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Formation of the definitive endoderm in mouse is a Smad2-dependent process

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SUMMARY

TGFβ growth factors specify cell fate and establish the body plan during early vertebrate development. Diverse cellular responses are elicited via interactions with specific cell surface receptor kinases that in turn activate Smad effector proteins. Smad2-dependent signals arising in the extraembryonic tissues of early mouse embryos serve to restrict the site of primitive streak formation and establish anteroposterior identity in the epiblast. Here we have generated chimeric embryos using *lacZ*-marked Smad2-deficient ES cells. Smad2 mutant cells extensively colonize ectodermal and mesodermal populations without disturbing normal development, but are not recruited into the definitive endoderm lineage during gastrulation. These experiments provide the first evidence that TGFβ signaling pathways are required for specification of the definitive endoderm lineage in mammals and identify Smad2 as a key mediator that directs epiblast derivatives towards an endodermal as opposed to a mesodermal fate. In largely Smad2-deficient chimeras, asymmetric *nodal* gene expression is maintained and expression of *pitx2*, a *nodal* target, is also unaffected. These results strongly suggest that other Smad(s) act downstream of Nodal signals in mesodermal populations. We found *Smad2* and *Smad3* transcripts both broadly expressed in derivatives of the epiblast. However, *Smad2* and not *Smad3* mRNA is expressed in the visceral endoderm, potentially explaining why the primary defect in Smad2 mutant embryos originates in this cell population.

Key words: TGFβ signaling, Smad, Definitive endoderm

INTRODUCTION

Recent studies demonstrate that signals originating in extraembryonic populations of the early mouse embryo, namely the primitive visceral endoderm and extraembryonic ectoderm, establish the initial anteroposterior (AP) pattern in the underlying epiblast (reviewed Beddington and Robertson, 1998, 1999). Moreover, fate-mapping studies show that allocation of epiblast cells to form the three germ layers during gastrulation depends on their relative position along the AP/proximodistal axes (reviewed Lawson et al., 1991). Thus epiblast cells on the prospective proximal anterior side mostly contribute to ectoderm derivatives, while cells delaminating from the epiblast at the edges of the primitive streak contribute to mesoderm or definitive endoderm lineages in a temporally and spatially dependent fashion. The earliest cells to delaminate through the streak largely form posterior extraembryonic mesoderm while those delaminating slightly later from more distal locations in the streak contribute to both embryonic mesoderm and definitive endoderm derivatives. However, the local molecular cues causing these distal cells to adopt an endodermal versus a mesodermal fate have yet to be elucidated.

TGFβ-related growth factors regulate tissue interactions at multiple stages during early mouse development (reviewed Hogan, 1996; Whitman, 1998). For example, Nodal is required for primitive streak formation, anterior patterning and left-right (LR) axis formation (reviewed Schier and Shen, 2000). TGFβ ligands transmit their signals via distinct cell surface serine-threonine receptor kinases classified as type I or type II according to structural and functional criteria (reviewed Massague and Weis-Garcia, 1996). The diverse ligand binding and signaling properties of TGFβ receptor kinases have been extensively analyzed (reviewed Massague, 1998). A family of intracellular effector molecules, the Smad proteins, act downstream of TGFβ receptors. Moreover, functionally distinct type I receptors have been shown selectively to phosphorylate different Smad proteins (reviewed Massague, 1998; Whitman, 1998). Consequently, these receptor-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), OAZ (Hata et al., 2000) or Mix family members (Germain et al., 2000) controlling target gene expression (reviewed Derynck et al., 1998; Wrana, 2000).

Two functionally distinct Smad signaling pathways have been described. For example, in *Xenopus* explant assays Smad2 mimics TGFβ/activin and induces dorsal mesoderm (Baker and Harland, 1996; Graff et al., 1996) whereas Smad1 elicits BMP-like responses, namely the production of ventral mesoderm (Graff et al., 1996; Thomsen, 1996). Recent gene targeting

experiments have demonstrated important roles played by Smad family members during early mammalian development. Mouse embryos lacking Smad4, a protein common to all TGFβ signaling pathways, die shortly after implantation due to defects in primitive endoderm formation (Sirard et al., 1998; Yang et al., 1998). Loss of Smad5 causes multiple defects in embryonic and extraembryonic tissues beginning at day 8 of development (Chang et al., 1999; Yang et al., 1999a), a phenotype consistent with disruption of the functions of multiple BMP family members (Winnier et al., 1995; Zhang and Bradley, 1996; Solloway and Robertson, 1999). In contrast, Smad3, thought to act downstream in the TGFβ/activin pathway, is not essential for embryonic development. Rather adult Smad3-deficient mice develop immunological defects and colon tumors (Datto et al., 1999; Yang et al., 1999b; Zhu et al., 1998).

Another family member Smad2 closely resembles Smad3 and similarly transduces TGFβ/activin signals. However, Smad2-deficient embryos become abnormal shortly after implantation and entirely lack tissues of the embryonic germ layers (Heyer et al., 1999; Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998). Smad2 activity is not required for mesoderm production per se. Rather, in the absence of Smad2, the entire epiblast adopts an extraembryonic mesodermal fate giving rise to a normal yolk sac and fetal blood cells (Heyer et al., 1999; Waldrip et al., 1998). We previously showed that Smad2-dependent signals contributed by the extraembryonic tissues are essential for establishing AP identity within the epiblast. Consistent with this, Smad2-deficient visceral endoderm forms normally, but fails to express anterior visceral endoderm (AVE) markers. The early onset of these defects precluded further studies of possible Smad2 functions in derivatives of the epiblast.

To examine possible Smad2 requirements in the later embryo here we studied the behavior of *lacZ*-marked Smad2-deficient ES cells in chimeric embryos. ES cell derivatives lacking Smad2 can extensively colonize ectodermal and mesodermal populations without disturbing normal development. However, Smad2-deficient cells are very rarely found in the definitive endoderm lineage. The fact that gastrulation and LR axis formation proceed normally in strongly chimeric embryos argues that Smad2 is not required to transduce Nodal signals in the embryo proper. Rather another Smad protein, possibly the highly related family member Smad3, may mediate Nodal activities. Consistent with this suggestion, we found *Smad2* and *Smad3* transcripts both broadly expressed in derivatives of the epiblast. However, *Smad2* and not *Smad3* mRNA is expressed in the visceral endoderm, potentially explaining why the primary defect in Smad2 mutant embryos originates in this cell population. Collectively, our findings provide the first evidence that TGFβ signaling pathways are required for specification of the definitive endoderm lineage in mammals, and identify Smad2 as a key mediator that directs epiblast derivatives towards an endodermal as opposed to a mesodermal fate. In addition these findings suggest that distinct molecular pathways are responsible for the formation of the primitive and definitive endoderm lineages in mammals.

MATERIALS AND METHODS

Mouse strains and ES cell derivation

The *Smad2^{Robm1}* allele was maintained on an inbred 129/Sv//Ev background and animals genotyped by PCR as described (Waldrip et al., 1998). Animals carrying the ROSA26 gene trap integration (Friedrich and Soriano, 1991) were obtained from the Jackson Laboratories (Tg(Rosa26)RSor strain, Jackson Laboratories, Bar Harbor) and maintained by backcrossing to 129/Sv//Ev partners. ES cell lines were isolated using standard procedures (Robertson, 1987) from delayed blastocysts obtained from *Smad2^{Robm1}*/+ females crossed to *Smad2^{Robm1}*/+, *Rosa26*/+ males. Individual cell lines were genotyped with respect to the *Smad2* locus by Southern blot analysis. Genotyping for the ROSA26 allele was assessed by staining cells for β-galactosidase activity (Hogan et al., 1994). The experiments presented here were carried out using one *lacZ*+ wild-type ES cell line (KT4), two independent *lacZ*+ *Smad2^{Robm1}* heterozygous ES cell lines (KT1, KT 2), two independent *lacZ*+, *Smad2^{Robm1}* homozygous ES cell lines (KT11, KT 15), and a single *Smad2^{Robm1}* homozygous ES cell line (KT5). Cell lines were routinely grown on STO feeder layers and induced to differentiate using suspension culture techniques as described (Robertson, 1987).

