

Spatio-temporal activation of Smad1 and Smad5 in vivo: monitoring transcriptional activity of Smad proteins

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Summary

Signaling by bone morphogenetic proteins is essential for a wide variety of developmental processes. Receptor-regulated Smad proteins, Smads 1 and 5, are intracellular mediators of bone morphogenetic protein signaling. Together with Smad4, these proteins translocate to the nucleus and modulate transcription by binding to specific sequences on the promoters of target genes. We sought to map transcriptional Smad1/5 activity in development by generating embryonic stem cell lines carrying a Smad1/5-specific response element derived from the *Id1* promoter coupled to β -galactosidase or luciferase as reporters. Three independent lines (BRE-lac1, BRE-lac2 and BRE-luc) have shown the existence of an autocrine bone morphogenetic

protein signaling pathway in mouse embryonic stem cells. Reporter activity was detected in chimeric embryos, suggesting sensitivity to physiological concentrations of bone morphogenetic protein. Reporter activity in embryos from transgenic mouse lines was detected in tissues where an essential role for active bone morphogenetic protein signaling via Smads 1 or 5 had been previously established. We have thus generated, for the first time, an in vivo readout for studying the role of Smad1/5-mediated transcriptional activity in development.

Key words: BMP responsive element, Smad1/5, Reporter mice, Embryonic stem cells

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF β) superfamily of secreted ligands that fulfil multiple functions during the development of vertebrate as well as non-vertebrate species (Hogan et al., 1996). Specific heteromeric type I/type II serine/threonine kinase receptor complexes mediate BMP action. Type I receptors, also termed ALKs (activin-receptor-like kinase) act downstream of type II receptors and determine the specificity within the receptor complex. The type I receptors, in turn, activate their intracellular downstream targets, known as Smads (Heldin et al., 1997). Smad proteins can be subdivided into three categories: receptor-regulated Smads (R-Smads), which transiently interact with type I receptors and become phosphorylated at their C-terminal motif SXS; common partner Smads (Co-Smads), which associate with R-Smads and translocate them to the nucleus and modulate transcription of target genes; and inhibitory Smads (I-Smads), which can interfere with TGF β /Activin/BMP signaling by binding to activated R-Smad proteins or targeting type I receptors for degradation (Chang et al., 2002). TGF β (via type I receptor ALK5) and activin (via type I receptor ALK4) signaling is mediated by R-Smads 2 and 3, while BMP-dependent signaling (via type I receptors ALK2, ALK3 and ALK6) is mediated specifically by R-Smads 1, 5 and 8 (Derynck and Zhang, 2003; ten Dijke et al., 2003). Activation of Smad1 and

Smad5 by BMPs has been described in endothelial cells (Valdimarsdottir et al., 2002), although TGF β can also activate Smads 1 and 5 through ALK1 in these cells (Goumans et al., 2002). The Smad complexes recognize specific sequences in the promoters of target genes that, in combination with co-factors, effectively determine the transcriptional regulation of target genes (Hata et al., 2000; Zwijsen et al., 2003). Recently, a number of short promoter sequences have been identified that, when multimerized, can elicit transcription of reporter genes in response to TGF β superfamily ligands. TGF β , activin and BMP activate transcription of a luciferase reporter, driven by Smad-binding elements (SBE) derived from the *c-jun* promoter (Jonk et al., 1998), while SBE sequences derived from the *PAI-1* promoter are responsive to ALK5 and ALK4, but not ALK1-, ALK2-, ALK3- or ALK6-mediated signaling (Dennler et al., 1998). Similar sequences (including SBEs) were found in the promoter of *Id1* (inhibitor of differentiation 1), a BMP target gene (Hollnagel et al., 1999; Korchynskiy and ten Dijke, 2002), and shown to be responsive to BMP, but not to TGF β or activin in C2C12 myoblasts (Katagiri et al., 2002; Korchynskiy and ten Dijke, 2002; Lopez-Rovira et al., 2002).

