Mice exclusively expressing the short isoform of Smad2 develop normally and are viable and fertile

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Smad2 and Smad3 are closely related effectors of TGFβ/Nodal/Activin-related signaling. Smad3 mutant mice develop normally, whereas Smad2 plays an essential role in patterning the embryonic axis and specification of definitive endoderm. Alternative splicing of Smad2 exon 3 gives rise to two distinct protein isoforms. The short Smad2(Δexon3) isoform, unlike full-length Smad2, Smad2(FL), retains DNA-binding activity. Here, we show that Smad2(FL) and Smad2(Δexon3) are coexpressed throughout mouse development. Directed expression of either Smad2(Δexon3) or Smad3, but not Smad2(FL), restores the ability of Smad2-deficient embryonic stem (ES) cells to contribute descendants to the definitive endoderm in wild-type host embryos. Mice engineered to exclusively express Smad2(Δexon3) correctly specify the anterior–posterior axis and definitive endoderm, and are viable and fertile. Moreover, introducing a human Smad3 cDNA into the mouse Smad2 locus similarly rescues anterior–posterior patterning and definitive endoderm formation and results in adult viability. Collectively, these results demonstrate that the short Smad2(Δexon3) isoform or Smad3, but not full-length Smad2, activates all essential target genes downstream of TGFβ-related ligands, including those regulated by Nodal.

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Transforming growth factor β (TGFβ)-related ligands comprise one of the largest groups of cytokines encoded in the vertebrate genome (Venter et al. 2001). They govern fundamental cell fate decisions in the embryo and are intimately involved in the maintenance of tissue homeostasis throughout postnatal life (for review, see Whitman 1998; Massagué et al. 2000). At the cell surface, the concentration of available ligand is interpreted by a receptor complex containing type I and type II serine–threonine kinases. Ligand-dependent activation of the type I receptor kinase triggers phosphorylation of a family of intracellular effector proteins termed Smads. The activated receptor-regulated Smads (R-Smads) then oligomerize with a structurally related collaborator, Smad4, and this heteromeric complex moves to the nucleus to regulate the transcription of target genes.

Smad2 and Smad3 function as R-Smads downstream of prototypical TGFβ ligands, Nodal, Activin, and some growth and differentiation factors (GDF) (Wall et al. 2000; Miyazawa et al. 2002; Cheng et al. 2003; Shi and Massagué 2003). These two molecules share 92% amino acid identity overall and display even greater similarity in the C-terminal MH2 protein–protein interaction domain (Fig. 1B). Flies and worms, unlike vertebrates, have only one Smad2/3-related gene, dSmad2/Smox and sma-2, respectively. It appears that gene duplication events within the chordate phyla gave rise to Smad2 and Smad3 (Newfeld et al. 1999; Dehal et al. 2002). In mice and humans, these loci show striking similarities in their linkage and genomic organization, with a near one-to-one correspondence in exon size [Fig. 1A]. However, the prevailing model holds that Smad2 and Smad3 have significantly diverged and are nonredundant.

Recent work demonstrates that Smad2 and Smad3 exist in distinct oligomeric complexes at steady state. Thus, Smad2 is found mostly as a monomer within the cytoplasm, while Smad3 constitutively forms higher-order complexes (Jayaraman and Massagué 2000). After ligand-dependent phosphorylation, Smad2 and Smad3 are released from cytoplasmic retention proteins such as SARA and assemble into multimeric complexes with Smad4, and this activated complex is next imported into...
the nucleus [Tsukazaki et al. 1998; Di Guglielmo et al. 2003]. Smad2 interacts directly with nucleopore components via its MH2 domain, whereas Smad3 uses the classical importin-β-dependent nuclear transport pathway (for review, see Reguly and Wrana 2003; Xu and Massagué 2004). Smad3 binds DNA with low affinity via the novel β-hairpin DNA-interaction motif within the MH1 domain [Shi et al. 1998]. In contrast, Smad2 cannot bind DNA due to the presence of a unique 30-amino acid insert that lies just N-terminal to the β-hairpin [Dennler et al. 1999; Yagi et al. 1999]. Interestingly, the inability of Smad2 to interact with itself or other proteins at steady state or to use the importin-β nuclear transport pathway has been attributed to the presence of this unique insert encoded by the alternatively spliced exon 3 (Fig. 1A; Jayaraman and Massagué 2000; Kurisaki et al. 2001).

Heteromeric Smad complexes associate with an array of tissue-specific transcription factors, coactivators, and corepressors [for review, see Massagué and Wotton 2000; Miyazawa et al. 2002; Liu 2003]. Consistent with their different mechanisms of nuclear import and DNA binding, several distinct transcriptional partners have been identified that specifically interact with either Smad2 or Smad3. For example, Smad3 associates with HNF4 to transactivate the apolipoprotein C-III promoter, while Smad3–FoxO protein complexes directly regulate the p21Cip1 growth inhibitory gene to achieve TGFβ-dependent cytostasis [Chou et al. 2003; Seoane et al. 2004]. In addition, Smad2 acts in combination with the forkhead transcription factor FoxH1 (FAST) and Smad4 to activate the goosecoid promoter, while Smad3-containing complexes inhibit expression [Labbé et al. 1998]. Taken together, these observations strongly suggest that Smad2 and Smad3 play unique roles downstream of TGFβ-related growth factors.

