Expression of the Inhibitory Smad7 in Early Mouse Development and Upregulation During Embryonic Vasculogenesis

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ABSTRACT SMAD proteins are downstream targets of serine/threonine kinase receptors of the transforming growth factor β (TGF β) superfamily. Ligands activating these receptors regulate cell growth, differentiation and development in many tissues of various organisms. In mammals eight different *Smad* genes are known, each with different roles in mediating signalling between plasma membrane and nucleus. Smad6 and Smad7 are inhibitors of TGF β family signalling. They are both expressed in human adult vascular endothelial cells, particularly after these cells have been subjected to shear stress (Topper et al. [1997] Proc Natl Acad Sci USA 94: 9314–9319). Here we show by reverse transcriptase polymerase chain reaction and in situ hybridization that *Smad7* mRNA is highly expressed in the developing vascular system of the mouse embryo but is also detectable much earlier in preimplantation embryos and during gastrulation. We also demonstrate by transient transgenesis that overexpression of *Smad7* in mouse zygotes inhibits development beyond the 2-cell stage. This confirms earlier conclusions of similar, but complementary, experiments using a dominant negative type II TGF β receptor demonstrating that TGF β signalling is required for normal preimplantation development. © 2000 Wiley-Liss, Inc.

Key words: BMP, endothelium; in situ hybridisation; mouse development; mRNA; RT-PCR; Smad7; TGF β ; vasculogenesis

INTRODUCTION

The signal transduction pathway of the TGF β superfamily is highly conserved from invertebrates, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, to vertebrates. Over 40 ligands that may activate this pathway have now been identified, with the TGF β s, activins/inhibins and bone morphogenetic proteins (BMPs) forming three major subfamilies. A genetic

screen for maternal enhancers of weak alleles of decapentaplegic (*dpp*), a BMP homologue in *Drosophila*, identified *Mothers against dpp* (*Mad*) as part of the *Dpp* signalling pathway while subsequent analysis of *C. elegans* mutants identified *sma-2*, *3*, and *4* as genes giving phenotypes similar to mutations in *daf-4*, a receptor for a BMP-like ligand in the nematode (reviewed in Kawabata et al., 1998). The similarities in function and in protein structure, together with sequence homology, were the first strong suggestions that *Mad* and *sma* form part of a conserved, intracellular signalling pathway for the TGF β superfamily.

Eight *Smad* genes were subsequently identified in mammals and grouped into three classes based on their structure and function (reviewed in Massagué et al., 1998; Heldin et al., 1997). Pathway-restricted or receptor-regulated SMADs (R-Smads) are directly phosphorylated by type I receptors following transient interaction. Smad2 and 3 are substrates for activated TGF β and activin type I receptors, whereas Smad1, 5, and 8 interact with BMP type I receptors. Phosphorylated R-Smads oligomerize with Smad4, which belongs to the class of common-mediator or Co-Smads. These *Smad* complexes accumulate in the nucleus where they participate in the regulation of transcription of a specific set of responsive genes. Smads of the third class antagonize TGF β /activin and/or BMP signalling by preventing the activation of the R-Smads or their subsequent oligomerization with the Co-Smads (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997; Hata et al., 1998). Two of these inhibitory Smads (I-Smads or anti-Smads), Smad 6 and 7, have been identified to date. Both inhibit BMP signalling but Smad7 is more potent in inhibiting TGF β /activin signals than

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Fig. 1. RT-PCR amplification of *Smad7* mRNA in pre- and postimplantation (A, B, D) mouse embryos. One (B, D) or two (A) rounds of PCR were performed on cDNA from embryonic stages between one-cell zygote and expanded blastocyst (A), pregastrulation and early headfold stages (D) and separated tissue layers of the yolk sac (B) to amplify *Smad7* mRNA (top panels). RT-PCR amplification of β -actin transcripts (bottom panels) served as control for RNA extraction and cDNA synthesis. cDNA from embryonic stem (ES) cells was used as positive control (+) for amplification. Water served as a negative control (-). *AFP* and *PECAM-1* expression were used to verify separation of the yolk sac

layers (B). The upper transcripts in (A) represent first round carry-over products. (C) Expression of *Smad7* in postimplantation mouse embryos. A Northern blot with polyA⁺ RNA was hybridized to the *Smad7* probe. Rehybridization with *GAPDH* confirmed equal mRNA loading in each lane. Abbreviations: 2c, 4c, 8/16c: 2-, 4- and 8 to 16-cell stage respectively; BI, blastocyst; C, cDNA; CM, compacted morula; e, embryonic region; ex, extraembryonic region; End, yolk sac endoderm; ExBI, expanded blastocyst; L, DNA ladder; Mes, yolk sac mesoderm; R, RNA; Zg, zygote.