Examination of BMP ligand, receptor and Smad expression and the effects of gene ablation in mice have demonstrated that BMP signaling is essential in many developmental processes (Jones et al., 1991; Dick et al., 1998; Flanders et al., 2001). Mice lacking BMP4, the BMP type II receptor (BmpRII) or

the type I receptor BmpRIa (ALK3) show defects in mesoderm differentiation or fail to initiate gastrulation (reviewed in Chang et al., 2002). BMP4 and BMP8b were shown to be necessary for allocation of cells to the germline, because homozygous null mutants for these genes have no primordial germ cells (PGCs) (Lawson et al., 1999; Ying et al., 2000). Deletion of Smad1 in mice results in defective extra-embryonic development, reduced numbers of PGCs and embryonic lethality around embryonic day 10.5 (E10.5) (Hayashi et al., 2002; Lechleider et al., 2001; Tremblay et al., 2001). BMP2 is essential for heart formation: BMP2 knockout mice lack or have retarded or malformed hearts (Zhang and Bradley, 1996). Both BMP4 and Smad5 null mice show defects in heart looping (Chang et al., 1999; Chang et al., 2000; Fujiwara et al., 2002). Tissue-specific deletion of ALK3 in cardiomyocytes, which circumvents early lethality, has shown that signaling by BMPs via ALK3 in these cells is essential for atrioventricular cushion formation and septal morphogenesis, although no defects were observed in the outflow tract (Delot, 2003; Gaussin et al., 2002). Interestingly, mice carrying a hypomorphic allele of the BmpRII lack septation in the outflow tract, but have no other heart defects (Delot et al., 2003). BMPs are also involved in limb and bone development, as well as in formation of teeth and whisker follicles (Dick et al., 1998; Dudley and Robertson, 1997; Flanders et al., 2001; Hogan, 1996; Jones et al., 1991; Lyons et al., 1995).

It is, however, important to realize that the response to BMPs depends not only on the availability of ligands but also on the presence of appropriate receptor combinations, extracellular antagonists such as noggin, chordin or follistatin (Balemans and Van Hul, 2002) and intracellular inhibitors, such as I-Smads (Chang et al., 2002). Availability of specific co-activators and co-repressors in different tissues also modulate the transcriptional response to BMPs mediated by Smad1/5/8 (Zwijnsen et al., 2003). The outcome of this complex interplay reflects the actual transcriptional activity elicited by BMP signaling via Smads1, 5 and 8. To map active Smad1/5 transcriptional activity throughout development, we designed constructs harboring the BMP-responsive element (BRE) identified previously in the *Id1* promoter (Korchynskiy and ten Dijke, 2002) and used them to generate transgenic reporter mice. We used mouse embryonic stem (ES) cells to select clones with the highest response to BMP in vitro for blastocyst injection to optimize the chances of generating mice reporting net transcriptional activity mediated by Smad1/5 proteins, in vivo. Of the three ES reporter cell lines selected, two expressed β -galactosidase (BRE-lac1 and BRE-lac2) and one expressed luciferase (BRE-luc) under the control of the BRE, here referred to collectively as Smad1/5 reporter ES cell lines. These ES lines all showed an autocrine BMP-activated signal transduction pathway while undifferentiated. In addition, the BRE- β -galactosidase transgenic mouse lines established from these cells showed reporter expression patterns that mapped to sites previously associated with active Smad1/5 signaling. Transgenic mice are now available that report Smad1- and Smad5-dependent transcriptional activity in vivo.

Materials and Methods

Generation of reporter constructs

The BMP-responsive construct BRE-luc has been described

previously (Korchynskiy and ten Dijke, 2002). To generate the BRE- β -galactosidase construct, an *NcoI/BamHI* fragment containing the luciferase coding sequence of the BRE-luc was replaced by a *NcoI/BamHI* fragment from PSDKlacZpA (gift from T. Oosterveen) containing the β -galactosidase sequence and a poly(A) sequence.

Generation of reporter ES cells

IB10 ES cells, a subclone of E14 cells, were co-electroporated with *DraIII* linearized BRE-luc or *XmnI* linearized BRE- β -galactosidase reporter and a *XhoI* linearized PGK-hygromycin resistance cassette (te Riele et al., 1990). Electroporation conditions are described elsewhere (Goumans et al., 1998). After 8 days in culture in the presence of hygromycin (150 μ g/ml), single resistant colonies were picked and DNA isolated for genotyping by PCR using primers 5' CCTTTCGCTATTACGCCAG 3' (sense) and 5' TTAAG-TTGGGTAACGCCAGG 3' (antisense) for the BRE- β -galactosidase construct and 5' CACACAGTTCGCCTCTTTGA 3' (sense) and 5' AAGATGTTGGGGTGTGGAG 3' (antisense) for the BRE-luciferase construct. Based on the response to 5 ng/ml of BMP4 (R&D systems), two independent β -galactosidase reporters (BRE-lac1 and BRE-lac2) and one luciferase reporter line (BRE-luc) were selected for further characterization.