Smad2 and Smad3 are further distinguished by their loss-of-function phenotypes. Smad3 mutant mice are viable and fertile [Zhu et al. 1998; Datto et al. 1999; Yang et al. 1999]. In contrast, Smad2 mutant embryos fail to form the specialized extraembryonic signaling center known as the anterior visceral endoderm [AVE] [Waldrip et al. 1998; Heyer et al. 1999]. Consequently, these embryos lack anterior–posterior (A-P) polarity and are highly disorganized by embryonic day 8.5 (E8.5). The dramatic phenotypic difference between Smad2 and Smad3 mutant mice results from the expression of Smad2 and not Smad3 in the AVE [Tremblay et al. 2000]. Recent experiments with Smad2-deficient embryonic stem (ES) cells and studies of conditional gene inactivation within the early epiblast reveal a second, focal requirement for high levels of Smad2 in the specification of definitive endoderm during gastrulation [Tremblay et al. 2000; Vincent et al. 2003]. Similarly, loss of Smad3 in the context of one wild-type Smad2 allele results in a failure to specify anterior primitive streak derivatives during gastrulation [Dunn et al. 2004]. Consequently, these mutant embryos develop anterior truncations that are identical to those observed in embryos with decreased levels of the Nodal ligand [Vincent et al. 2003]. From this more refined genetic analysis, Smad3 emerges as an essential component of the Nodal signal transduction pathway [Liu et al. 2004]. Biochemical studies also show that Smad2 and Smad3 associate with the activated Alk4 type 1 Nodal receptor [Lebrun et al. 1999]. Furthermore, in transcriptional activation assays Smad3 can regulate the ASE autoregulatory enhancer element from the Nodal gene, previously characterized as Smad2–FoxH1 dependent [Osada et al. 2000; Saijoh et al. 2000; Dunn et al. 2004]. These data therefore support an opposing model that Smad2 and Smad3 are to a large degree functionally interchangeable.

Here, we investigate potentially shared or unique functional activities of Smad2 and Smad3 in the embryo. We have previously shown that descendants of Smad2-deficient ES cells are greatly compromised in their ability to colonize the definitive endoderm in chimeric mouse embryos [Tremblay et al. 2000]. In reconstitution experiments, the Smad2Δexon3 splice variant and Smad3, but not full-length Smad2, can compensate for loss of Smad2 and restore these activities. Remarkably, germline deletion of exon 3 from the Smad2 locus results in 100% viability, indicating that expression of full-length Smad2 is dispensable. Finally, replacing

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**Figure 1.** Smad2 and Smad3 structural comparisons. (A) Mouse Smad2 and Smad3 genomic organization. The alternatively spliced Smad2 exon 3 and the corresponding 30-amino acid insert it encodes are shown in red. Noncoding exons are depicted as open rectangles. Smad2 exons 4–11 and Smad3 exons 2–9 are nearly identical in size. (B) Smad2 and Smad3 protein alignments showing the highly conserved N-terminal and C-terminal Mad Homology domains 1 [MH1] and 2 [MH2], respectively, and the intervening proline-rich linker region. The Smad2 MH1 domain also contains an N-terminal glycine-rich 10-amino acid insert [yellow] that is not present in Smad3. Smad2 and Smad3 possess distinct C-terminal SS(V/M)S phosphorylation motifs. The sequences corresponding to those protected by RPA probes are indicated as black lines.
Smad2 coding sequences with Smad3 also results in a homozygous viable phenotype. These results demonstrate unequivocally that the short Smad2(Δexon3) isoform or Smad3 activate all essential TGFβ signaling pathways.

Results

Expression of Smad2 isoforms in the mouse

Alternatively spliced Smad2 transcripts have been previously documented in various human cell lines (Takenoshita et al. 1998; Yagi et al. 1999), and in frogs both Smad2(Δexon3) and Smad2(FL) are coexpressed in unfertilized eggs and during development (Faure et al. 2000). To investigate the expression of Smad2 splice variants in mice, we used a ribonuclease protection assay (RPA) probe spanning the ATG-containing exon 2 and alternatively spliced exon 3 that distinguishes Smad2(FL) and Smad2(Δexon3) transcripts (Fig. 1B). The shorter Smad2(Δexon3) transcript is strongly expressed in ES cells and mouse embryos at all stages examined as well as in adult tissues (Figs. 2A, 4D [below]). Smad2(FL) transcript levels are consistently higher than Smad2(Δexon3), but the FL:Δexon3 ratio decreases at later stages (Fig. 2A). By late gestation (E16.5) the ratio is nearly 1:1 (data not shown). In adults, Smad2(FL) is more abundant in spleen and thymus, while Smad2(Δexon3) is barely detectable in liver (Fig. 4D, below). Among the diverse tissues analyzed here, the FL:Δexon3 ratio is never greater than 3.5:1. These RNase protection assays were repeated several times analyzing independent RNA samples to ensure that the results were reproducible. Our findings contrast with previous RT–PCR results that estimate FL:Δexon3 expression ratios to be 10:1 in human cell lines (Takenoshita et al. 1998; Yagi et al. 1999). Taken together, these results show that the Smad2(Δexon3) splice form is dynamically regulated and represents a much higher proportion of transcripts than previously estimated by RT–PCR. One possibility is that alternative Smad2 isoforms play unique roles in the transduction of TGFβ-related signals in the mouse embryo and adult.

