5 μm) and stained by hematoxylin and eosin. Embryos from wild-type crosses were stained with a rabbit polyclonal anti-Smad1 antibody (Zymed) at 1:500 dilution in phosphate-
buffered saline, washed, and detected with horseradish peroxidase
coupled secondary and diaminobenzamidine (Vector Labs) and
counterstained with hematoxylin. Flk-1 expression was analyzed
by immunohistochemistry using a commercially available anti-
body (Chemicon). Whole-mount immunostaining for VCAM-1 was
performed basically as described (Kwee et al., 1995). Briefly, E8.5
embryos were fixed for 5 min in Dent’s fixative (80% methanol/
20% DMSO), rehydrated, blocked in BSA for 2 h, and incubated
overnight at 4°C with anti-VCAM-1 monoclonal antibody (Phar-
mingen). Following extensive washing in neutral buffered saline
with Tween 20, embryos were incubated overnight with secondary
antibody, washed, and incubated with colorimetric substrate.

Whole-mount in situ hybridization. Embryos at 8.5 dpc were
prepared and hybridized as described (Lowe and Kuehn, 2000). A
500-bp probe to the coding region of the mouse
Vcam1
gene was
used. Antisense controls showed no hybridization (data not
shown). Embryos were photographed with a Leica MZ-12 stereomi-
croscope fitted with a standard 35-mm camera.

Cell culture and growth inhibition assays. Embryos at 9.5 dpc
were dissected free of maternal structures and minced in DMEM
containing 10% fetal bovine serum and antibiotics. Cells were
allowed to expand and surviving colonies were genotyped by both
PCR and Southern blot analysis, confirming genotypes as either
wild-type or homozygous null. For growth inhibition assays, 4
3 10^4 cells per well were plated in 24-well plates, treated as indicated
with BMP2 or TGF-β overnight, and [3H]thymidine incorporation
determined as described previously. Each experiment was repeated
at least once with similar results. Results are presented as the
mean ± the SEM of triplicate wells from a single experiment.

RESULTS

Creation of a Smad1 targeting vector and generation of
ES cell heterozygotes. A Smad1 genomic clone was ob-
tained from a 129 Sv/Ev ES cell genomic library using a 5'
TABLE 1
Genotypes of Viable Offspring of Smad1−/− Intercrosses

<table>
<thead>
<tr>
<th>Background</th>
<th>Genotypea</th>
</tr>
</thead>
<tbody>
<tr>
<td>129Sv/Ev</td>
<td>+/+</td>
</tr>
<tr>
<td>129/Black Swiss</td>
<td>+/+</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
</tr>
</tbody>
</table>

Note. Numbers in parenthesis are percent of total viable off-
spring. a As determined at weaning.

TABLE 2
Genotypes at Various Stages of Development

<table>
<thead>
<tr>
<th>Stagea</th>
<th>Genotypeb</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>+/−</td>
</tr>
<tr>
<td>−/−</td>
<td>Resorbed</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stagea</th>
<th>Genotypeb</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5</td>
<td>8 (31)</td>
</tr>
<tr>
<td></td>
<td>12 (46)</td>
</tr>
<tr>
<td></td>
<td>6 (23)</td>
</tr>
<tr>
<td></td>
<td>0 (0)</td>
</tr>
<tr>
<td>9.5</td>
<td>16 (26)</td>
</tr>
<tr>
<td></td>
<td>36 (60)</td>
</tr>
<tr>
<td></td>
<td>6 (10)</td>
</tr>
<tr>
<td></td>
<td>2 (3)</td>
</tr>
<tr>
<td>10.5</td>
<td>11 (39)</td>
</tr>
<tr>
<td></td>
<td>13 (46)</td>
</tr>
<tr>
<td></td>
<td>2 (7)</td>
</tr>
<tr>
<td></td>
<td>2 (7)</td>
</tr>
<tr>
<td>11.5</td>
<td>5 (24)</td>
</tr>
<tr>
<td></td>
<td>12 (57)</td>
</tr>
<tr>
<td></td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>4 (19)</td>
</tr>
</tbody>
</table>