Cell culture, transient transfections and western blots

ES cells were routinely cultured on a monolayer of irradiated primary mouse embryonic fibroblasts in complete medium (CM): GMEM (BHK-21, Invitrogen) supplemented with 2 mM L-glutamine, 100 mM sodium pyruvate, non-essential amino acids (1:100, Invitrogen), 10% fetal calf serum (FCS), Leukemia inhibitory factor (LIF, 10^3 U/ml) and 0.1 mM β -mercaptoethanol. Alternatively, ES cells were cultured in the absence of feeder cells in BRL-conditioned medium [BRL CM: DMEM medium conditioned in Buffalo rat liver cells, supplemented with 2 mM L-glutamine and non-essential amino acids (1:100) and 20% FCS] as described previously (Mummery et al., 1990). HepG2, C2C12 and MDA MB468 cells were cultured in DMEM supplemented with 10% FCS. For transfection, HepG2, C2C12 and MDA MB468 cells were seeded at 1.5×10^4 cells/cm² in 12-well plates, grown overnight (o/n) then transiently transfected with the BRE-luc reporter (150 ng/well; 300 ng/well for HepG2) in the absence or presence of expression plasmids (150 ng/well each one). pcDNA3 plasmid was used to keep the total amount of transfected DNA constant (500 ng of total plasmid DNA/well, 1 μ g for HepG2 cells). Transfections were carried out using FuGene6 transfection reagent (Roche) following the manufacturer's protocol. β -Galactosidase plasmid co-transfection (50 ng/well) was used as an internal control to normalize transfection efficiency. 16 hours before lysis, cells were exposed to growth factors (BMP2, 4, 6, 7, or TGF β 1; EGF and FGF, purchased from Peprotech, USA) at concentrations indicated in the figures. Luciferase and β -galactosidase activity were quantified using the Luciferase assay (Promega) with Victor luminometer (Wallac) as described previously (Jonk et al., 1998). Cultures were maintained in a humidified chamber in a 5% CO₂/air mixture at 37°C.

To investigate the response of the Smad1/5 reporter lines to BMP or noggin, these ES cells were cultured in BRL CM in the absence of feeders, supplemented with 0.1 mM β -mercaptoethanol and either 20% or 5% FCS. On day 1, 2.5×10^4 cells/cm² were seeded in gelatinized 12-well plates and allowed to grow for 24 hours. On day 2, cells were washed once with phosphate buffered saline (PBS) and fresh BRL CM supplemented with 5% FCS and BMP4 and noggin (R&D systems) was added. On day 3, BRE-lac1 and BRE-lac2 ES cells were lysed with 100 μ l of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1% NP-40, 0.5% deoxycholate, 2 mM EDTA, 25 mM β -glycerophosphate, 1 mM Na₃VO₄, 100 mM NaF and 20 μ g/ml aprotinin, 40 μ g/ml leupeptin, 0.75 mM PMSF) and BRE-luc ES cells

with 200 μ l of luciferase lysis buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EDTA, 1% Triton X-100). Reporter activity was measured in 10 μ l and 50 μ l of whole cell lysates for β -galactosidase and luciferase, respectively. β -Galactosidase activity was measured with the Galacto Plus kit (AB systems) and luciferase with LucLite substrate (Perkin-Elmer) in a luminometer (Packard), following the manufacturer's instructions. Western blot with an antibody raised against the phosphorylated C-terminal peptide SSVS of Smads 1, 5 and 8 (PSmad1/5/8), PS1 (Persson et al., 1998) and anti-Id1 (Santacruz Biotechnology, 1:500 dilution) was performed on BRE-lac1 and BRE-lac2 ES cell lysates essentially as described (Faure et al., 2000). BCA protein assay kit (PIERCE Biotechnology) was used for protein quantification. 10 μ g total protein was loaded in each lane. Both PSmad1/5/8 and Id1 proteins were probed in the same blot; each experiment was repeated at least twice.

Genomic DNA isolation, RNA isolation, cDNA synthesis and PCR

Genomic DNA was isolated using standard techniques (Sambrook et al., 1989). RNA was isolated with Ultraspec (Biotecx) following manufacturer's instructions and subjected to DNase treatment. 1 μ g of total RNA was used to synthesize cDNA with M-MLV-RT (Superscript, Isogen). 1/20 of the cDNA was used for PCR. When appropriate, RNA was used as negative control. PCR conditions were as follows: after 5 minutes of denaturation at 94°C, 40 amplification cycles were performed, each including denaturation at 94°C, 15 seconds, annealing at 30 seconds at the primer's specific temperature (see below) and 45 seconds extension at 72°C. These cycles were followed by a 7 minutes extension at 72°C. Samples were analyzed on 2% agarose gels. Primers for the following genes were used for RT-PCR: BMP2, BMP4, BMP7 (60°C annealing temperature), described elsewhere (Roelen et al., 1997), Oct4 (55°C annealing) (Levenberg et al., 2002) and β -actin (56°C annealing) (Roelen et al., 1997).