Generation and analysis of chimeric embryos

Chimeras were generated by blastocyst injection as described (Bradley, 1987). Blastocysts were collected from matings between C57BL/6J (Jackson Laboratories, Bar Harbor) or outbred ICR strain females (Taconic) and C57BL/6J males. Between 10 and 15 ES cells were injected into the blastocoel cavity. Following transfer into pseudopregnant foster females, the manipulated embryos were recovered at the desired stage of development, fixed in X-gal buffer (PBS, 5 mM EGTA, 2 mM MgCl:6H_2O, 0.2% NP-40, 0.2 mM deoxycholate) containing 1% formaldehyde and 0.2% gluteraldehyde, washed three times in X-gal buffer and stained overnight at 37°C in X-gal buffer containing 5 mM of both potassium ferricyanide and potassium ferrocyanide and 0.5 mg/ml X-gal. After washing three times in X-gal buffer, embryos were then postfixed overnight at 4°C in 4% paraformaldehyde. Embryos were then washed three times in PBS, dehydrated and embedded in paraffin wax for sectioning. 7-8 μm sections were dewaxed using standard procedures, mounted in Cytoseal and photographed under Nomarski optics.

Section and whole-mount in situ hybridization

Radioactive (Jones et al., 1991) and whole-mount (Wilkinson, 1992) in situ hybridization were performed as described. The probes used for radioactive in situs include a full-length *Smad2* cDNA probe (P. Hoodless, unpublished) and a 424 bp *Smad3* EST corresponding mostly to 5′UTR (GenBank accession no. AA023641). Sections were photographed using a Leitz DMR microscope and Fujichrome Velvia color slide film. The whole-mount probes used to examine *nodal* (Varlet et al., 1997) and *Pitx2* (Ryan et al., 1998) were as described.

Embryoid body formation and analysis

Embryoid bodies were harvested following 4 or 9 days of suspension culture. Marker gene expression was analyzed either by RT-PCR or by radioactive in situ hybridization of serially sectioned embryoid bodies. For RT-PCR, 1 μg of total RNA was reverse transcribed using MMLV reverse transcriptase (Gibco/BRL). 0.5-2.0 ml of the RT reaction were subject to PCR using several sets of primers for 28-32 cyles. The primers used were directed toward *HNF-3α* (forward 5′ GTAGACAGTAGGGGCTC, reverse 5′ GGGGAATCCTTAAACGG) (Farrington et al., 1997), *Hex* (forward 5′ GTTCTCCAACGA-CCAGACCG, reverse 5′ GGAGGGTGAACACTGCGAAC), *IFABP* (forward, 5′ GGAAAGGAGCTGATTGCTGCTC, reverse 5′ CTTTGACAAGGCTGGAGACCAG). The primer-pair used as a control to normalize the ethidium-bromide stained agarose gel was *β-actin*. The *β-actin* forward primer was 5′ GTGATGGTGGGAATG-GGTCA 3′ and the reverse primer was 5′ TTTGATGTCACG-CACGATTTCC 3′ (Dick et al., 1998). The probes used for in situ hybridization were: *Cer-l* (Thomas et al., 1997), *Hex* (Bedford et al.,

1993), *HNF3β* (Sasaki and Hogan, 1994), *Lim1* (Barnes et al., 1994) and *Otx2* (Ang et al., 1994).

5′ RACE, northern and western blots

5′RACE was performed using the Marathon cDNA amplification kit (Clonetech). Poly(A)$^+$ RNA from 13.5 dpc embryos as well as wild-type and Smad2 mutant ES cells was subjected to reverse transcription using a modified oligo(dT) primer. Following second-strand synthesis, the double-stranded cDNA was blunt ended and ligated to Marathon cDNA Adaptors (Clonetech). 5′RACE was then performed with these cDNA pools. The primers used were: AP1(Adaptor primer) 5′ CCATCCTAATACGACTCACTATAGGGC 3′; Sm2Race2 5′ GGCA-AGACTGGGGTCTCAACTCTCTGG 3′; Sm2Race3 5′ GACAGTT-CAGCCGGAGAGCCTGTGTCC 3′. The PCR conditions were 94°C for 30 seconds, 5 cycles at 94°C for 5 seconds and 73°C for 1 minute 15 seconds, followed by 5 cycles at 94°C for 5 seconds and 71°C for 1 minute 15 seconds, and finally 27 cycles at 94°C for 5 seconds and 69°C for 1 minute 15 seconds. Northern blots were prepared by electrophoresis of 2 μg of poly(A)$^+$ RNA on a 1.3% agarose-formaldehyde gel followed by transfer onto GeneScreen Plus (NEN research products). Filters were hybridized with ^{32}P-random primed full-length *Smad2* cDNA probe (P. A. H., unpublished data). For western blot analysis, ES cell extracts (75 μg/lane), in TNTE (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 0.5% (v/v) Triton X-100), were separated on a 9% SDS-PAGE gel and transferred to nitrocellulose. Control extracts were from mock-transfected COS cells, or COS cells transfected with constructs encoding full-length Smad2, or the MH2 domain (amino acid 241 to COOH terminus). Membranes were incubated with Smad2 antibody (Santa Cruz Biotechnology, N-19 or E20, specific for the MH1 and MH2 domain respectively, 1/1000 dilution) in 5% non-fat dry milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20).

RESULTS

Generation and characterization of Smad2 homozygous ES cells

Smad proteins contain highly conserved amino- and carboxy-terminal regions referred to as the MH1 and MH2 domains, respectively, that have distinct functional properties. For example, the MH1 domain stably binds to DNA while the MH2 domain acts as a transcriptional activator (reviewed Whitman, 1998). A number of Smad2 mutant alleles have been generated (Heyer et al., 1999; Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998) and the mutant phenotypes seem to differ according to the targeting strategies used. Smad2 alleles in which the 5′ exons encoding the MH1 domain were left intact caused early arrest resulting in embryo failure prior to gastrulation (Nomura and Li, 1998; Weinstein et al., 1998). Moreover, one of these Smad2 mutant alleles was shown to have a pronounced heterozygous defect and aproximately 10% of heterozygous embryos die in utero (Nomura and Li, 1998). In marked contrast, two independent mutant alleles generated by deletion of the first coding exon give rise to Smad2 mutant embryos that survive longer and form mesoderm (Heyer et al., 1999; Waldrip et al., 1998). These differences could potentially be attributed to genetic background effects. Alternatively, it is also possible that expression of a truncated MH1 domain-containing Smad2 protein acts in a dominant-negative fashion disrupting the function of other Smad family members and causing the more severe defects associated with the strategies used by Noruma and Li (1998) and Weinstein et al. (1998). Finally, deletion of exon 1 coding information potentially generates a hypermorphic allele, due to recognition of a downstream in-frame initiation codon(s) and production of a truncated protein containing the carboxyl-terminal MH2 domain.