Reconstitution of Smad2 signaling in Smad2-deficient ES cells and colonization of definitive endoderm in chimeric mouse embryos

We previously engineered the Smad2Robm1–/– loss-of-function mutation in which the ATG containing exon 2 is deleted (Waldrip et al. 1998). Two independent homozygous mutant ES cell lines KT11 and KT15 were subsequently derived that constitutively express the Rosa26 lacZ reporter gene (Tremblay et al. 2000). These lines efficiently colonize ectodermal and mesodermal lineages when injected into wild-type host blastocysts, but are generally compromised in their ability to contribute to the definitive endoderm (Heyer et al. 1999; Tremblay et al. 2000).

To assess the unique or common activities of Smad2(FL) and Smad2(Δexon3) isoforms during development, we developed a strategy to restore Smad2 signaling in these Smad2-deficient ES cell lines in gain-of-function transfection experiments. Thus, full-length cDNAs encoding N-terminally Flag-tagged human (h) Smad2(FL), Smad2(Δexon3), or the closely related Smad3 proteins were inserted into a modified version of the pCAGGS vector, which directs robust expression in ES cells and early embryos and confers hygromycin resistance (Fig. 3A; Niwa et al. 1991; Yagi et al. 1999). Since the parental KT11 and KT15 ES cells lack endogenous Smad2(FL) and Smad2(Δexon3) proteins (Tremblay et al. 2000; Dunn et al. 2004), drug-resistant clones were initially analyzed by flow cytometry (FACS) with an anti-Smad2/3 antibody to evaluate hSmad protein levels (Fig. 3A). Smad2(FL) and Smad2(Δexon3) transfected clones display expression levels approximately equal to endogenous Smad2 in wild-type ES cells, while Smad3 transformants exhibit increased levels, which reflects the contribution of endogenous Smad3 (Fig. 3A). Expression of introduced human Smad proteins was also examined by Western blot analysis using the anti-Flag antibody (Fig. 3B). Finally, transfected ES cells were also treated with TGFβ1 or Activin and analyzed with an anti-phospho-Smad2 antibody to verify that hSmad proteins were efficiently phosphorylated by native receptor complexes in ES cells (data not shown). Consistent with findings by Parisi et al. (2003), we find that wild-type CCE ES cells express ActRIIA and ActRIIB type II receptors as well as the Alk2, Alk4, and Alk5 type I receptors (L. Oxburgh, N.R. Dunn, and E.J. Robertson, unpubl.). Collectively these experiments demonstrate that Flag-tagged human Smad2(FL), Smad2(Δexon3), and Smad3 proteins stably expressed in KT11/KT15 ES cell clones potentially function as effectors downstream of Nodal signals.

Transformants were next tested for their ability to
contribute to the definitive endoderm in chimeric embryos [Table 1]. As expected, wild-type lacZ-expressing R26.1 ES cells injected into wild-type host embryos efficiently colonize all embryonic germ layers, whereas Smad2-deficient KT15 ES cell descendants are specifically excluded from the definitive endoderm [Fig. 3C; Varlet et al. 1997; Tremblay et al. 2000]. Transformants expressing hSmad2(Δexon3) or hSmad3 behave similarly to wild type, with ES cell derivatives [blue cells] interspersed among host wild-type cells along the length of the E9.5 gut epithelium [Table 1; Fig. 3C]. It is particularly interesting that hSmad3 expression rescues Smad2 signaling because endogenous Smad3 expression is not compromised in Smad2-deficient ES cells or embryos [Dunn et al. 2004]. Thus, increased Smad3 levels appear to compensate for the loss of Smad2 and permit gut colonization. Despite extensive chimerism throughout the embryo, none of the five KT11 subclones expressing hSmad2(FL) contribute to the definitive endoderm [Table 1; Fig. 3C]. Thus, specification of definitive endoderm is rescued by expression of Smad2(Δexon3) or Smad3 in cells of the anterior primitive streak (APS), but Smad2(FL) expression on its own alone fails to restore Nodal-dependent signaling pathways.