a Days post coitum (dpc).
b As determined by PCR of embry or yolk sac.
fragment of murine Smad1 cDNA as a probe. The targeting vector was constructed to interrupt the third exon of Smad1 at the SmaI site by insertion of the neomycin resistance gene driven by the PGK promoter in the reverse orientation (Fig. 1). We call this allele Smad1<sup>tm1</sup>. Approximately 8.5 kb of 5' flanking DNA and 4.4 kb of 3' flanking DNA were cloned into the targeting vector pPNT. Following electroporation and selection in G418, 180 ES cell clones were analyzed by Southern blotting of HindIII digests of genomic DNA. Proper targeting would yield an 8.3-kb fragment for the mutant allele and a 6.5 fragment for the wild-type (Fig. 1). Twelve clones with likely targeting events were identified. Two of these clones were confirmed to have correct targeting events by repeat Southern blotting following digestion with Apal and use of a 3' flanking probe (not shown). Both Smad1<sup>+/−</sup> clones were injected into C57/B6 blastocysts to generate chimeric mice. Subsequent analyses did not reveal any differences between the two clones.

**FIG. 2.** Gross phenotype of the Smad1 null embryos. (A) +/− and (B) −/− embryos at 8.5 dpc. Each embryo had seven somites. The allantois is indicated (arrowhead). (C) Embryos at 9.5 days, genotypes as indicated. The mutant embryo is smaller and developmentally delayed. Note the bulbous, vascular allantois on the mutant embryo (arrow). The heterozygous embryo has normal umbilical vasculature. Normal (D) and mutant (E) 9.5-dpc embryos with intact yolk sacs, genotypes as indicated. Embryos were dissected away from maternal tissues and the yolk sacs cleared in glycerol. The mutant allantois (white arrows) in (E) is indicated. Note the failure of the mutant embryo (E) to form umbilical vessels, which are clearly present in the normal embryo (arrows in D). al, allantois. Bar, 1 mm.
The targeting construct used could theoretically produce a truncated form of Smad1 lacking the MH2 domain which could potentially act as a dominant interfering or activating mutant. RT-PCR analysis showed that no transcript spanning the insertion site was present in RNA extracted from mutant embryos, but that a truncated message that could potentially be translated into a C-terminally truncated protein was still produced (Fig. 1C). In order to determine whether any truncated or alternatively spliced protein product was produced, we performed immunoblots on protein extracts purified from wild-type, heterozygous, and null embryos. Using a Smad1 monoclonal antibody, we confirmed that, although both the wild-type and heterozygous embryos expressed Smad1, no full-length Smad1 could be detected in extracts from the Smad1−/− embryos. To confirm that the antibody could recognize a C-terminal truncated Smad1 product, we created a Smad1 expression vector (Smad1 1–212) which contains only the N-terminal amino acids and represents the potential truncated protein produced. The Smad1 antibody recognized this truncated construct, while no truncated product was detected in extracts from the heterozygous or null embryos. This confirms that

FIG. 3. Expression of Smad1 at 8.5 days. (A) Low- and (B) high-power view of transverse sections of wild-type 8.5-dpc embryos stained with a Smad1 antibody. Black arrows show positive Smad1 staining cells, white are negative. v, vessel; b, blood; al, allantois; a, amnion; ys, yolk sac; n, neural fold. Scale bar, 25 μm (A), 100 μm (B).