To characterise product(s) arising from our *Smad2^{Robm1}* exon 1 deletion allele (Waldrip et al., 1998), we generated homozygous Smad2 mutant ES cells from blastocysts obtained from Smad2 heterozygous females crossed to Smad2 heterozygous males carrying the ROSA-26 *lacZ* gene-trap integration, which is ubiquitously expressed in the developing embryo (Friedrich and Soriano, 1991). A panel of 20 cell lines was genotyped by Southern analysis (Fig. 1A) and by X-gal staining. Three independent homozygous mutant cell lines

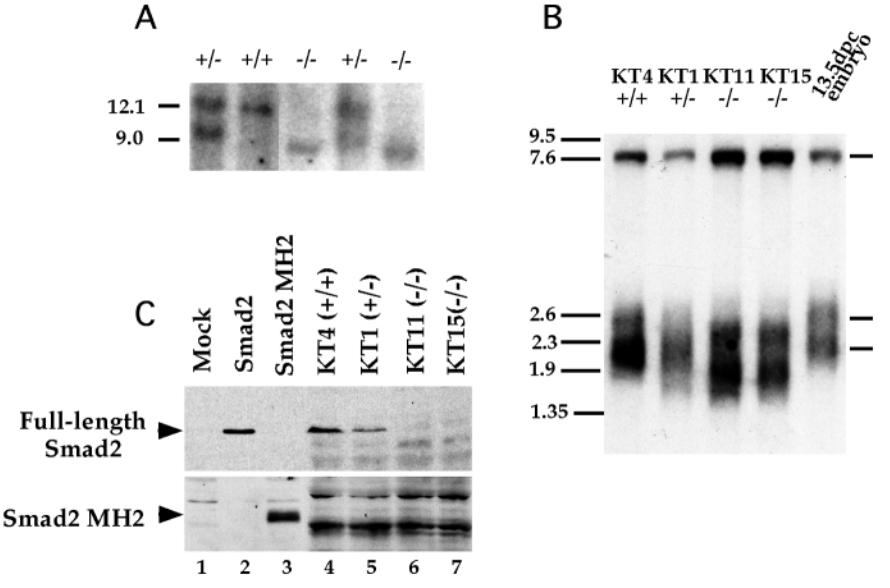

Fig. 1. Molecular characterization of Smad2-deficient ES cell lines. (A) Southern blot analysis of mutant (–/–), heterozygous (+/–) and wild-type (+/+) ES cells. The 5′ probe hybridized to *EcoR*I-digested DNA identifies a 12.1 kb wild-type and a 9.0 kb mutant fragment (Waldrip et al., 1998). (B) Northern blots were probed with a full-length *Smad2* cDNA (P. A. H., unpublished). The probe recognizes three transcripts of 7.6, 2.4 and 2.0 kb (indicated on the right of the gel) in the wild-type samples. The 2.4 and 2.0 kb transcripts are slightly smaller in the mutant samples. (C) Western blot probed with the E-20 antibody raised against the MH2 domain (amino acids 186-273). Lysates were prepared from COS cells transfected with a full-length *Smad2* cDNA (Smad2), or a vector encoding the Smad2 MH2 domain (241-end), or non-transfected ES cell lines as indicated. The positions of the intact or truncated Smad2 protein are indicated by the arrowheads. The E-20 antibody is predicted to show reactivity towards a 25 kDa truncated protein resulting from a cryptic translation start site in the linker region. Note that the lower molecular weight bands present in all ES cell samples represent non-specific background.

(KT5, KT11 and KT 15), two heterozygous cell lines (KT1 and KT2), and a single wild-type cell line (KT4) were further analysed. Homozygous Smad2 mutant ES cells contain transcripts approximately 300 bp smaller than wild type as expected if transcripts arise by splicing around the PGKneomycin cassette replacing the first coding exon (Fig. 1B). This predicted configuration was confirmed by RT-PCR and 5′RACE analysis (data not shown). These transcripts contain 22 initiation codons, only 3 of which are in-frame and could, in theory, generate a truncated protein. The largest of these including part of the linker region together with the entire MH2 domain has a predicted size of 25 kDa. To test for Smad2 expression, we probed western blots with MH1 domain (deleted in the *Smad1*^{Robm1} allele) and MH2 domain-specific antibodies. There was no product detectable using either antibody (Fig. 1C and data not shown). Thus our *Smad2*^{Robm1} mutation represents a null allele, a conclusion similarly reached by Heyer et al. (1999) who used the same strategy to disrupt Smad2 function.

Smad2$^{-/-}$ ES cells differentiate extensively and appear morphologically indistinguishable from wild type cells. To test for functional restrictions, embryoid bodies were examined using radioactive in situ hybridization techniques for the expression of cell-type-specific markers, including genes expressed in the AVE not detected in Smad2-deficient embryos. Patches of cells expressing *Cer1*, *HNF3β*, *Lim1*, *Hex* or *Otx2* transcripts were detected in *Smad2*$^{-/-}$ embryoid bodies (data not shown). Similarly RT-PCR analysis demonstrated the presence of *Hex*, *HNF3α* and *IFABP* transcripts in Smad2 mutant samples after 9 days of culture. Under these in vitro conditions, we clearly observe Smad2-independent expression of endoderm-specific marker genes. In contrast, Heyer et al. (1999) failed to detect *Hex* mRNA expressed by Smad2-deficient embryoid bodies, possibly due to a lack of *Hex*-expressing endothelial cells formed only after longer term culture.

Smad2-deficient cells contribute extensively to embryonic mesoderm and ectoderm but rarely colonize the definitive endoderm

When introduced into blastocysts, ES cells predominantly colonize the embryonic epiblast, allowing the generation of chimeric embryos in which the extraembryonic tissues are derived exclusively from the host, while both host and ES cell derivatives contribute to the embryonic region (Beddington and Robertson, 1989). This developmental bias offers the opportunity to dissect TGFβ signaling activities in embryonic and extraembryonic cell lineages (Gu et al., 1998, 1999; Sirard et al., 1998; Varlet et al., 1997). For example, studies of chimeras generated by injecting wild type ES cells into mutant blastocysts demonstrate Smad2 signals arise in extraembryonic tissues, since massive colonization of the epiblast by wild type cells fails to rescue the developmental defects characteristic of Smad2 mutant embryos (Waldrip et al., 1998). However, the possibility remains that Smad2 may also function in epiblast derivatives. To test this, we generated reciprocal chimeras by injecting *lacZ*-marked *Smad2*$^{-/-}$ ES cells into wild type host blastocysts. As shown in Fig. 2A, at 9.5 dpc strongly colonized embryos are phenotypically normal. All 29 chimeras, obtained using two independent Smad2 mutant ES cell lines, develop overtly normal AP and LR axes.