Smad6 (Imamura et al., 1997; Nakao et al., 1997; Ishisaki et al., 1998, 1999; Hata et al., 1998; Nakayama et al., 1998a; Casellas and Hemati-Brivanlou, 1998). mRNAs for both *Smad6* and *Smad7* are also induced by BMPs and TGF β although with different kinetics; *Smad6* is induced three hours after ligand stimulation and is maintained for more than 48 hours, whereas *Smad7* induction is more rapid but only transient (Afrakhte et al., 1998; Takase et al., 1998). Together the findings suggest that I-Smad genes are induced by R-Smads and may regulate their activity by a negative feedback loop.

Smad7 was identified among genes upregulated in a differential screen of vascular endothelial cells subjected to shear stress; in adults it manifested an endothelial selective expression pattern (Topper et al., 1997). Since TGF β signalling in endothelial cells also plays a crucial role in the pathology of several vascular diseases (McAllister et al., 1994; Johnson et al., 1996), and is essential for normal vasculogenesis of the yolk sac in mouse development (Dickson et al., 1995; Goumans et al., 1999), we examined *Smad7* mRNA expression in mouse development. The results described here show that *Smad7* is indeed highly ex-

pressed in the endothelial cells of the developing vascular system, as in adults, but is also detectable much earlier in preimplantation embryos and in gastrulation. In addition by overexpressing *Smad7* in zygotes, we confirm a functional requirement for TGF β signalling in preimplantation development, first observed in embryos expressing dominant negative TGF β receptors (Roelen et al., 1998), later demonstrated in certain strains of mice lacking TGF β 1 (Kallapur et al., 1999).

RESULTS

Smad7 Expression in Early Development

Expression of *Smad7* mRNA during early mouse development was determined by RT-PCR. *Smad7* was not present as maternal transcripts in the zygote but became detectable in embryos at the 2-cell stage, when embryonic transcription begins; expression was maintained throughout preimplantation development (Fig. 1A). After implantation, *Smad7* mRNA was not detectable by RT-PCR in prestreak embryos at E6.0 (Fig. 1D), but transcripts were observed in both embryonic and extraembryonic regions from E6.5 onwards (Fig. 1D). Since type I receptors for TGF β (*ALK5*), activin (*ALK4*) and BMPs (*ALK2*, *ALK3*, and *ALK6*) are expressed

Fig. 2. Overexpression of *Smad7* causes embryos to become developmentally arrested at the two-cell stage. Fertilized oocytes were injected with 10 fg of the constructs indicated. After 3 days of culture, the percentages of embryos at the 2-cell stage, 3–7 cell stages, 8 cell-morula and blastocyst stages were scored. N = 25 successfully injected embryos for each construct or combination of constructs. The data shown are from one of two identical experiments giving similar results. Positive (*dn-TβRII*) and negative (*caALK-5*) controls reproduce previous findings (Roelen et al., 1998).

during these stages (Roelen et al., 1997a, 1997b; Gu et al., 1998), they are candidates for negative regulation by *Smad7*.

***Smad7* Overexpression Inhibits First Cleavage**

We have demonstrated previously that if TGFβ signalling in the zygote is inhibited by overexpression of *dn-TβRII*, lacking the intracellular kinase domain, the embryos do not develop beyond the 2-cell stage (Roelen et al., 1998). This effect was rescued by co-expression of a constitutively active TGFβ type I receptor (*ca-ALK5*). Since *Smad7* can also inhibit TGFβ signalling, we injected a DNA construct encoding *Smad7*, driven by the *PGK-1* promoter, into the male pronuclei of fertilized eggs. The embryos were subsequently cultured for three days and scored for their developmental stage. After 24 hours, the embryos that had not developed to the 2-cell stage were discarded (these were primarily unfertilized oocytes); there was no difference in the percentage of embryos remaining as one cell between the experimental groups (data not shown). After three days in culture, more than 70% of the embryos injected with empty vector had developed to blastocysts (Fig. 2); this compares with ~80% of non-injected controls (data not shown). By contrast less than 30% of the embryos injected with the *Smad7* cDNA had reached this stage and 45% did not develop beyond the 2-cell stage. There was no evidence of apoptosis in the embryos remaining at the 2-cell stage (data not shown). These results compare with 60% of embryos injected with a *dn-TβRII* construct remaining at the 2-cell stage (Fig. 2). Coinjection of *ca-ALK5*, that mimics ligand-activation (see Roelen et al., 1998) largely rescued the *Smad7*-induced

inhibition of cleavage, but itself had no effect on the efficiency of development (Fig. 2).