FIG. 4. Allantoic and placental phenotype of Smad1−/− mutants. (A, B) Microscopic view of Smad1−/− allantois at 9.5 dpc from two different embryos. The arrows in (A) indicate the collection of cells at the amnion. Arrowheads demonstrate likely vascular structures (C–F). Wild-type (C, D) and mutant (E, F) placentas. The brackets indicate the chorionic plate, and the boxes in (C) and (E) are the areas represented in (D) and (F). Horizontal arrows identify maternal blood vessels, vertical arrows identify embryonic vessels, absent in (F). Scale bar, 100 μm (A, B, D, F), 500 μm (C, E).
the failure to identify Smad1 in the mutant extracts was due to lack of protein and not inability of the antibody to recognize the truncated protein. The lack of full-length transcript as demonstrated by RT-PCR and the failure to detect a truncated protein by immunoblotting demonstrate that we have correctly targeted the Smad1 gene in the mouse, and that genetic targeting leads to a complete loss of Smad1 protein production.

**Loss of Smad1 leads to embryonic lethality in early to midgestation due to a failure of chorioallantoic fusion.** Smad1 heterozygous animals were generated by crossing the germ line chimeras into both 129Sv/Ev and Black Swiss backgrounds. Heterozygotes from these matings were intercrossed and the relative proportion of Smad1 genotypes was determined by PCR. From these matings, no Smad1−/− animals were recovered at weaning (Table 1). Analysis of the intercrosses in the 129 background revealed disproportionately few heterozygous animals, although the cause for this has not been determined. Smad1 heterozygous adults were healthy and fertile, and no abnormalities were observed in mice kept for up to 6 months.

Analysis of timed pregnancies from Smad1−/− crosses revealed Mendelian ratios of all three genotypes through 8.5 dpc (Table 2). Beginning at 9.5 dpc, resorptions and loss of Smad1−/− embryos were evident. By 10.5 dpc, no viable Smad1−/− embryos were identified, although occasional small, poorly developed embryos were recovered. Gross morphological analysis of Smad1−/− embryos at 8.5 dpc revealed no obvious defects (Fig. 2B). Smad1−/− embryos had comparable numbers of somites as wild types at 8.5 dpc, and embryos recovered at this stage were generally of similar size to wild-type or heterozygous embryos. Smad1−/− embryos appeared to have a somewhat shortened allantois compared to wild-type or heterozygous embryos, although precise measurements of allantois length were not made. Although mutant embryos recovered at this stage had failed to develop a fully fused chorioallantoic placenta, the same was true for a subset of wild-type and heterozygous embryos. Smad1−/− embryos appeared to have a somewhat shortened allantois compared to wild-type or heterozygous embryos, although precise measurements of allantois length were not made. Although mutant embryos recovered at this stage had failed to develop a fully fused chorioallantoic placenta, the same was true for a subset of wild-type and heterozygous embryos. Smad1−/− embryos appeared to have a somewhat shortened allantois compared to wild-type or heterozygous embryos, although precise measurements of allantois length were not made.

**Smad1 protein expression and chorioallantoic placenta defects.** Immunohistological analysis of Smad1 at 8.5 dpc showed widespread expression, with particularly high expression in the yolk sac (Fig. 3), and expression in nearly all cells in the allantois. Microscopic analysis of the mutant allantois at 9.5 dpc revealed a disorganized collection of cells with some nests or channels of what appeared to be hematopoietic cells (Figs. 4A and 4B). The mutant allantois penetrated the amnion, although there was often a small collection of cells around the area of penetration through the amnion (Fig. 4A). In some embryos, the fusion of the allantois and amnion was more complete (Fig. 4B), with a large collection of cells at the junction of the allantois and amnion. Despite this apparent disorganized proliferation, the mutant allantois still appeared to organize into structures consistent with blood vessels (Fig. 4B).

Failure of chorioallantoic fusion lead to defects in the placenta. The placenta is a combined maternal–fetal organ that requires coordinated invagination by embryonic components into maternal tissue. In particular, vessel ingrowth from the embryonic allantois is required for proper maternal–fetal oxygen and nutrient exchange. The first event in placental development is the fusion of the chorion and allantois that occurs around 8.5 dpc. As noted, Smad1−/− embryos failed to form proper chorioallantoic fusion. Examination of placentas revealed further defects in placental development (Figs. 4C–4F). By 9.5 dpc, normal placenta had developed a significant labyrinthine layer, with extensive vessel growth from the site of fusion into the maternal component. In contrast, although maternal vessels can be seen in the labyrinthine layer of the mutant placenta as evidenced by maternal, unucleated erythrocytes, no vessels derived from the embryo are evident, and the chorionic plate is much smaller. The defect in vascularization is not a primary defect in vasculogenesis, as evidenced by vessels visible in the mutant allantois (Figs. 4B, 5A, and 5B), but is directly related to the failure of allantoic fusion.