To investigate potential biases in tissue colonization patterns, a large panel of experimental and control chimeras were serially sectioned. We observe that Smad2-deficient cells contribute extensively to all mesodermal derivatives including the heart, limb buds, somites and notochord as well as

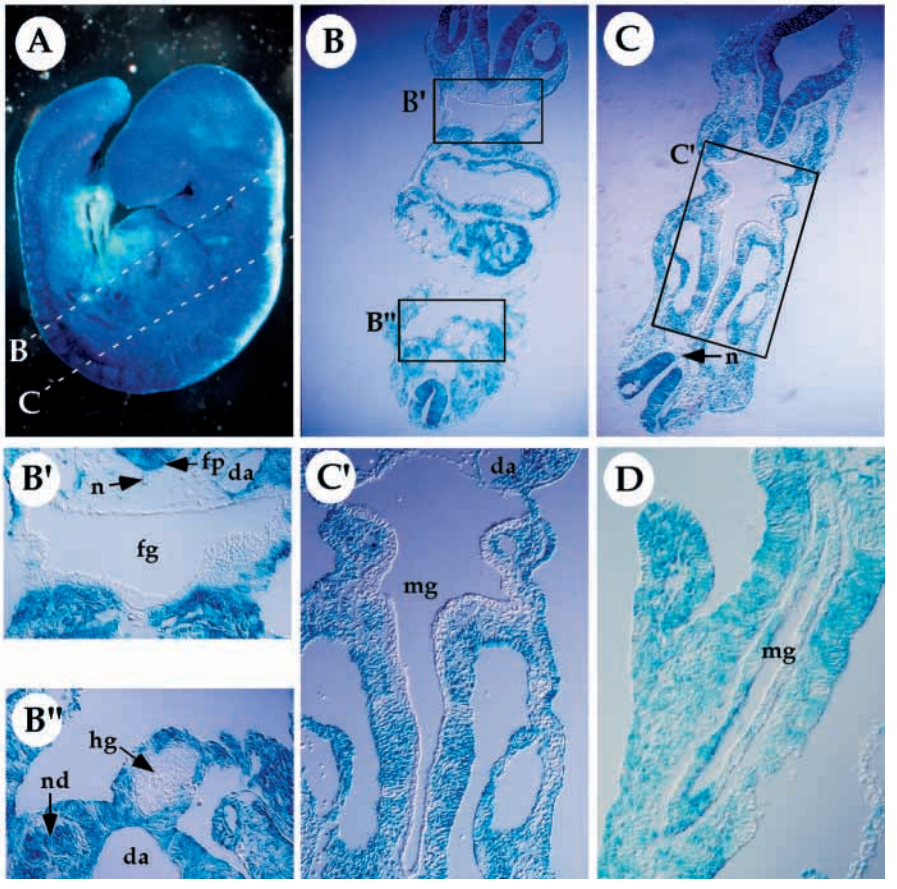

Fig. 2. Smad2-deficient cells effectively contribute to the formation of mesoderm and ectoderm derivatives. Chimeras generated by injecting *lacZ*-marked ES cells into wild-type blastocysts were stained for β-galactosidase activity. The chimeras in A, B and C contain Smad2-deficient ES cell derivatives, whereas the chimera in D was generated using Smad2 heterozygous ES cells. (A) 9.5 dpc chimeric embryos composed mainly of Smad2-deficient cells are phenotypically normal. The planes of sections through two independent 9.5 dpc embryos are indicated. (B) Smad2-deficient cells are found in all embryonic derivatives except for the foregut and hindgut (B′ and B″, respectively). (C,C′) Smad2-deficient cells are absent from the midgut. (D) Smad2 heterozygous cells can contribute to all embryonic derivatives. n, notochord; fp, floorplate; fg, foregut; da, dorsal aorta; nd, nephrogenic duct; hg, hindgut; mg, midgut.

ectodermal derivatives including the CNS, neural tube and surface ectoderm. These findings strongly suggest that Smad2 activities are not essential for axis formation, nor are they required for the processes that pattern the mesodermal and ectodermal derivatives. In striking contrast, Smad2 mutant ES cell derivatives rarely contribute to the definitive endoderm lineage. Thus careful examination of serial sections through 15 embryos demonstrated that, in contrast to the remainder of the tissues, the foregut (Fig. 2B′), midgut (Fig. 2C′) and hindgut (Fig. 2B″) of *Smad2*$^{-/-}$ chimeras contained very few if any mutant Smad2 ES cell derivatives.

The definitive endoderm is initially formed during early gastrulation from epiblast cells within and just anterior to the primitive streak (Lawson et al., 1991; Lawson and Pedersen, 1987). Nascent definitive endoderm emerges at the midline and migrates medially and anteriorly to contribute to the anterior foregut tissue (Thomas and Beddington, 1996). As gastrulation proceeds, epiblast cells are continually recruited to the anterior steak and give rise to both mesodermal cell populations and endodermal derivatives that are laid down along the forming axis in an anterior to posterior fashion. Thus the hindgut is the last definitive endoderm population to be specified. The inability of Smad2 mutant ES cells derivatives to colonize gut tissues at day 9.5 dpc potentially reflects a requirement for Smad2 during initial specification of the endoderm lineage. For example, local TGFβ signals may promote development of endoderm progenitors. Alternatively, Smad2 may be required for cell growth and proliferation (Alexandrow and Moses, 1995; Roberts and Sporn, 1993) rather than endoderm formation per se. In this case, Smad2 mutant endoderm cells may be progressively under-represented as development proceeds. To examine this possibility, we assessed colonization patterns at earlier developmental stages. We observed that *Smad2*$^{-/-}$ ES cell derivatives were frequently specifically excluded from the definitive endoderm of chimeric embryos (*n*=31) examined at 8.5 dpc (Fig. 3). Interestingly, when Smad2-deficient cells were detected in endodermal tissues in strongly colonized embryos, their distribution along the AP axis was markedly nonuniform. Thus *Smad2*$^{-/-}$ cells were most frequently detected in the foregut (Fig. 3C, C') and only very rarely found in the midgut (Table 1). However, in all chimeras examined the contribution of mutant ES cell derivatives to the definitive endoderm was considerably lower than the ES contribution to the mesodermal and ectodermal tissues. Interestingly, heterozygous *Smad2*$^{+/-}$ ES cell lines similarly gave a graded pattern of gut colonization, albeit to a lesser extent. As expected, wild type cells contribute equally well to the gut endoderm as compared with other tissues (Table 1).

The simplest possibility is that the failure to contribute to definitive endoderm reflects spatially restricted colonization of the epiblast by Smad2-deficient cells during gastrulation. To test this, we assessed the distribution of mutant cells in mid-streak to early head-fold-stage chimeras. Smad2-deficient cells were found uniformly distributed throughout the epiblast but were specifically excluded from the superficial distal regions corresponding to the emerging definitive endoderm from mid streak stages onwards. This was the case even in chimeras where the epiblast comprises greater than 90% Smad2-deficient cells (Fig. 4A, B). In contrast, Smad2 mutant cells can clearly contribute to axial mesoderm populations including