***Smad7* Expression in Postimplantation Development to Mid-Gestation**

We also used in situ hybridisation to map the distribution of *Smad7* in sections of embryos during postimplantation development (E6.5–E12.5). The specificity of the probe was verified by Northern blot analysis of polyA⁺ RNA from mouse embryos. *Smad7* transcripts of 4.4 and 2.4 kb are present throughout mouse development (Fig. 1C). In situ hybridisation confirmed that *Smad7* is weakly expressed in the epiblast at E6.5 (Fig. 3A). At E7.5, high levels of transcripts were observed in the extraembryonic mesodermal cells of the allantois (Fig. 3C). The yolk sac (extraembryonic mesoderm and visceral endoderm) was weakly positive for *Smad7* (Fig. 3C), but we confirmed by RT-PCR that the signal was specific and above background (Fig. 1B). A *Smad7* transcript was detected in both the endoderm and mesoderm components of the yolk sac, while RT-PCR to detect *α-fetoprotein (AFP)* and *platelet endothelial cell adhesion molecule 1 (PECAM-1)*, which are markers for endoderm and mesoderm respectively, confirmed that the two tissue samples had been cleanly separated (Fig. 1B). The inner layer of the deciduum and the trophoblast giant cells were also positive, both at E7.5 and at E8.0 (Fig. 2B–G). In the embryo proper at E7.5, the *Smad7* mRNA level was low in all three embryonic germ layers (Fig. 3B), and similar to that observed in the epiblast at E6.5 (Fig. 3A); again, however, RT-PCR data confirmed that the signal did not represent non-specific background (Fig. 1D).

Distinct, regionalized expression of *Smad7* in the embryo proper was first observed from E8.0 onwards. Lateral plate mesoderm (splanchno- and somatopleure) (Fig. 3G) and cardiac mesoderm (Fig. 3E) expressed higher levels of *Smad7* transcript than the endoderm, the neuroectoderm, and the paraxial and cephalic mesoderm (Fig. 3D–G).

At E12.5, when primordia of most organs have been established, most prominent expression was in aorta and arteries, the outflow tract and regions involved in heart septation (Fig. 4C–E). Expression was somewhat higher in arteries than in veins (e.g., thoracic artery positive, with the adjacent thoracic vein negative). The mouse embryo then adopted the expression pattern described for adult human endothelium (Topper et al., 1997). Multiple members of the TGFβ family have been implicated in cardiogenesis and differentiation of endothelium. Partial co-localisation of their type I receptors *ALK1*, *ALK3*, *ALK5*, and *ALK6*, and *Smad7* occurs at those sites (Roelen et al., 1994, 1997b; Dewulf et al., 1996).

Smad7 was also expressed in precartilage condensations, the urogenital system, distinct neuroectodermal tissues and derivatives of the lateral plate mesoderm (summarized in Table 1; Fig. 4).

Fig. 3. Expression of *Smad7* in early postimplantation embryos. Sections of embryos in decidua at E 6.5 (A; sagittal), E7.5 (B, C; transverse) and E8.0 (D–G; transverse). Abbreviations: al, allantois; am, amnion; b, body wall; c, cephalic mesoderm; d, deciduum; e, ectoderm; en, endoderm; epc, ectoplacental cone; f, entrance to foregut diverticulum; h,

heart primordium; i, intraembryonic coelomic cavity; m, mesoderm; ne, neuroectoderm; ng, neural groove; pe, parietal endoderm; pm, paraxial mesoderm; so, somatopleura; sp, splanchnopleura; t, trophoblast giant cells; ys, yolk sac. Scale bars = 100 μ m.