**Mutant mice exclusively expressing Smad2(Δexon3) develop normally**

Results above strongly suggest that Smad2(Δexon3) but not Smad2(FL) plays an essential role during embryonic
ES cells injected into host wild-type blastocysts, the endoderm. Their descendants were specifically excluded from the definitive endoderm. expressing clones extensively contributed to the embryo, but did not play any overt late-onset abnormalities up to 9 mo of age. Adult homozygotes fail to dis- play any overt hypomorphic phenotypes resulting from Smad2exon3; Smad3null double heterozygous progeny were obtained in Mendelian numbers and then intercrossed. To our surprise, Smad2exon3; Smad3null pups were recovered at the predicted Mendelian frequency at weaning (data not shown). These animals are viable and fertile and display no overt haploinsufficiency or Smad3null homozygous adult mice (Datto et al. 1999; Yang et al. 2001; data not shown). We next characterized Smad2exon3; Smad3null protein levels and activation in Smad2exon3; Smad3null homozygous mutant mice. As expected, Smad2exon3; Smad3null homozygotes lack phospho-Smad2(FL), but Smad2exon3 accumulates and is appropriately phosphorylated in response to TGFB1 (Fig. 4F). Collectively, these results show that the Smad2exon3 alone is fully capable of transducing the spectrum of TGFB-related signals in the intact animal.

Human Smad3 expressed under the control of the mouse Smad2 locus partially rescues viability

Smad3 is not normally expressed within the visceral endoderm, a tissue in which Smad2 is required to pattern the AVE. If Smad2exon3 and Smad3 are functionally interchangeable, we reasoned that ectopic expression of Smad3 within the visceral endoderm should lead to normal establishment of embryonic A-P polarity and postnatal viability. To test this possibility, we designed a targeting vector to simultaneously delete coding sequences within exon 2 and introduce the hSmad3 cDNA into the Smad2 locus (Fig. 5A). To be able to distinguish the products arising from the manipulated and wild-type loci and to confirm that the introduced cDNA is appropriately expressed under control of the endogenous regulatory elements, and perhaps more importantly that there is no read-through expression of the endogenous wild-type product, we designed our knock-in alleles using Flag-tagged human Smads that were previously proven to produce biologically active proteins and developed a panel of RNase protection probes that readily distinguish Flag-tagged human and endogenous mouse Smad transcripts. To avoid disruption of cis-acting regulatory elements that neighbor exon 2, we included the first 44 bp of exon 2 within the 5’ homology arm, while the 3’ homology arm overlaps with last 50 bp of exon 2. ES cells carrying the initial knock-in allele [Smad2hSmad3Kneo+] were identified by Southern blot analysis. Derivative subclones lacking the neomycin selection cassette [Smad2hSmad3K+] were recovered following transient transfection with a vector driving expression of Cre recombinase (Fig. 5A–C). Smad2hSmad3K heterozygotes were intercrossed to

Table 1. Transfected ES cells expressing Smad2(exon3) or Smad3, but not Smad2(FL), contribute to the definitive endoderm in chimeric embryos

| Transfected ES cell clone | Chimeras analyzed | Percent colonizationa | >10% β-gal positive cells in guta |
|---------------------------|------------------|------------------------|---------------------------------|
| N-Flag-hSmad2(FL)b        |                  |                        |                                 |
| AA3                       | 4                | 5%–60%                 | 0                               |
| BB2                       | 14               | 5%–80%                 | 0                               |
| BB6                       | 4                | 80%–90%                | 0                               |
| BB7                       | 5                | 10%–90%                | 0                               |
| CC1                       | 4                | 5%–70%                 | 0                               |
| N-Flag-hSmad2(exon3)b     |                  |                        |                                 |
| BB2                       | 6                | 5%–50%                 | 1                               |
| BB10                      | 9                | 30%–95%                | 8                               |
| CC1                       | 1                | 20%                    | 1                               |
| CC3                       | 4                | 30%–90%                | 4                               |
| N-Flag-hSmad3c           |                  |                        |                                 |
| GS                        | 16               | 30%–90%                | 14                              |
| H12                       | 8                | 40%–90%                | 6                               |

aRescue indicates >10% of cells within the gut epithelium were derived from lacZ-expressing ES cell derivatives as assessed in serial sections of chimeric embryos.

bThe parent ES cell line was KT11. We analyzed a total of 31 serially sectioned chimeric embryos obtained from five independent hSmad2(FL)-expressing clones. As for Smad2-deficient ES cells injected into wild-type blastocysts, the hSmad2(FL)-expressing clones extensively contributed to the embryo, but their descendants were specifically excluded from the definitive endoderm.

cThe parent ES cell line was KT15.

development. To further evaluate functional contributions made by the short form of Smad2, we engineered a novel Smad2 allele lacking the alternatively spliced exon 3. Smad2exon3+/+ mice were derived and subsequently intercrossed to generate animals exclusively producing the Smad2exon3 transcript (Fig. 3A–C). Surprisingly, Smad2exon3 homozygotes were born at Mendelian ratios, and matured to viable and fertile adults (data not shown). Adult homozygotes fail to display any overt late-onset abnormalities up to 9 mo of age.