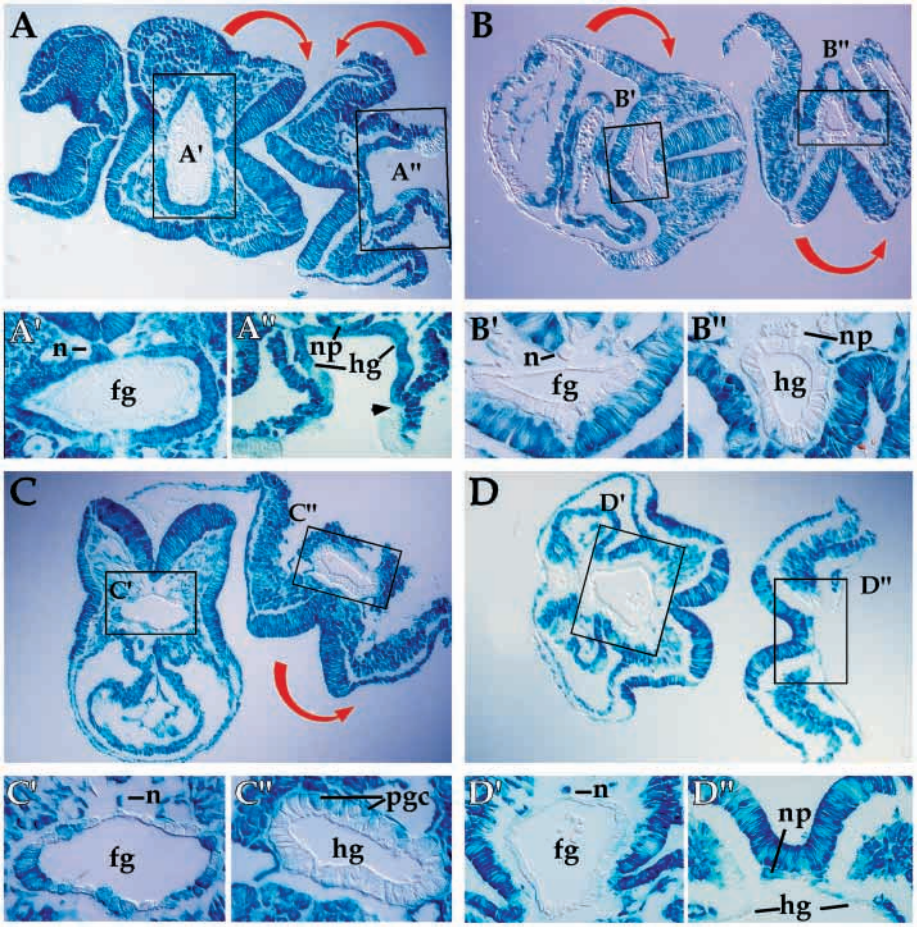

Fig. 3. Smad2-deficient cells fail to contribute efficiently to the definitive endoderm. Transverse sections through 8.0-8.5 dpc wild-type (A) or mutant (B-D) chimeric embryos created by injecting *lacZ*-positive ES cells into wild-type embryos and staining for β-galactosidase activity. (A) Wild-type ES cells efficiently colonise all embryonic derivatives including the foregut and hindgut endoderm (A′ and A″, respectively). The arrowhead (A″) indicates the demarcation between the definitive (blue, ES cell derived) and the visceral (white, blastocyst derived) endoderm. (B,D) Smad2 mutant ES cells contribute to all lineages except endoderm where there is a specific exclusion of mutant cells from both the foregut (B′,D′) and hindgut (B″,D″). (C) In chimeras composed of more than 95% mutant cells, mutant cells are occasionally observed in the foregut (C′), while the non-columnar *lacZ*-positive cells observed in the hindgut (C″) are likely to be primordial germ cells (pgc). Red arrows indicate the direction of embryonic turning which is undisturbed in chimeras comprised largely of Smad2 mutant cells. n, notochord; np, notochordal plate.

Table 1. Colonization of gut tissues by ES cell derivatives in 8.5 dpc chimeric embryos*

Genotype ES cells	Chimeras scored by serial section	Gut colonization pattern				Equivalent contribution to all tissues§
		No contribution to gut	Foregut only‡	Foregut and midgut‡	Foregut, midgut and hindgut‡	
–/–	31	18 (58%)	11 (36%)	2 (6%)	0	0
+/–	9	0	2 (22%)	0	5 (56%)	2 (22%)
+/+	8	0	0	0	2 (25%)	6 (75%)

*All chimeras composed of >75% ES cell derivatives.
‡Some derivatives found in indicated regions, but these regions are less well colonized than all other tissues in the embryo.
§Contribution to gut tissue is equivalent to mesoderm and ectodermal tissues.

the head process, notochord and prechordal plate. These observations indicate that Smad2 signals are essential for the initial specification of the definitive endoderm lineage.

Smad2 independent Nodal signaling during establishment of the LR axis

A proportion of *Smad2* and *nodal* doubly heterozygous embryos develop anterior patterning and LR axis defects (Nomura and Li, 1998; E. J. R., unpublished data) consistent with the idea that Smad2 acts downstream of Nodal (Collignon et al., 1996; Varlet et al., 1997). Moreover, a proportion of embryos derived from *Smad2*$^{-/-}$ ES cells used in conjunction with tetraploid wild type blastocysts develop holoprosencephaly and randomized heart looping, phenotypes suggesting attenuation of Nodal signaling (Heyer et al., 1999). In contrast here, in extensively chimeric embryos (n=60) generated using two independent Smad2-deficient cell lines, we observe no overt abnormalities, arguing against an essential role for Smad2 in LR axis formation or patterning of the anterior CNS. Alternatively these differences in experimental outcome may relect an important role for Smad2-dependent signals arising from the definitive endoderm since, in our experiments, this tissue is formed almost exclusively from wild type cells.

To further test whether Nodal pathways are compromised in Smad2-deficient tissues, we examined target gene expression patterns in early somite stage chimeras. We have previously shown that asymmetric *nodal* expression is maintained via a discrete intronic enhancer (Norris and Robertson, 1999), the activity of which is dependent on the FAST transcription factor (Saijoh et al., 2000). FAST cooperates with Smad2 to transduce Nodal signals (Saijoh et al., 2000) suggesting that Nodal may regulate its own expression in the lateral plate. It was therefore of interest to test whether embryos composed largely of Smad2-deficient cells display normal *nodal* mRNA expression domains. As shown in Fig. 5, with only a single exception, chimeric embryos (n=12) maintained robust asymmetric *nodal* mRNA expression. In the exceptional embryo, *nodal* mRNA was bilaterally expressed (Fig. 5D), suggestive of a mid-line defect (Meno et al., 1998). Left-sided *pitx2* mRNA expression was similarly unaffected (Fig. 5E, F). These data, as well as the finding that Smad2 is not required for primitive streak formation (Heyer et al., 1999 and data presented here), indicate that Smad2 is not required to mediate Nodal signaling in