DISCUSSION

In the present study we have shown that *Smad7* is widely expressed throughout mouse development, not only at sites of embryonic vasculogenesis, but also during preimplantation development and gastrulation. This is largely in agreement with studies in *Xenopus laevis* (Casellas and Hemmati-Brivanlou, 1998; Na-

kayama et al., 1998b). Restricted expression in the embryo proper was first observed at E8.0 where it was most evident in cells and tissues of mesodermal origin. By E12.5 it had adopted an expression pattern essentially as described for that of adult human endothelium being particularly prominent in the outflow tract of the heart, tissues involved in its septation, in the aorta and

Fig. 4. Expression of *Smad7* in E12.5 embryos. Transverse sections through head (A, B), heart (D, E) and abdomen (G). Sagittal sections through heart (C) and abdomen (F). Abbreviations: a, lumen of atrium; aa, arch of aorta; ap, aortico-pulmonar spiral septum; as, arytenoid swelling; av, atrioventricular cushion tissue; c, choroid plexus; ca, carotid artery; cb, cerebral artery; d, dorsal aorta; di, diaphragm; f, fourth ventricle; g, gonad; gt, genital tubercle; h, hypothalamus; l, liver; lf, left fourth

arch artery; lp, leaflets of pulmonary valve; lu, lung; m, mesonephros; mc, Meckel's cartilage; o, outflow tract; p, precartilage primordium; r, rib shaft; rf, right fourth arch artery; rl, rhombic lip; s, stomach; sp, spinal cord; t, thoracic aorta; th, third ventricle; tr, trachea; to, tongue; tp, tooth primordium; v, lumen of ventricle; vi, vibrissae; vn, vomeronasal organ. Scale bars = 250 μ m.

in other major arteries. *Smad7* was originally identified as a gene induced in endothelial cells subjected to shear stress (Topper et al., 1997). The higher expression in arteries versus veins in the embryo may therefore reflect differences in shear stress to which they are exposed as the circulation develops.

Smad7 has also been documented as being upregulated in several cell types exposed to ligands of the TGF β superfamily, thus forming a negative feedback loop in the response to ligand. We have demonstrated

in the present study that *Smad7* expression is upregulated at the onset of embryonic transcription; no transcripts were detectable by RT-PCR in zygotes but expression was clearly evident at the 2-cell stage. TGF β receptor expression is the exact converse: mRNAs for both *T β RII* and *T β RI* (*ALK5*) are detectable in zygotes and their levels decrease so that neither is detectable by the 4-cell stage (Roelen et al., 1998). Since receptor expression precedes that of *Smad7*, it is conceivable that TGF β signalling is responsible for its initial up-

TABLE 1. *Smad7* Expression in E12.5 Mouse Embryos as Determined by In Situ Hybridisation

Tissue	mRNA	Tissue	mRNA
Nervous system		Stomach/intestines	
Brain	+	Mucosal-epithelium	—
Spinal cord	+/-	Submucosal layer	+
DR ganglia	—	Muscle layer	—
Meninges	+/-	Lung	
Cerebral peduncle	—	Mesenchyme	—
Choroid plexus	+	Epithelium	—
Trigeminal ganglion	+	Liver	—
Left vagus nerve	—	Pancreas	—
Optic nerve	—	Pituitary	+
Optic chiasma	—	Thymic primordium	—
Vestibulocochlear nerve	+	Adrenal primordium	+/-
Nephrogenic system		Vomeranasal organ	+
Mesonephros	+/-	Olfactory lobe	+ ^m
Paramesonephric duct	+	Cochlea	+
Metanephros	+/-	Tooth primordium	+
Gonad	+	Infundibulum	+
Cardiovascular system		Tongue	—
Heart		Vibrissae	+
Atrium	—	Oesophagus	—
Ventricle	—	Trachea	+
Pericard	—	Pharynx	—
Leaflets of pulmonary valve	++	Eye	
Atrio-ventricular cushion	++	Lens	—
Blood vessels		Cornea	—
Dorsal aorta	++	Retina	—
Thoracic aorta	++	Notochord	—
Carotid arteries	+	Erythrocyte	—
Cerebral arteries	+	Genital tubercle	+
Vertebral arteries	+	Meckel's cartilage	—
Spinal cord artery	—	Intervertebral discs	—
Umbilical cord	+	Dermomyotome	+/-
Segmental arteries	+	Pinna primordium	—
Hyoid vessels	—	Body wall	—
Jugular veins	—	Diaphragm	+
Inferior vena cava	+	Precartilage primordium	+

^mmesenchyme only, —: no expression, +/-: either background, or very weak expression, +: moderate expression, ++: high expression.