To further characterize the Smad2exon3 mutant allele, total RNA was isolated from the organs of adult littermates and tested for expression of the Smad2(FL) and Smad2(exon3) transcripts. As expected, Smad2exon3 heterozygotes show increased levels of Smad2(exon3) compared to wild type, and Smad2(FL) expression is undetectable in Smad2exon3 homozygotes (Fig. 4D). Furthermore, loss of Smad2(FL) expression has no noticeable effect on endogenous Smad3 expression (Fig. 4E; Dunn et al. 2004). We next assessed Smad2(FL) and Smad2(exon3) activation upon TGFB1 stimulation. The anti-phospho-Smad2 antibody detects low levels of endogenous Smad2(exon3) protein in wild-type T lymphocytes, whereas this isoform is barely detectable in splenic B cells and HepG2 hepatoma cells (Fig. 4F). Stimulation with TGFB1 leads to prominent Smad2(FL) phosphorylation. Despite roughly equal amounts of Smad2(exon3) mRNA expressed by embryonic and adult tissues (Figs. 2A, 4D), the predominant species detectable by Western blot analysis is invariably Smad2(FL) (Fig. 4F), suggesting that Smad2 variants are subject to significant post-transcriptional regulation.

We also evaluated Smad2exon3 protein levels and function in the absence of Smad3. First, Smad2exon3+/+ and Smad3null/+/+ mice were mated. Smad2exon3, Smad3null double heterozygous progeny were obtained in Mendelian numbers and then intercrossed. To our surprise, Smad2exon3; Smad3null homozygous adults (Datto et al. 1999; Yang et al. 2001; data not shown). We next characterized Smad2exon3 and activation in Smad2exon3; Smad3null homozygous mutant mice. As expected, Smad2exon3; Smad3null homozygotes lack phospho-Smad2(FL), but Smad2(exon3) accumulates and is appropriately phosphorylated in response to TGFB1 (Fig. 4F). Collectively, these results show that the Smad2(exon3) alone is fully capable of transducing the spectrum of TGFB-related signals in the intact animal.
generate Smad2<sub>hSmad3ki</sub> homozygous mice. Homozygotes were recovered at a sub-Mendelian frequency (10% vs. expected 25%) at weaning (Table 2), but those that survive to adulthood are viable and fertile.

To determine whether introduction of the <i>hSmad3</i> cDNA cassette and accompanying pA signal dysregulates transcription at the <i>Smad2<sub>Δexon3</sub></i> locus, we designed RPA probes that distinguish mouse and human <i>Smad3</i>. As expected, a 3′-probe derived from the Flag-<i>hSmad3</i> cDNA specifically detects <i>hSmad3</i> transcripts produced by the <i>Smad2<sub>hSmad3ki</sub></i> allele [Fig. 5D]. Introduction of the triply repeated SV40 poly[A] cassette into the <i>Smad2</i> locus greatly attenuates transcription of the remaining downstream <i>Smad2</i> exons. Thus, residual <i>Smad2<sub>Δexon3</sub></i> transcripts are barely detectable in <i>Smad2<sub>hSmad3ki/hSmad3ki</sub></i> adult tissues (Fig. 5D). Importantly, in <i>Smad2<sub>hSmad3ki/+</sub></i> ES cells and viable adults, <i>hSmad3</i> transcripts arising from the manipulated locus are expressed at levels approximately equal to endogenous levels of <i>Smad2</i> [Fig. 5D].

To investigate the onset of lethality, <i>Smad2<sub>hSmad3ki</sub></i> homozygous embryos were collected at various develop-
mental stages. From mid- to late gestation, abnormal embryos were easily recognized due to varying degrees of microcephaly, holoprosencephaly, or anencephaly (Fig. 5F). Histological analysis of six representative mutant embryos at E15.5 and E16.5 reveals limited forebrain and hindbrain development, with no associated defects in the situs of internal organs (data not shown). These anterior patterning defects closely resemble those arising from modulation of Nodal signaling within the APS (Nomura and Li 1998; Song et al. 1999).

One plausible explanation that accounts for the hypomorphic nature of the Smad2hSmad3ki allele is that the presence of the Flag epitope impacts the DNA binding or nuclear import functions within the MH1 domain, or alternatively augments the autoinhibitory interactions between the MH1 and MH2 domains (Hata et al. 1997). Additionally, Smad2 contains a 10-amino acid glycine-rich insert within the extreme N-terminal MH1 domain not found in Smad3 (Fig. 1B) that potentially regulates selection of DNA-binding partners. Finally, the intro-

Table 2. Summary of phenotypic disturbances observed in Smad2hSmad3ki intercross progeny

| Age  | +/+ | Smad2hSmad3ki/+ | Smad2hSmad3ki/3ki | RS/EDa | AADb |
|------|-----|----------------|-----------------|-------|------|
| E9.5 | 15  | 16             | 6               | 4     | 0    |
| E15.5| 4   | 11             | 4               | 0     | 2    |
| E16.5| 15  | 28             | 8               | 6     | 4    |
| Total (%) | 34 (32%) | 55 (51%) | 18 (17%) |       |      |
| Weanlings (%) | 33 (32%) | 60 (58%) | 10 (10%) |       |      |

a The presence of resorption sites (RS) or empty decidua (ED) at the time of dissection suggests that some mutants die with more severe developmental abnormalities. Insufficient material for genotype determination.