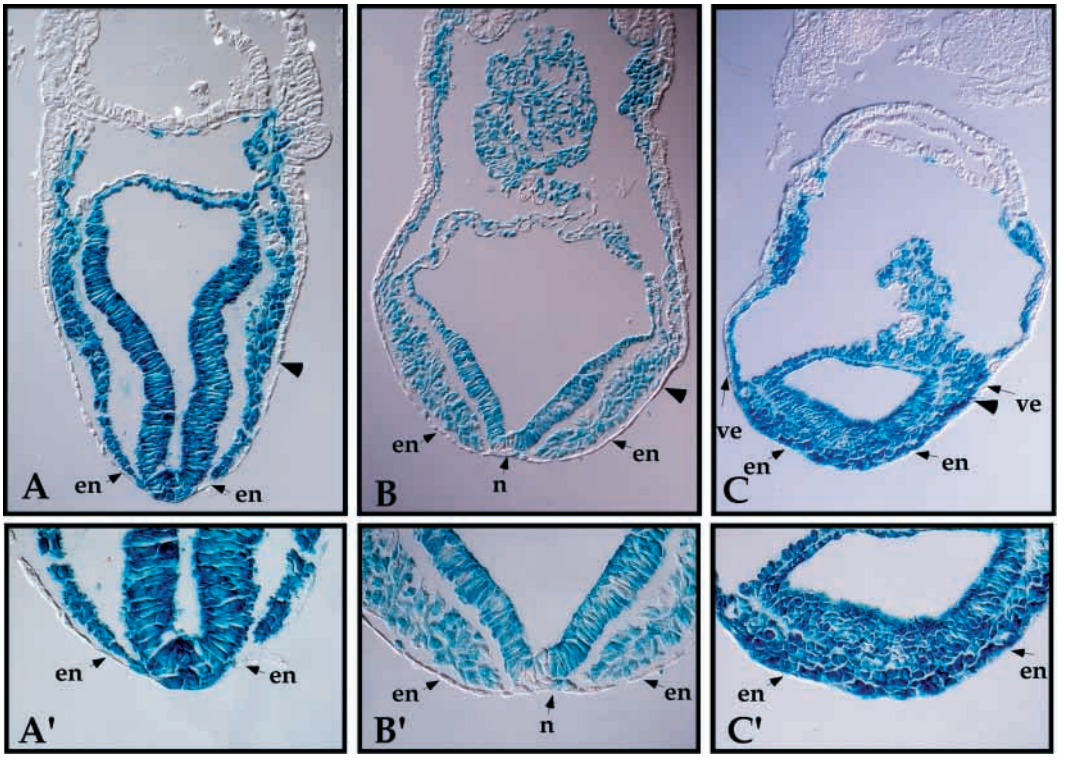

Fig. 4. Formation of the definitive endoderm during gastrulation requires Smad2 function. Frontal sections of Smad2-deficient (A,A′,B,B′) or Smad2 heterozygous (C,C′) chimeric embryos. (A,A′) Although this 7.5 dpc chimera is composed almost entirely of Smad2-deficient cells, β-galactosidase positive Smad2-deficient cells are not found in the newly formed definitive endoderm (en). (B,B′) Smad2 mutant ES cell derivatives were identified in the notochord (n) of this 8.0 dpc embryo, but not in the nascent definitive endoderm. (C,C′) Wild-type ES cells efficiently colonize the nascent endoderm. The demarcation between visceral endoderm (ve) and definitive endoderm is indicated by the arrowhead.

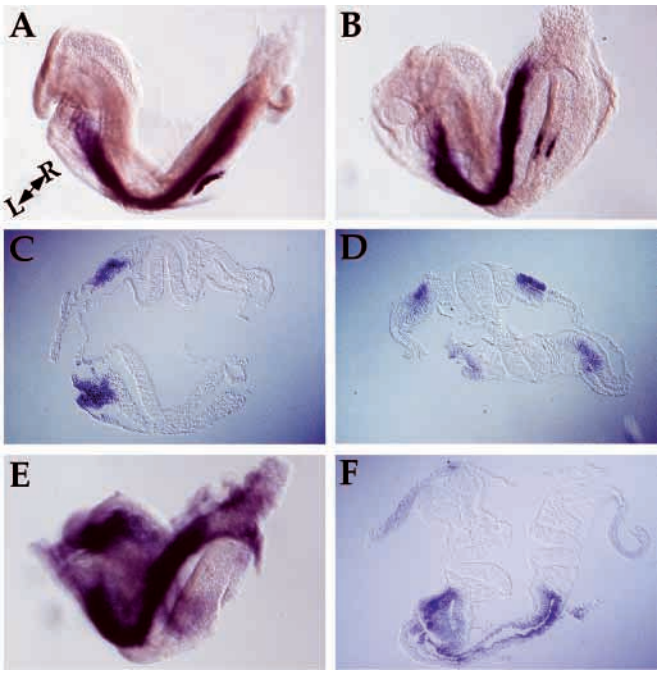

Fig. 5. Asymmetric *nodal* and *pitx2* expression in the absence of Smad2 activity. Individual chimeras composed mainly of Smad2-deficient cells were identified by staining a portion of their yolk sacs for β-galactosidase activity. These embryos were then analysed for *nodal* (A-D) or *pitx2* (E,F) mRNA expression by whole-mount in situ hybridization. *nodal* expression in wild-type (A) and in Smad2-deficient chimeras (B-D) is seen both in the node as well as in the left-lateral plate mesoderm. Transverse sections (C,D) through stained Smad2-deficient chimeras reveals robust *nodal* expression throughout the left lateral-plate mesoderm. (D) A single embryo displayed atypical bilateral expression of *nodal* in the lateral plate mesoderm. (E) *Pitx2* expression is observed in the left-lateral plate mesoderm and head mesenchyme of Smad2-deficient chimeras. (F) In transverse section, expression of *Pitx2* can be observed throughout the left lateral-plate mesenchyme in largely Smad2-deficient embryos.

mesoderm populations. The LR defects seen in Nodal and Smad2 doubly heterozygous embryos are more likely to be secondary to disturbances of midline structures, known to be important for maintenance of asymmetric gene expression patterns (Izraeli et al., 1999; Meno et al., 1998; Meyers and Martin, 1999). The anterior truncations, and microprosencephaly and holoprosencephaly phenotypes often accompanying LR defects could also be explained due to abnormalities affecting anterior midline populations responsible for patterning the ventral regions of the CNS (Bachiller et al., 2000; Dale et al., 1997; Foley et al., 1997).

Distinct Smad2 and Smad3 expression patterns in extraembryonic tissues but not in the embryo proper

Results above indicate Smad2-deficient epiblast cells can efficiently give rise to mesoderm and ectoderm derivatives. A likely possibility is that Smad3, and/or other closely related TGFβ/activin mediator(s), potentially compensates for the loss of Smad2 activity. Whole-mount in situ hybridization experiments have shown that *Smad2* is broadly expressed in the postimplantation stage embryo from day 6.5 onwards (Waldrip et al., 1998) but *Smad3* mRNA expression patterns have not as yet been described at early stages of mouse development. We used radioactive in situ hybridization techniques to assess *Smad3* and, as a control, *Smad2* expression domains in the postimplantation embryo. As expected, we found *Smad2* mRNA ubiquitously expressed in the embryonic and extraembryonic tissues at all stages examined (Figs 6, 7). By contrast, *Smad3* mRNA expression is spatially restricted. Thus, at the earliest stage examined (5.5 dpc), *Smad3* expression is confined to the extraembryonic ectoderm. One day later at perigastrulation stages, strong expression is observed in the extraembryonic ectoderm, but there is no detectable signal in the epiblast and primitive endoderm. By late-streak stages, *Smad3* is abundantly expressed in embryonic and extraembryonic mesoderm, as well as in the definitive endoderm and in the extraembryonic ectoderm component of the chorion (Fig. 5F). Transverse sections through gastrulating embryos confirm that *Smad3* mRNA is absent from primitive endoderm populations (Fig. 5H). By day 9.5, both genes are ubiquitously expressed throughout the embryo and in particular, strong expression is observed within the developing foregut, midgut and hindgut (Fig. 6). In contrast, the visceral yolk sac (VYS) endoderm lacks *Smad3* transcripts. Thus, closely related *Smad2* and *Smad3* transcripts show overlapping expression domains within the tissues of the embryo proper from gastrulation stages onward, supporting the notion that these molecules act interchangeably in transducing TGFβ-like signals in early embryos. However, the primitive endoderm appears to exclusively express *Smad2* mRNA. Thus the primary defect observed in Smad2-deficient embryos likely reflects unique expression of *Smad2* in this tissue (Waldrip et al., 1998).

DISCUSSION

Much has been learned over recent years about TGFβ signals that determine cell fate and establish the body plan during early vertebrate development. A family of downstream mediators, the Smad proteins, have been shown to transmit TGFβ signals from the cell surface to the nucleus. *Smad2* expression in extraembryonic tissues was previously found to be essential for specification of the initial AP axis of the mouse embryo (Waldrip et al., 1998). Thus Smad2 functions to restrict the site of primitive streak formation and maintain the prospective anterior region of the epiblast fated to give rise to ectodermal derivatives. *Smad2* overexpression in *Xenopus* explants results in extensive formation of dorsal mesodermal types including notochord, and can also cause axis duplications (Baker and Harland, 1996; Graff et al., 1996). In contrast Smad2 is clearly not required for mesoderm formation during early stages of mouse development. Here we show that Smad2-deficient ES cells extensively colonize ectodermal and mesodermal cell populations of the embryo proper but rarely contribute to the definitive endoderm lineage.