Generation of teratomas

Teratomas were generated by injecting approximately 5×10^4 ES cells (in PBS) in the testis capsule of 129/Ola mice. Briefly, ES growing on feeders were washed with PBS, trypsinized, resuspended in CM medium, seeded in gelatinized wells and allowed to recover for 1 hour at 37°C. Attached ES cells were resuspended in CM medium, counted, centrifuged and resuspended in PBS (150–200 μ l). Following injection, mice were monitored weekly for swelling and signs of discomfort. At 4 weeks after injection, teratomas were isolated in PBS and fixed in 4% paraformaldehyde (PFA) for 2–4 hours at 4°C and stained overnight with X-Gal for β -galactosidase activity. After washing with PBS, teratomas were post-fixed in 4% PFA overnight at 4°C, dehydrated and embedded in paraffin. 7 μ m sections were stained with Haematoxylin and Eosin or with Neutral Red and mounted in DEPEX.

Chimeric embryos and transgenic mice

BRE-lac1, BRE-lac2 or BRE-luc ES reporter cells were injected into the inner cell mass of C57BL/6 blastocysts. These were implanted in pseudopregnant C57BL/6xCBA recipients and allowed to develop to term. Male chimeras were crossed with C57BL/6xCBA females and the F1 progeny genotyped for germline transmission of the transgene. To generate chimeric embryos for analysis, BRE-lac1 ES cells were injected into GFP-expressing blastocysts (Hadjantonakis et al., 1998), which were implanted in pseudopregnant C57BL/6xCBA recipients and allowed to develop until E9.5. Noon of the day of the vaginal plug is E0.5.

BRE-lac1 and BRE-lac2 F1 transgenic males were crossed with C57BL/6xCBA females to obtain transgenic embryos at E9.5.

Chimeric and transgenic embryos were isolated in ice-cold PBS, fixed in 0.4% PFA for 30 minutes at 4°C and stained overnight with X-Gal. All animal experiments were in accordance with Dutch Law.

β -galactosidase staining and immunohistochemistry

Before staining, ES cells were fixed briefly with 0.1% glutaraldehyde, 0.5% formaldehyde in PBS, followed by three washes with PBS. Samples were stained for 2 hours to o/n at 30°C in X-gal staining solution (200 mM X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS). After staining, samples were washed three times with PBS and post-fixed for 30 minutes with 2% PFA at room temperature. Embryos were isolated in ice cold PBS, fixed in 0.4% PFA in PBS and stained for β -galactosidase o/n at 30°C in X-gal staining solution, as described (Nagy et al., 2003). After staining, the embryos were washed in PBS, fixed o/n in 4% PFA at 4°C, dehydrated and embedded in plastic or paraffin. Serial sections of 6 μ m were cut and counterstained with Neutral Red or with Haematoxylin. For anti-PSmad1/5/8 antibody (PS1) staining, paraffin sections of X-gal stained E9.5 transgenic embryos were rehydrated, treated with 1.2% hydrogen peroxide for 15 minutes and boiled in 10 mM Tris, 1 mM EDTA, pH9 for 20 minutes for antigen retrieval. After washing with PBS, sections were blocked with 0.05% BSA in PBS for 30 minutes and incubated with PS1 (Cell Signaling) 1:200 in blocking solution, o/n at 4°C. After o/n incubation, PowerVision™ Poly-HRP-Conjugates (ImmunoVision Technologies) was used as secondary antibody with the Fast 3,3'-diaminobenzidine tablet set (DAB, SIGMA).

Whole mount photomicrographs were taken on a Camedia C3030 digital camera mounted on an Olympus SZX9 microscope. Sections were photographed with a Nikon DMX1200 digital camera on a Nikon eclipse E600 microscope and in a MC100 Spot camera mounted on a ZEISS axioplan microscope.