The allantois in Smad1−/− mutants forms vessels but fails to express VCAM1. The allantois gives rise to the umbilical vessels, and is one of the few sites in the embryo that develops vessels through vasculogenesis (Downs et al., 1998). In order to determine whether the structures seen in the mutant allantois represented vessels and hematopoietic cells, we stained the mutant allantois for flk1. Flk1 is a receptor for VEGF, and is an early marker for hematopoietic and vascular endothelial cells. The Smad1−/− mutant clearly expressed flk1 as determined by immunohistochemistry (Figs. 5A and 5B). Tubular structures lined by cells resembling endothelial cells stained positively for flk1, as did occasional cells within these structures. This demonstrates that the mutant allantois is able to support vasculogenesis, despite the proliferative defect and failure to fuse with the chorion.

To start to determine the molecular mechanism of the failure of chorioallantoic fusion, we examined expression of VCAM-1. The phenotype resulting from the loss of Smad1...
expression closely resembles that of the loss of the intercellular adhesion molecule VCAM-1. VCAM-1 in the adult is expressed on endothelial cells and activated leukocytes. In the embryo, VCAM-1 is expressed in the developing allantois, heart, and yolk sac, beginning about 7.5 dpc. Loss of VCAM-1 expression leads to embryonic lethality at approximately E9.5 from lack of proper placental circulation, similar to the phenotype seen with Smad1 (Kwee et al., 1995; Gurtner et al., 1995). In order to determine whether VCAM-1 expression was altered in the Smad1 knockout mice, we performed whole-mount immunostaining of 8.5-dpc embryos with an antibody to VCAM-1. As expected, VCAM-1 was expressed in the heart, allantois, and yolk sac of both +/+ (not shown) and −/− embryos (Fig. 5C). In contrast, VCAM-1 expression was lost in the Smad1−/− embryos, but loss of expression was restricted to only the allantois (Fig. 5D). Expression levels in the heart and yolk sac were comparable to that found in the wild-type or heterozygous animals. Whole-mount in situ hybridization, however, suggested that VCAM-1 message was still expressed (Fig. 5E), but that this expression was variable. Similarly, mTwist expression was also assayed by whole-mount in situ hybridization, but no differences between wild-type and mutant embryos were observed (data not shown). These data suggest that, although we cannot detect VCAM-1 protein in the mutant allantois, it may still be present, as suggested by in situ hybridization results. Thus, the role of VCAM-1 expression in the observed phenotype is unclear.

**Loss of Smad1 does not lead to loss of BMP2 responsiveness.** Studies using Smad deficient cells have demonstrated that Smad signaling is essential for growth-inhibitory and some transcriptional responses activated by both TGF-β and BMP family ligands (Sirard et al., 2000; Yang et al., 1999). To determine whether Smad1 was necessary for BMP responses in cultured cells, wild-type and Smad1−/− fibroblasts were cultured from 9.5-dpc embryos and their responses to TGF-β family ligands determined. Although TGF-β and BMPs can stimulate growth of mesenchymal cells under certain conditions, under the growth conditions employed in these experiments, both ligands were growth inhibitory. Treatment of both wild-type and Smad1−/− fibroblasts with 1 ng/ml of TGF-β caused a significant growth inhibition (Fig. 6). Surprisingly, treatment of both wild-type and Smad1−/− fibroblasts with BMP2 also resulted in a significant growth inhibitory response, with essentially equal IC50 values in both cell lines. Similar results were obtained when transcriptional induction of PAI-1 was examined, but the absolute induction in either wild-type or mutant cells by BMP2 was lower than that observed with TGF-β (data not shown). This finding suggests that Smad1 is not a critical mediator of the BMP2 growth inhibitory response, and that other Smad proteins or auxiliary pathways can mediate this response. They further suggest that the defect seen in the Smad1 mutant embryos is a very specific one which cannot be corrected by Smad5.