Two temporally and spatially distinct populations of so-termed ‘endoderm’ are sequentially induced during early mouse development. Concomitant with implantation, the primitive, or extraembryonic, endoderm initially delaminates from the blastocoelic surface of the inner cell mass. This progenitor pool gives rise to both the parietal endoderm and

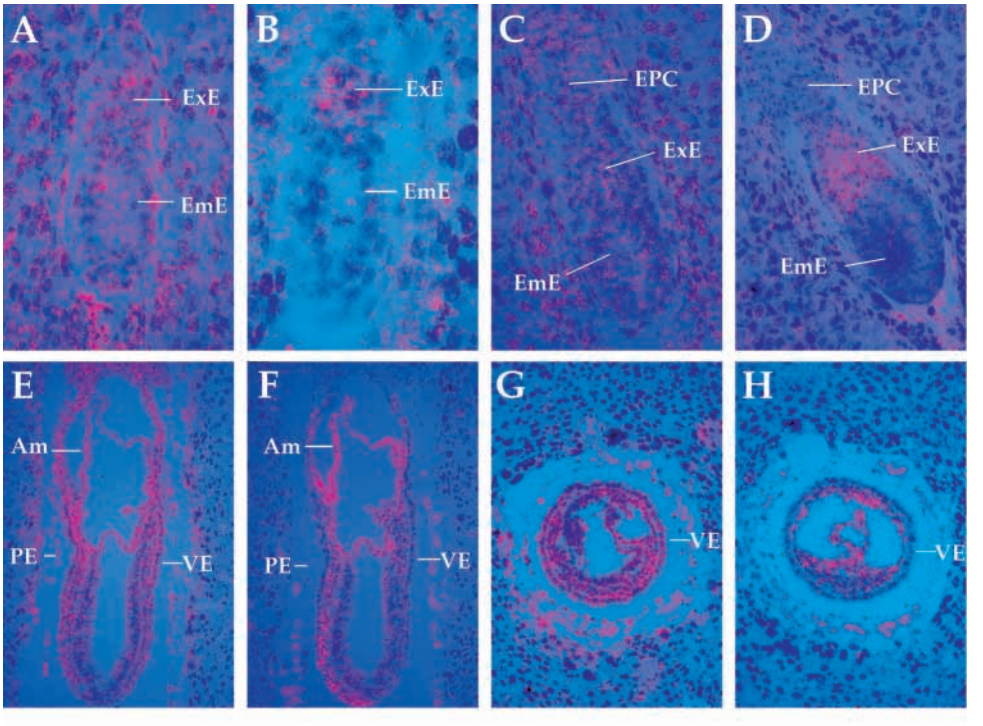

Fig. 6. *Smad2* and *Smad3* show distinct mRNA expression patterns in extraembryonic tissues at early postimplantation stages. Adjacent sagittal sections through 5.5 dpc (A,B), 6.25 dpc (C,D) and 7.25 dpc (E,F) embryos and transverse sections through 7.25 dpc embryos (G,H) were hybridized using *Smad2* (A,C,E,G) or *Smad3* (B,D,F,H)-specific probes. *Smad2* is ubiquitously expressed throughout the embryo and extraembryonic tissues at all times examined (A,C,E,G). In contrast, *Smad3* transcripts were localized to the extraembryonic ectoderm at 5.5 dpc (B) and 6.25 dpc (D). (F) *Smad3* mRNA expression is detectable throughout the embryo and in the amniotic folds (Am) as well as the allantoic rudiment but not in the visceral endoderm (VE) shortly after the initiation of gastrulation. Dark-field and bright-field images are superimposed, resulting in a pink hybridization signal. ExE, extraembryonic ectoderm; EmE, embryonic ectoderm; EPC, ectoplacental cone; PE, parietal endoderm.

the visceral endoderm. These extraembryonic cells are important for the formation of highly specialised structures including the VYS necessary for supporting growth of the embryo within the uterine environment. In addition the visceral endoderm gives rise to the AVE, a discrete population of endoderm cells responsible for imparting anterior pattern to the underlying epiblast (reviewed Beddington and Robertson, 1998, 1999). The second endoderm population, the definitive endoderm, derives from the epiblast during gastrulation and gives rise to the gut tube of the embryo (reviewed Wells and Melton, 1999).

Despite their different origins, both the visceral and definitive endoderm cell populations are known to express many common gene products reflecting their overlapping physiological functions (reviewed Zarat, 1999). Members of the HNF and GATA families of transcriptional regulators are required for survival of the primitive endoderm (reviewed Bielinska et al., 1999). However, their potential involvement during formation and maintenance of the definitive endoderm has yet to be established. Interestingly Smad4 is known to be required for the formation and/or maintenance of the primitive endoderm lineage (Sirard et al., 1998; Yang et al., 1998). In contrast, the visceral endoderm in Smad2 mutant embryos proliferates normally and supports the formation of a well-differentiated VYS containing hematopoetic and endothelial cells (Waldrip et al., 1998). However, as shown here, Smad2 activity is critically important for the specification of definitive endoderm. Distinct signaling pathways are therefore responsible for specification of the definitive as

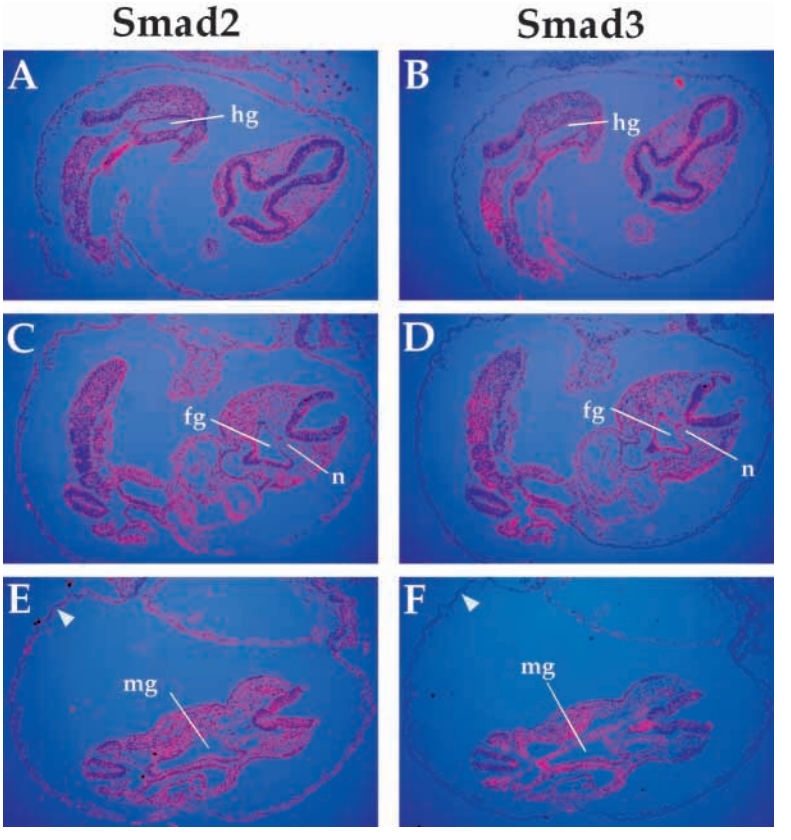

Fig. 7. Both *Smad2* and *Smad3* are strongly expressed throughout the embryo at 9.5 dpc. Adjacent serial sections through a 9.5 dpc embryo hybridized with either a *Smad2* (A,C,E) or *Smad3* (B,D,F)-specific probes. *Smad2* and *Smad3* are ubiquitously expressed in the embryo proper at 9.5 dpc. The arrowhead in E and F points to the yolk-sac endoderm which expresses *Smad2* (E) but not *Smad3* (F).

opposed to the primitive endoderm lineage of the mammalian embryo.