b Number of Smad2hSmad3ki/3ki embryos with abnormal anterior development (AAD).

c Percentage of total embryos or weanlings genotyped. A loss of more than half of Smad2hSmad3ki homozygotes was observed across more than three generations of breeding. The surviving homozygotes show normal fertility, and in homozygous crosses, ∼50% of the embryos develop normally, whereas the others die in utero or at birth due to anterior patterning defects. Since the Smad2hSmad3ki mice were maintained on a mixed [C57BL/6J × 129Sv/Ev] background, these distinct phenotypes may be due to genetic background differences.
duction of the human versus mouse Smad3 cDNA by homologous recombination could lead to subtle functional differences, as has been recently illustrated with mice humanized for the EGF receptor [Sibilia et al. 2003]. Nevertheless, the fact that a significant number of Smad2hSmad3k allele homozygotes are viable provides compelling evidence that Smad2(Δexon3) and Smad3 are the key regulators of TGFβ-signaling pathways.

Discussion

Smad2(FL) is nonessential for viability

Recent transcriptional activation assays comparing Smad2-deficient and Smad3-deficient mouse embryonic fibroblasts (MEFs) have identified distinct Smad2 and Smad3 downstream target genes. For example, Smad7 expression requires Smad3, whereas matrix metalloproteinase 2 regulation is dependent on Smad2 [Pick et al. 2001]. Moreover, transcriptional profiling of these mutant MEFs reveals that in response to TGFβ stimulation, Smad3 activates a set of immediate-early genes that encode signal transducers and transcriptional regulators, while Smad2 appears to negatively modulate a number of these same genes [Yang et al. 2003]. Similarly, morpholino knock-down experiments in HaCaT keratinocytes demonstrate that Smad3 serves as the principal effector of TGFβ-mediated growth arrest, while loss of Smad2 has no detectable effect [Kretschmer et al. 2003]. Thus, considerable evidence suggests that the closely related Smad2 and Smad3 effectors are not redundant but, rather, have distinct activities and transcriptional targets. This difference is likely due to the presence of the unique 30-amino acid insert found just N-terminal to the β-hairpin in the Smad2 MH1 domain.

Here, we find quite unexpectedly that replacing the mouse Smad2 gene with a Flag-tagged human Smad3 cDNA results in adult viability. Thus, Smad3 expressed under the control of the endogenous Smad2 locus correctly regulates the panoply of target genes that lies downstream of TGFβ-related signals and is required for patterning the early embryo. This result is especially surprising since some of these targets such as Nodal and Ptk2 were previously characterized by in vitro assays to be Smad2-dependent [Saina et al. 2000; Shiratori et al. 2001]. In particular, we have shown that Smad2 is absolutely required in the visceral endoderm to specify the AVE [Waldrip et al. 1998]. Smad3 is not normally expressed in this tissue. The ectopic expression of Smad3 by the Smad2hSmad3k allele restores Smad2 signaling and unequivocally demonstrates that Smad3 can activate the genetic program that specifies the AVE signaling center. Thus, irrespective of well-documented differences in DNA binding, nuclear import, oligomeric states, and interacting partners, the present experiments provide clear evidence that Smad2 and Smad3 are functionally interchangeable in the intact animal. In particular, it is the Smad2(Δexon3) transcript generated by alternative splicing that contributes essential activities, whereas Smad2(FL) is nonessential.

Smad2 and Smad3 genetic interactions revisited

Smad2+/−; Smad3−/− mouse embryos display anterior patterning defects identical to conditional loss of Smad2 within the epiblast [Vincent et al. 2003; Dunn et al. 2004]. These strikingly similar phenotypes result from the loss of anterior definitive endoderm and prechordal plate that emerge from the APS during early gastrulation, and suggest that Smad2 serves as the predominant intracellular effector of Nodal signaling in the early embryo [Dunn et al. 2004]. In other words, Smad3 levels within the epiblast of Smad2 conditional mutant embryos as well as in chimeras mostly composed of Smad2-deficient ES cell descendants are inadequate to compensate for the loss of Smad2. We were unable to precisely determine the expression ratios of Smad2(FL), Smad2(Δexon3), and Smad3 within the small population of epiblast cells that ingresses through the APS, but our results nevertheless demonstrate that the Smad2(Δexon3) isoform acting alone can correctly specify APS derivatives that come to underlie and pattern the anterior neural plate. Our results therefore suggest that the failure to pattern the APS in these mutant contexts results from the specific loss (or decreased levels) of Smad2(Δexon3).

Several elegant studies with Activin and TGFβ1 have shown that the number of occupied cell surface receptors is proportional to the nuclear concentration of activated Smad2 [Shimizu and Gudon 1999; Bourillot et al. 2002; Inman et al. 2002]. Decreased concentrations of intracellular Smads are therefore predicted to significantly impact target gene regulation. Consistent with this, our previous work shows that the population of cells most sensitive to fluctuating Nodal levels resides within the APS [Norris et al. 2002; Vincent et al. 2003; Dunn et al. 2004]. In the wild-type embryo, coexpression of the short Smad2(Δexon3) isoform and Smad3 may serve to selectively amplify Nodal signals in these discrete cell types.