regulation. In later development, trophoblast giant cells, yolk sac mesoderm, the heart, choroid plexus, submucosal layer of the stomach, and the kidneys express both *Smad7* and *TβRII* (Roelen et al., 1994; Goumans et al., 1999), so again, *Smad7* could be up-regulated by TGFβ signalling. The effects may be mediated by Smad4 in combination with either Smad2 or Smad3 since both are detectable in the embryo proper by E7.5 (Zwijnsen et al., 1999). In the light of the restricted expression pattern of *TβRII*, particularly at early postimplantation stages, however (Roelen et al., 1994), other ligands are also likely to be involved in regulating Smad7. For example, activin (or a ligand binding to its receptors) could be responsible for the observed upregulation of *Smad7* between E7.5 and E8.5, in precartilage condensations, the brain and in the urogenital system (Feijen et al., 1994; Verscheuren et al., 1995; see Mummery and van den Eijnden-van Raaij, 1999 for a review of receptor/ligand expression patterns).

In a previous study, we have shown by transient expression of dnTβRII in zygotes that development

beyond the 2-cell stage requires an intact TGFβ signalling pathway although we do not know the molecular basis for this effect. Here, we have demonstrated that overexpression of *Smad7*, which would also inhibit TGFβ signalling but in a manner different from that following ectopic expression of a dominant negative receptor, results in a similar failure to proceed beyond the 2-cell stage of development. Taken together, the results demonstrate that fine-tuning of TGFβ signalling via proteins that may include Smad7 could be crucial to very early normal development. Gene ablation studies and conditional or tissue specific overexpression should provide the clues on whether *Smad7* has a function at other sites of expression during later stages of development.

EXPERIMENTAL PROCEDURES

Collection, Microinjection, and Culture of Embryos

For reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridisation analysis, mouse embryos from F1 crosses of C57Bl6 × CBA strains were

collected and processed as described before (Dewulf et al., 1996, Roelen et al., 1997b). For zygote injection, *Smad7* cDNA was cloned into an expression vector containing the mouse *phosphoglycerate kinase-1* (*PGK*) promoter sequence (Adra et al., 1987). This vector was linearized and the DNA dissolved in injection buffer at a final concentration of 2–8 ng/μl, as described previously (Roelen et al., 1998). Approximately 5 pl of plasmid DNA was injected into the male pronucleus of one-cell zygotes; embryos undergoing successful injection of either *Smad7* plasmid DNA or empty vector, monitored by nuclear swelling, were transferred to M16 medium for culture. Three days later, when uninjected control embryos had developed to blastocysts, all embryos were scored for developmental stage (Roelen et al., 1998). A construct encoding dominant negative type II TGFβ receptor (*dn-TβRII*) or coding for a constitutively phosphorylated type I TGFβ receptor (*ca-ALK5*) were injected, either to act as negative controls or to rescue the phenotype, respectively (Roelen et al., 1998).

RNA Isolation, cDNA Synthesis, and PCR

RNA isolation, subsequent cDNA synthesis and PCR were as described (Roelen et al., 1997a). 5'-CCTCCTCCTTACTCCAGATA-3', 5'-TCTTGGACACAGTAGAGCCT-3' and 5'-ACGCACCAGTGTGACCGATC-3' were used as forward, reverse, and internal reverse primer (annealing temperature: 58°C), to amplify the region corresponding to nucleotides 929–1148 and 929–1095 of the mouse *Smad7* sequence (Nakao et al., 1997). Primers used for β-actin and for *PECAM-1* and *AFP* are described in Roelen et al. (1997a) and Goumans et al. (1999), respectively. Groups of 15–20 preimplantation and 10 (microdissected) postimplantation embryos were pooled to extract RNA. PCR was carried out using 1 embryo equivalent. Experiments were carried out on 3–4 independent samples.

Northern Blotting and In Situ Hybridisation

A *Smad7* specific 260 bp-long *HindIII-EcoRV* cDNA fragment (Nakao et al., 1997), subcloned into pBS SK+, was used as probe. A mouse embryo poly-A⁺ RNA blot (Clontech) was hybridized with random-primed *Smad7* probe to check for its specificity. Equal loading of mRNA was confirmed by rehybridisation of the blot with *GAPDH*. In vitro transcription with T3 or T7 RNA Polymerase yielded ³⁵S-labelled single-stranded antisense (*EcoRI*) or sense (*NotI*) *Smad7* riboprobes. The *Smad7* antisense probe did not show signal above background by in situ hybridization at any developmental stage tested (data not shown).

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