The definitive endoderm intially appears as a discrete cell layer at the most distal end of the primitive streak (Lawson et al., 1986; Lawson and Pedersen, 1987) displacing visceral endoderm into the extraembryonic regions (Lawson and Pedersen, 1987; Tam and Beddington, 1992; Thomas and Beddington, 1996). However, a well-defined progenitor pool giving rise to this cell lineage has not been identified. In fact, single-cell-labeling experiments have shown that sharp boundaries in cell fate do not exist in the pregastrulation/early streak stage embryo, since greater than 75% of cells labelled in the region of the anterior streak contribute to multiple germ layers (Lawson et al., 1991). The present experiments demonstrate that Smad2 signals promote recruitment of epiblast cells to form the gut endoderm. In those chimeras in which the epiblast is composed almost exclusively of Smad2-deficient cells, we observe that the superficial cell layer corresponding to nascent definitive endoderm is generated from wild type cells. However, Smad2 mutant cells are readily incorporated into node-derived axial midline structures including the prechordal plate, notochordal plate, notochord and prospective floor plate. These data suggest that only a small number of precursor cells give rise to the definitive endoderm and imply that endoderm formation is probably controlled by highly localised TGFβ signal(s) causing epiblast derivatives to form the outermost population of endoderm rather than the deeper mesoderm cell populations.

Interestingly the few Smad2 mutant cells contributing to the gut tube in strongly chimeric embryos are found almost exclusively at anterior levels as expected if a subpopulation of cells lying anterior to the forming streak gives rise to descendants in multiple anterior mesendoderm tissues including the anterior notochord and foregut endoderm (Lawson et al., 1986; Lawson and Pedersen, 1987). Moreover, these occasional Smad2 mutant cells are not always confined to either the dorsal or ventral sides of the gut tube but often are dispersed, consistent with extensive cell mixing as previously documented (Lawson and Pedersen, 1987). In contrast, we found the hindgut tissue is consistently devoid of Smad2-deficient cells (Table 1). DiI labeling of late gastrulation primitive streak regions has shown that progenitors of the hindgut endoderm likely represent a distinct lineage (Wilson and Beddington, 1996). The present data demonstrate that formation of this population is strictly Smad2 dependent.

It is well known in *Xenopus* that endoderm derives from the yolk-rich vegetal region of the blastula. During gastrulation, the vegetal cells internalize and elongate to form the gut tube and its derivatives including the liver, lung and pancreas. The signals responsible for specification of the endoderm germ layer in *Xenopus* remain enigmatic. Moreover, TGFβ/activin signals induce both mesodermal and endoderm cell types (reviewed Harland and Gerhart, 1997). Gain-of-function experiments have identified several transcription factors having the ability to induce endoderm marker gene expression in animal cap explant assays (Ecochard et al., 1998; Henry and Melton, 1998; Hudson et al., 1997; Lemaire et al., 1998). VegT, a maternally supplied T-box transcription factor regulating both mesoderm and endoderm formation (Zhang et al., 1998), thought to induce zygotic TGFβs including Derriere and Xnr1-2 (Osada and Wright 1999; Piccolo et al., 1999; Sun et al., 1999) that promote mesoderm formation, also signals endoderm formation via the activation of specific target genes including Mix and Sox-related transcription factors (Clements et al., 1999; Kofron et al., 1999). VegT homologs have yet to be identified in mammals, but conserved molecular pathways appear to regulate endoderm formation in zebrafish. Thus injection of a constitutively active form of the activin receptor TARAMA-A diverts cells into endodermal fates (Peyrieras et al., 1998) while mutant embryos lacking expression of both of the Nodal homologs Cyclops and Squint, display defective mesoderm and endoderm formation (Feldman et al., 1998).

In the mouse, Nodal is the only candidate endoderm-inducing TGFβ-related activity identified to date. *nodal* is expressed in the posterior epiblast coincident with the onset of gastrulation, and also transiently expressed throughout the visceral endoderm (Varlet et al., 1997). The putative *nodal* antagonists *lefty1* (Chazaud et al., 1999; Meno et al., 1998) and *cer1* (Belo et al., 1997; Piccolo et al., 1999) are also expressed in visceral endoderm on the anterior side of the epiblast potentially attenuating Nodal signals from this tissue. Enhanced Nodal signaling in posterior regions may thus selectively induce endoderm formation. Consistent with this suggestion, recent findings suggest that formation of the definitive endoderm may be regulated by a balance of TGFβ/activin and BMP signals. Thus embryos lacking both Noggin and Chordin, secreted BMP antagonists expressed in the distal streak, node and its derivatives, show a marked reduction in definitive endoderm at early gastrulation which leads to a marked reduction in pharyngeal endoderm at later stages (Bachiller et al., 2000). Similarly, formation and/or migration of definitive endoderm cells is impaired in embryos lacking Furin/SPC1, a serine protease controlling proteolytic maturation of TGFβ and related proteins including BMP4 and Nodal precursors (Constam and Robertson, 2000).

What are the targets of the Smad pathway that specifically divert epiblast derivatives to an endoderm fate? Recent studies demonstrate that double mutants lacking both HNF3β and Lim1 functions phenocopy Smad2-deficient embryos (Perea-Gomez et al., 1999), suggesting that these transcription factors act downstream of Smad2 signals in primitive endoderm. Interestingly tetraploid chimera experiments have shown that although a hindgut is present, HNF3β-deficient embryos lack anterior and midgut tissues (Dufort et al., 1998). However, Lim1-deficient ES cells contribute efficiently to the gut in chimeric embryos (Shawlot et al., 1999). Thus Lim1 is unlikely to be an important target of Smad2 signaling in definitive endoderm. Experiments in *Xenopus* have identified discrete regulatory elements in the the HNF3β homolog XFKH1, that are directly activated by Xsmad2 (Howell and Hill, 1997). Moreover, an activin response element (ARE) is present in Xlim1 (Rebbert and Dawid, 1997). Recent studies in *Xenopus* have shown that transcription factors of the Mix family form complexes with Smad2/Smad4 in response to activin-like signals, to regulate target genes during mesendoderm formation (Germain et al., 2000). It will be interesting to learn whether Mix homologs (Ecochard et al., 1998; Henry and Melton, 1998; Lemaire et al., 1998), as well as Sox family members (Hudson et al., 1997) similarly participate in endoderm formation and patterning in mouse embryos.

Considering that loss of Smad2 results in highly restricted tissue defects, other closely related Smad family member(s)

probably mediate essential TGFβ signals during mesoderm induction and patterning. In support of this idea, we find that *Smad3*, the only other TGFβ/activin receptor Smad identified to date, is ubiquitously expressed together with *Smad2* mRNA in the embryo proper. In contrast, only *Smad2* mRNA is exclusively expressed in the primitive endoderm. Interestingly, although Smad2 and Smad3 are over 90% identical at the amino acid level (Zhang et al., 1996), recent studies suggest these molecules may in part have unique activities. For example, although both form complexes with Smad4 and function as transcriptional activators in some reporter assays (Lebrun et al., 1999), it was recently shown that Smad3 suppresses transcriptional activity from the FAST-dependent ARE present in the goosecoid promoter, while Smad2 enhanced activation (Labbe et al., 1998). Both the positive and negative regulatory effects mediated by distinct Smad proteins in response to TGFβ signals are likely to reflect their interactions with other DNA-binding factors (reviewed Wrana, 2000). Clearly further studies are needed to understand the specificity of Smad signaling pathways in vivo and to identify Smad2 target genes responsible for specification of the endodermal lineage.

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