**DISCUSSION**

The TGF-β superfamily is a large family of growth and differentiation factors that play diverse roles during mammalian development. Despite the large number of ligands and the diversity of responses they elicit, the signaling pathway from the cell surface receptors to the nucleus is alarmingly simple. To date, only five R-Smads have been identified: Smad1, Smad2, Smad3, Smad5, and Smad8. It is apparent, therefore, that individual Smad proteins will have specific roles based on both the signals that lead to their activation and their subsequent participation in specific transcriptional complexes. Additional levels of control will include temporal and spatial regulation of expression, as well as positive and negative regulation by other signaling pathways.

When overexpressed in biochemical assays, Smad1 and Smad5 have been shown to be largely homologous. Both proteins are phosphorylated by BMP family receptors, and can mediate signals from these receptors to activate gene transcription of artificially constructed reporter constructs. Differences in the abilities of these two closely related transcription factors to recognize or regulate target genes in situ have not been demonstrated. It is clear from genetic studies in the mouse, however, that Smad1 and Smad5 have distinct but partially overlapping functions during mammalian development. The defect observed in Smad1−/− animals, failure of choioallantoic fusion, is quite specific. Although one group observed milder allantois defects in Smad5 null mice, these were not seen in mice generated in the laboratory of one of the authors of this report (C. Deng). Unlike the defect described by Chang et al. (1999) for mice mutant at the Smad5 locus, Smad1 mutant mice rarely demonstrated full extension of the allantois to the chorion. This suggests that Smad1 plays a distinct role in regulating allantois growth and fusion with the chorion, perhaps at an earlier stage than Smad5. Similarly, Smad1 mutant embryos did not demonstrate the range or severity of defects seen in the Smad5 mutants reported by either group. For example, although some defects in yolk sac angiogenesis were observed in Smad1 mutants (compare the vitelline vessels in Fig. 2E with 2D), these were mild compared to the defects reported for Smad5 mutants. Similarly, no gross morphological or histological defects were observed in cardiac or foregut development in Smad1−/− embryos (data not shown), and defects in turning or cardiac left-right patterning were not observed in Smad1 mutants, unlike those seen in Smad5 null embryos (Chang et al., 2000). This is particularly surprising since surgical removal of the allantois has previously been shown to cause defects in tail sidedness in the developing rat embryo (Fujiwara and Baden, 1993; Fujinaga et al., 1995). These findings suggest distinct roles for these two genes during murine development.

The defect in Smad1 mutants does not exactly phenocopy other ligand, receptor, or Smad mutants. The phenotype partially resembles defects in Bmp4 null embryos, which fail to development an allantois on certain genetic
Type-II receptor mutants either singly as in BmprII mutants (Beppu et al., 2000) or in combination, as with ActrIIA;ActrIIB double mutants (Song et al., 1999), lead to defects in gastrulation, and not to specific allantois defects, while type-I receptor mutations either lead to pre- or
perigastrulation lethality or specific cardiovascular defects. Other Smad mutations are either lethal at about the time of gastrulation, as is the case for Smad2 and Smad4 (Goumans and Mummery, 2000), or lead to viable animals with defects in immune (Yang et al., 1999; Datto et al., 1999) (Smad3) or cardiac (Galvin et al., 2000) (Smad6) function. Thus it appears that Smad1, like the other Smad genes, plays a non-redundant role in regulating murine development.