Results

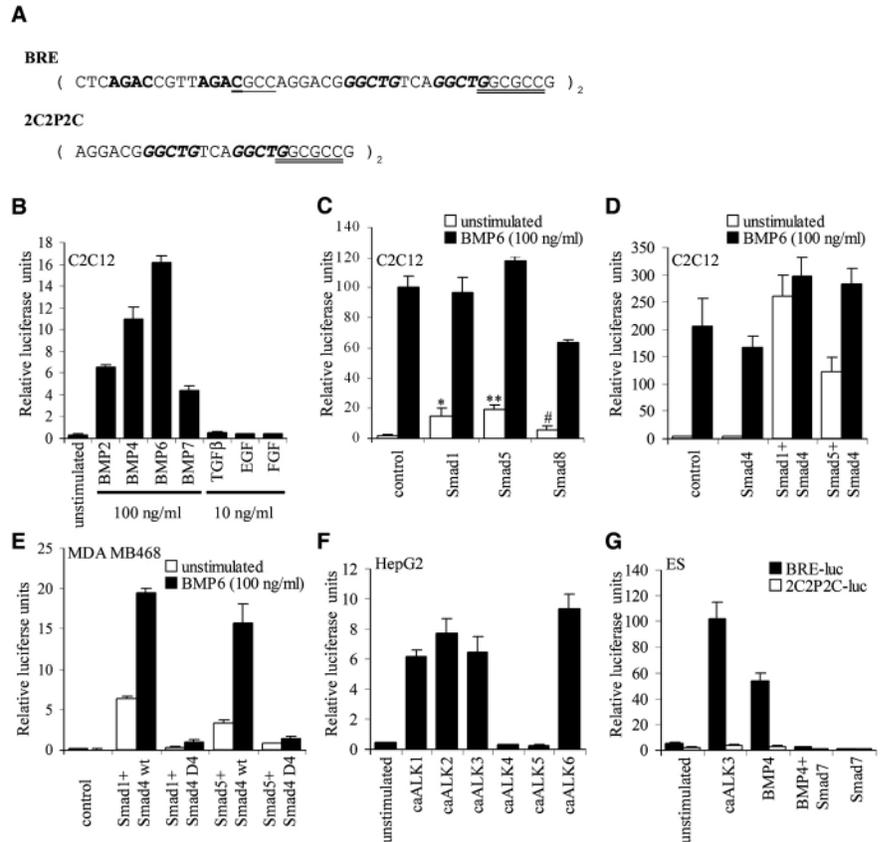
Specific activation of the BMP response element by Smad1/4 and Smad5/4 complexes

We used BMP-responsive elements (BRE) recently identified within the promoter of *Id1* (Katagiri et al., 2002; Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002) to generate reporter BRE- β -galactosidase and BRE-luciferase constructs (Fig. 1A). In C2C12 cells, addition of BMP2, 4, 6 or 7 increases the reporter activity, while addition of TGF β 1, epidermal growth factor (EGF) or fibroblast growth factor (FGF) had no effect (Fig. 1B). Activin also had no effect on reporter activity (Korchynskyi and ten Dijke, 2002). This showed that only BMPs specifically activated the BRE-luciferase reporter in these cells. We showed previously that co-operation between Smad1/5 and Smad4 and presence of both of their putative binding sites in the BMP target gene promoters is critically important for transcriptional activation of those genes (Korchynskyi and ten Dijke, 2002). Over-expression of Smad1 or Smad5 led to a highly significant increase of the BRE-luciferase reporter activity in C2C12 cells; Smad8 induced only a marginal increase (Fig. 1C). In all cases, however, reporter activity was increased upon addition of BMP6 (Fig. 1C). Co-transfection of Smad1 and Smad4 or Smad5 and Smad4 led to upregulation of the reporter activity in C2C12 cells (Fig. 1D), indicating that the combination of one R-Smad (Smad1 or Smad5) with Smad4 is sufficient for an efficient transcriptional activation of the reporter in the absence of ligand. The combination Smad8 and Smad4 was

Fig. 1. Specificity of the BMP response element

(BRE) to Smad1/5-mediated BMP signaling in vitro. (A) The BRE sequence consists of two repeats of 2×SBE (bold), 1×CGCC (underlined), 2×CAGC (bold, italic) and 1×GGCGCC (double underlined). 2C2P2C is a similar sequence that lacks 2×SBE + 1×CGCC binding sites and does not respond to BMPs in vitro (see Korchynskiy and ten Dijke, 2002). (B) In C2C12 cells, the BRE-luc reporter is specifically upregulated by BMP2, 4, 6 and 7 (100 ng/ml). 10 ng/ml of TGFβ, EGF or FGF do not activate reporter transcription. (C) BRE-luc reporter activity is induced by overexpression of either Smad1 or Smad5 and to a lower extent, by Smad8 in C2C12 cells. BRE-luc reporter activity is further increased upon addition of BMP6 to control cells or cells overexpressing Smad1, 5 or 8.