We also engineered Smad2hSmad2(FL) and Smad2hSmad2(Δexon3) knock-in mutations via the identical strategy described above for the construction of the Smad2hSmad3k allele. Disappointingly, both these alleles recapitulated the Smad2-null phenotype, with characteristic failure to establish A-P polarity and formation of abundant extraembryonic mesoderm at E8.5. Heterozygous mutant embryos were found to express Flag-hSmad2(FL) and Flag-hSmad2(Δexon3) proteins at levels equivalent to wild-type Smad2 [data not shown]. In contrast, microinjection experiments analyzing transfected Smad2-deficient ES cells expressing Flag-tagged Smad2 isoforms for their ability to reconstitute chimeras exploited a strong ubiquitous promoter to drive expression throughout development, and in this case rescue of gut colonization only requires Smad2 activity over a narrow time window and at restricted tissue sites. In contrast, complete rescue of normal embryonic development requires appropriate expression of knock-in alleles under control of the endogenous regulatory elements governing wild-type Smad2 levels at earlier egg cylinder stages in the visceral endoderm and the epiblast. The inability of Flag-tagged human Smad2 isoforms to rescue early em-
bryos is, indeed, quite perplexing. It seems most likely that then N-terminal Flag-epitope more strongly interferes with Smad2 than Smad3 functional activities. Nevertheless, the present positive results conclusively demonstrate that roughly half of the Smad2Δexon3 homozogotes develop normally and are viable and fertile. Thus, we conclude that Smad3 can activate all essential TGFβ/Nodal/Activin signaling pathways.

Evolution of vertebrate Smad2 and Smad3

Nodal homologs have been identified in both chordates and echinoderms (Morokuma et al. 2002; Yu et al. 2002; Duboc et al. 2004). In sea urchins, Nodal expression in the presumptive oral ectoderm is absolutely required for the development of oral structures and regulates Bmp4 expression in the extraembryonic ectoderm is positively regulated by Nodal signals emanating from the proximal epiblast (Brennan et al. 2001; Beck et al. 2002). Thus, we propose that Nodal and its requisite intracellular effector Smad2 are components of an evolutionarily conserved signaling paradigm for organizing the basic body plan. Indeed, the sea urchin genome project has recently identified a Smad2/3-related gene [GenBank Trace Repository, http://sugp.caltech.edu].

In mice, Smad2 is tightly linked to Smad4 and Smad7, which encodes an inhibitory Smad, on chromosome 18, while Smad3 is linked to Smad6, a second inhibitory Smad, on chromosome 9 [http://www.ensembl.org]. This parallel genomic organization is also conserved in humans (OMIM). It therefore appears that Smad3 arose in the vertebrate lineage due to a partial duplication of an ancestral “Smad” cassette. Since Smad3-null mutants exhibit phenotypes primarily confined to the regulation of the immune system, we therefore speculate that this duplication event coincides with the emergence of adaptive immunity among the jawed vertebrates (Datto et al. 1999; Yang et al. 1999; Kasahara et al. 2004). This prediction is also consistent with our dose-dependency model, which emphasizes the predominant role of Smad2 in axis specification and germ-layer formation in the early embryo, with amplifying or reinforcing activity provided by Smad3 in the primitive streak (Dunn et al. 2004). Indeed, a high percentage of chimeras derived from Smad2-deficient ES cells, whose definitive endoderm is wild type in origin, are overtly normal and fertile (our unpublished results).

Rare inactivating mutations in Smad2 have been identified in a proportion of human colorectal and lung cancers, whereas similar genetic lesions in Smad3 have not been characterized (Arai et al. 1998; Derynck et al. 2001; Siegel and Massagué 2003). It will be interesting to learn whether the tumor-suppressor activity of Smad2 is contributed by the short Smad2Δexon3 isofrom. In a recent study, reduced Smad3 levels were observed in more than one-third of gastric tumors (Han et al. 2004). The loss of TGFβ-dependent cell cycle arrest and promotion of tumorigenesis may therefore be accomplished by the combined levels of Smad2Δexon3 and Smad3 falling below a threshold that normally ensures the activation of the cyclin-dependent kinase inhibitors p15 and p21 and the maintenance of cytostasis (Feng et al. 2000; Pardali et al. 2000). Release from the antiproliferative effects of TGFβ may also occur by direct phosphorylation of Smad2Δexon3/Smad3 by the cyclin-dependent kinases, which inhibits their transcriptional activity (Matsuura et al. 2004).