The murine allantois is an extraembryonic mesodermal structure that originates from precursors in the proximal epiblast that are allocated to the allantois from the posterior primitive streak region at the late streak stage, about 7.0 dpc. The allantois fuses with another extraembryonic structure, the chorion, at about 8.5 dpc to form the chorioallantoic placenta which ultimately provides for nutrient and waste exchange in the developing animal. Specific loss of the allantois, or failure of allantois fusion with the chorion, has been reported for few other mouse mutants (Downs, 1998). Loss of the cell adhesion molecule VCAM-1 (Kwee et al., 1995; Gurtner et al., 1995) or its receptor, α4-integrin (Yang et al., 1995), leads to defects similar to those seen in Smad1 mice. Although we observed a loss of VCAM-1 protein expression in the allantois of Smad1 mutants, expression of VCAM-1 mRNA appeared unchanged. Whether this difference is due to the lack of sensitivity of the immunoassay, or represents a true difference is not discernible from our data. Another possibility is that expression of VCAM-1 is delayed due to a defect in proliferation of the allantois, or a defect in distal differentiation. This is not supported by the RNA expression nor by the fact that flk1 is expressed in the mutant allantois. Vascularization, and hence flk-1 expression, begins distally and extends proximally (Downs et al., 1998), suggesting that distal differentiation is at least initiated in Smad1 mutants even if it is not properly regulated. Expression of other factors is likely necessary for chorioallantoic fusion regulated by Smad1, since loss of VCAM-1 does not exactly phenocopy loss of Smad1 (Gurtner et al., 1995; Kwee et al., 1995).

The ligands and receptors that activate Smad1 during development of the allantois remain to be fully determined. Analysis of mutants homozygous null at both the Bmp7 and Bmp5 locus demonstrated a role for these ligands in allantois development (Solloway and Robertson, 1999). While loss of either ligand alone has no effect on development prior to organogenesis, embryos homozygous null for both ligands display defects in allantois development, somitogenesis, branchial arch formation, and heart and neural development (Solloway and Robertson, 1999). The cellular and molecular mechanisms of the failure of allantois development in these embryos may be unrelated to Smad1, however, as Bmp5;7 mutants showed defects in mTwist expression which we did not observe in Smad1 mutants. Bmp4 mutants can demonstrate a loss of the allantois on a mixed 129SV/Black Swiss background (Lawson et al., 1999). Our analysis was primarily performed on the same background, but analysis on a pure 129Sv/Ev background was similar. Interestingly, the reported Bmp4 null phenotype completely failed to form an allantois, while the Smad1−/− mutants demonstrate defects that are more consistent with a failure to properly proliferate and differentiate. Finally, mutants of Fgfr2 display failure of chorioallantoic fusion in approximately one-third of mutant embryos (Xu et al., 1998). In these animals, the molecular mechanism of the defect is not known, but VCAM-1 and α4-integrin expression appeared normal. Whether FGFR2 and Smad1 cooperate in a signaling pathway is not known, although synergy between receptor tyrosine kinase and Smad signaling pathways has been observed in other systems (de Caestecker et al., 1998). Fully determining the ligand and receptor pathways that are responsible for activation of Smad1 during allantois development and its role in other developmental processes awaits further studies.

**FIG. 6.** Smad1 is not essential for growth inhibition induced by BMP2. Fibroblasts from wild-type (Left) or mutant (Right) embryos were cultured as described. Cells were assayed for [3H]thymidine incorporation following a 24-h treatment with the indicated ligand. Points represent measurements from triplicate wells ± SEM. Each genotype was tested in at least two separate experiments and a representative experiment is shown.
ACKNOWLEDGMENTS

We thank S. Huang for communicating the genomic structure of murine Smad1 before publication, X.-F. Wang for the genomic Smad1 clone, L. Lowe for help with in situ hybridization, and M. Weinstein and X. Yang for helpful discussions and advice. This work was supported in part by USUHS Grant C075GA (to R.J.L.). The opinions and assertions contained herein are the private opinions of the authors and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the U.S. Department of Defense, or the Department of Health and Human Services.

REFERENCES


Received for publication August 1, 2001
Revised August 28, 2001
Accepted August 29, 2001
Published online October 25, 2001