(D) Overexpression of Smad1+Smad4 or Smad5+Smad4 is sufficient to induce BRE-luc activity in C2C12 cells. Smad4 alone does not influence inducibility by BMP6 (100 ng/ml). (E) Binding of Smad4 to the BRE sequence is critical for the reporter response to ligand-dependent and ligand-independent Smad1 or Smad5 overexpression. In the presence of the Smad4-D4 DNA-binding mutant (harboring the K81R and R88K mutations), neither Smad1 nor Smad5 are able to drive BRE-luc expression in MDA-MB468 cells. Expression of wild-type Smad4 is sufficient to restore reporter inducibility either in response to BMP6 or to co-expressed Smad1 or Smad5. (F) In HepG2 cells, constitutively active (ca) type I receptors caALK1, caALK2, caALK3 and caALK6 specifically induce BRE-luc reporter activity, while caALK4 or caALK5, do not. (G) Specific modulation of the BRE-luc reporter in response to activation or inhibition of BMP signaling in ES cells. 10 ng/ml of BMP4 and caALK3 induce while Smad7 blocks basal and BMP induced reporter activity in transient assays. As expected, the 2C2P2C-luc reporter showed no response to modulation. ** $P \leq 0.005$; * $P \leq 0.05$; # $P \leq 0.1$.



effective although much less efficient than Smad1+4 or Smad5+4 (4-fold compared with 60-90-fold, results not shown). Smad4 alone did not activate the BRE-luciferase reporter. Furthermore, addition of BMP6 showed lower induction of reporter when Smad4 was co-transfected compared with the control, suggesting that Smad4 is necessary, and that Smad1 or Smad5 are also required for efficient induction of the reporter activity. Interestingly, co-transfection of Smad1+4 was more efficient in activating the reporter than Smad5+Smad4 (Fig. 1D) whereas the additive effect on reporter activity observed after addition of BMP6 was similar for Smad1+4 and Smad5+4 combinations. In the light of the marginal response to Smad8 (2-fold) compared with induction by Smad1 or 5 (7-10 fold, see Fig. 1C), the BRE promoter is regarded predominantly as a Smad1/5 reporter and will be further referred to as such.

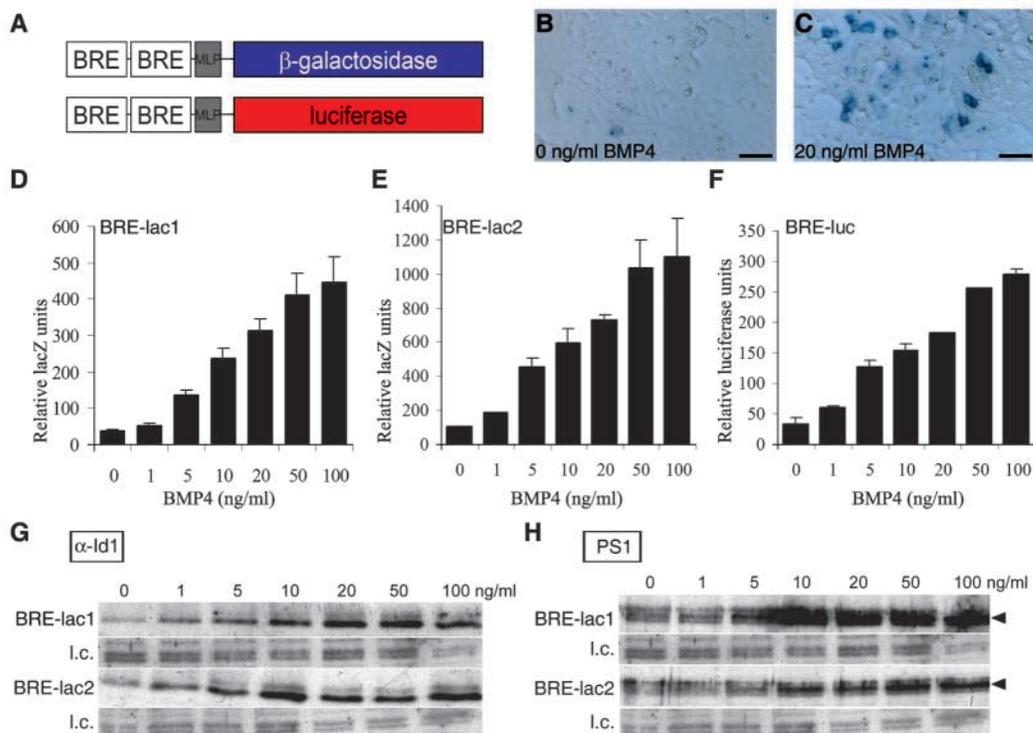
To explore whether intrinsic Smad4 DNA-binding activity is critically important to drive the BMP response, we used a Smad4-deficient cell line, MDA MB468. Wild-type Smad4, but not Smad4-D4, a DNA-binding-deficient mutant containing mutations K81R and R88K in the Smad4 DNA-binding domain (Moren et al., 2000), rescued inducibility of BRE-Luc reporter by BMP-6 in this cell line when co-transfected with Smad1 or Smad5 (Fig. 1E). Similar results were obtained by using single mutants, either K81R or R88K (results not shown).