Splicing among the Smad genes

To date, alternative splicing has been described for the Smad2, Smad3, Smad4, Smad5, and Smad8 genes (Takeno et al. 1998; Nikita et al. 1999; Pierreux et al. 2000; Kjellman et al. 2004). For example, six Smad4 splice variants have been identified that differ in the length of the linker region. Each of these isoforms retains its ability to interact with Smad2 and Smad3, but only three are capable of robust transcriptional activation (Pierreux et al. 2000). We speculate that structurally distinct Smad protein isoforms equip target cell populations with a versatile intracellular machinery to accommodate the array of extracellular TGFβ-related signals, and provide an important mechanism for modulating target gene activity (Lareau et al. 2004). A comprehensive understanding of the individual contributions of the growing Smad proteome awaits future genetic analysis.

Materials and methods

Derivation of ES cell lines and production of chimeric embryos

N-terminally Flag-tagged human (h) Smad2(FL), Smad2Δexon3, and Smad3 cDNA cassettes (Yagi et al. 1999) were subcloned into a modified version of pCAGGS (Niwa et al. 1991). Each expression vector was linearized and electroporated into either the KT11 or KT15 Smad2Robm1 homozygous ES cell lines that constitutively express the Rosa26 lacZ reporter (Tremblay et al. 2000). Hygromycin-resistant clones were initially screened for expression of the introduced construct by flow cytometry. For cytoplasmic staining, cells were treated with 10% formalin and extensively washed with PBS containing 0.1% saponin. STO feeder cells were eliminated from the analysis by appropriate gating. Primary antibodies [Ab] included mouse anti-Smad2/3 [Transduction], which cross-reacts with hSmad2/3, and, as a control, mouse anti-β-galactosidase [Promega]. The secondary reagent used was FITC-conjugated goat anti-mouse IgG (H + L; Caltag). Fluorescence was analyzed using a FACScan flow cytometer, and the data are displayed as cell number versus log fluorescence. The cell lines developed in this study [Table 1] as well as the control ES cell lines R26.1 [Varlet et al. 1997] and KT15 were separately injected into host ICR [Tacoma] blastocysts [Nagy et al. 2003]. Manipulated embryos were recovered between E9.0 and E10.0, X-gal stained in whole mount, embedded in paraffin wax, and serially sectioned according to standard procedures.

Generation of Smad2 mutant alleles

Deletion of Smad2 coding exon 3. A 488-bp KpnI–NcoI fragment of the Smad2 gene that contains the alternatively spliced
exon 3 was replaced with a loxP-flanked PGK-neomycin neo resistance cassette by homologous recombination in ES cells. The targeting vector contains 2.4-kb 5' and 1.3-kb 3' homology arms. Linearized vector was electroporated into CCE ES cells, and drug-resistant colonies were genotyped by Southern blot analysis using either a 5'-external 1.6-kb EcoRI-HindIII probe (Fig. 3B, Nomura and Li 1998) or a 3'-internal 962-bp Spel–EcoRI probe with KpnI digestion of genomic DNA. Four correctly targeted clones out of 2200 were identified.

Introduction of Smad3 coding sequences into the mouse Smad2 locus. The ATG-containing exon 2 of Smad2 was disrupted by homologous recombination with a vector containing 2.7 kb of 5' homology, followed by an N-terminally Flag-tagged human Smad3 cDNA [above], a triply repeated SV40 polyadenylation cassette to ensure transcriptional termination [Maxwell et al. 1989], a loxP-flanked PGK-neo cassette, and 1.86 kb of 3' homology. A PGK-dta cassette was used for negative selection. Linearized vector was electroporated into CCE ES cells, and drug-resistant colonies were genotyped by Southern blot analysis with a 5'-external probe as previously described (Waldrip et al. 1998) or a 3'-internal 587-bp EcoRV–Spel probe with Spel digestion of genomic DNA. Five correctly targeted clones out of 600 were identified.

For both Smad2 alleles, targeted clones were transiently transfected with Cre recombinase. Loss of the neo cassette was verified by Southern analysis with a 630-bp PstI–XbaI neo probe. At least two independently targeted ES cell clones were used to generate germline chimeric mice.

Mouse strains and genotyping procedures

\[ \text{Smad2}^{+/+} \] and \[ \text{Smad2}^{+/+} \] mice were outcrossed to ICR, and then each line was maintained by intercrossing. Outbred \[ \text{Smad2}^{+/+} \] and \[ \text{Smad2}^{+/+} \] mice were intercrossed. The PCR primers used to detect the \[ \text{Smad2}^{+/+} \] allele were \[ \Delta 2-1, 5'-GAACTGATCCTCCTGTTGAGAGA-3' \] and \[ \Delta 2-5, 5'-TTTACACGTCAGTTTGGG-3' \]; FLAG1, \[ 5'-CATGCTTCTTGATCAGTTG-3' \] and B5, \[ 5'-CAGTTATGGCCACCGCTGATACTTACG-3' \]; and \[ \text{Smad2}^{+/+} \] allele were \[ \text{Smad2}^{+/+} \] and \[ \text{Smad2}^{+/+} \] with a rabbit anti-phospho-smad2 (Cell Signaling), followed by HRP-conjugated donkey anti-rabbit secondary (Amersham). Blots were developed by chemiluminescence using ECL (Amersham).

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