Constitutively active type I receptors (caALKs) can activate specific downstream Smad proteins, mimicking the effect of ligand addition (Wieser et al., 1995). Because we showed that Smad1+4 and Smad5+4 complexes are critical for activation of the BRE-luciferase reporter, we expected it to be upregulated by type I receptors that specifically activate Smad1 and Smad5 (ALK1, ALK2, ALK3 and ALK6) but not receptors that activate Smad2 or Smad3 (ALK5 and ALK4) signaling. To test this hypothesis, we co-transfected caALK1 to 6 with the BRE-luciferase reporter in HepG2 cells. As expected, the BRE-luciferase reporter was specifically activated by caALK1, 2, 3 and 6, but not by caALK4 or caALK5 (Fig. 1F). Taken together, these results show that specific transcriptional activation of the BRE-luciferase reporter requires DNA binding of Smad1+4 or Smad5+4 complexes. These complexes bind to the BRE element after activation of Smad1/5 proteins by type I receptors ALK1, ALK2, ALK3 and ALK6 and are able to induce the expression of target genes.

Transient transfection of the BRE-luciferase reporter construct in ES cells showed specific activation by BMP4 and by caALK3. Co-transfection of either Smad7 or Smad6 blocked basal and BMP-induced reporter activity (Fig. 1G and results not shown, respectively). A BRE element containing GGCGCC and CAGC motifs, but lacking SBE-binding sites (2C2P2C-luc, see Fig. 1A) failed to respond to stimulation by

Fig. 2. Response of stable ES reporter cell lines to BMP4.

(A) Constructs used to generate transgenic ES cells. BRE- β -galactosidase or BRE-luc reporter constructs and a PGK-hygromycin cassette were co-electroporated in wild-type ES cells. Single colonies were picked from selection medium (150 μ g/ml hygromycin) and genotyped by PCR on genomic DNA. Transgenic ES cell lines were assayed for BMP-induced reporter expression. Basal reporter activity was observed in (B) unstimulated BRE-lac1 ES cells. (C) Reporter activity increased in response to 20 ng/ml BMP4. Bars, 50 μ m (B,C). Activity of the reporter gene in ES lines BRE-lac1 (D), BRE-lac2 (E) and BRE-luc (F) increases with increasing concentrations of BMP4. Western blots for Id1 (anti-Id1 antibody) (G) and for activated Smad1/5/8 (PSmad1/5/8, PS1 antibody) (H) were performed on the same extracts as in D and E; expression profile of these proteins show good correlation with the reporter activity. Note that the middle band in the triplet corresponds to phosphorylated Smad1/5/8 (black arrowheads). 10 μ g/ml of protein was loaded in each lane and confirmed by Ponceau S staining. Nonspecific bands are shown as loading control (l.c.). The results are represented as the mean of three independent experiments (mean \pm s.d.).



BMP4, or caALK3, highlighting the need for the presence of the SBE-binding sites and indicated that the BRE element is specifically activated by the BMP-signaling pathway. These results showed that the complete BMP signal transduction machinery, including type I and type II receptors and R-Smads, was present in undifferentiated ES cells, as described (Roelen et al., 1997; Wiles and Johansson, 1999; Ying et al., 2003).

Generation of Smad1/5 reporter embryonic stem (ES) cell lines

We used the BRE reporter coupled to β -galactosidase or luciferase (Fig. 2A) to generate stable mouse ES reporter cell lines. Because the BMP signal transduction machinery are present in ES cells (Ying et al., 2003), we could screen for the reporter lines most sensitive to activation by BMP. Transgenic lines showing a 2-4-fold activation of the reporter in response to 5 ng/ml of BMP4 (compared with the unstimulated controls) were selected for further analysis. Using this criterion we selected two ES reporter lines coupled to β -galactosidase (BRE-lac1 and BRE-lac2) and one reporter line coupled to luciferase (BRE-luc) for further study (Fig. 2A). Addition of 20 ng/ml of BMP4 markedly increases reporter activity compared with unstimulated cells (Fig. 2B,C). Surprisingly, not all cells expressed β -galactosidase upon stimulation by BMP4 and further increase in ligand concentration did not lead to uniform expression of β -galactosidase in the cultured cells, although the reporter activity was increased (see below). Southern blot analysis showed that the number of copies of the

construct integrated for the selected lines was less than 10 (results not shown).

Response of Smad 1/5 ES reporter cell lines to BMP is dose dependent

We investigated whether the response of the Smad1/5 reporter lines BRE-lac1, BRE-lac2 and BRE-luc to BMP was dose dependent. BMP4 was added in the range of 1 to 100 ng/ml, and reporter activity measured in whole cell lysates. All three Smad 1/5 reporter cell lines showed similar dose-dependent reporter activation upon BMP stimulation (Fig. 2D-F). Addition of 5 ng/ml of BMP4 to the reporter ES cells resulted in a 3- to 4-fold induction of the reporter, while at higher concentrations of BMP4 (e.g. 20 ng/ml), BRE-lac1 and BRE-lac2 reporter ES cells showed greater fold induction (7- to 8-fold) than BRE-luc ES cells (6-fold). We then investigated whether the reporter activity could be correlated with upregulation of Id1, a target of BMP in ES cells (Hollnagel et al., 1999; Korchynskyi and ten Dijke, 2002). Western blot analysis for Id1 in BRE-lac1 and BRE-lac2 whole cell extracts showed that Id1 protein levels increased in a dose-dependent manner (Fig. 2G) consistent with the measured reporter activity. In accordance, the increase in reporter activity was also accompanied by a dose-dependent increase in phosphorylation of Smad1/5/8 proteins (Fig. 2H). These experiments showed that there is a close correlation between different readouts of active BMP signaling (phosphorylation of Smad1/5/8, levels of Id1 protein) and reporter activity in Smad1/5 ES reporter lines.

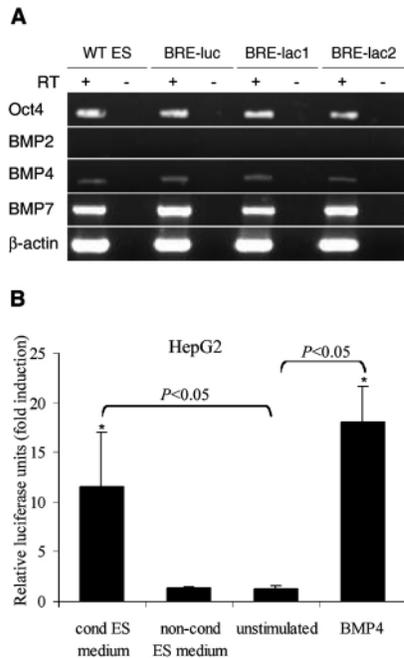


Fig. 3. ES cells express active BMP4 and 7 but not BMP2. (A) RT-PCR on cDNA from wild type and transgenic ES cell lines BRE-luc, BRE-lac1 and BRE-lac2. All the lines tested express BMP4 and BMP7, but not BMP2. Using the same primer sets, transcripts for BMP2, 4 and 7 were detected by RT-PCR on cDNA from E9.5 mouse hearts, as expected (results not shown). β -actin was a positive control for the reverse transcription reaction; expression of Oct4 indicates the undifferentiated state of the ES cells.

(B) Transactivation assay in HepG2 cells. Medium conditioned by ES cells induces BRE-luc reporter activity in HepG2 cells. Non-conditioned medium had no effect. As a positive control, 10 ng/ml BMP4 was added to transfected cells to activate the BRE-luc reporter. As a negative control, HepG2 cells were transfected with the BRE-luc reporter and no BMP was added. Results are presented as mean fold induction (\pm s.d.) of triplicates in relation to unstimulated cells, set to 1 ($*P \leq 0.05$).

ES cells express and respond to stimulation by BMPs in vitro

Basal levels of reporter activity were observed in unstimulated transgenic ES cells. Since R1 ES cells are known to express at least BMP4 (Gratsch and O'Shea, 2002; Johansson and Wiles, 1995), we investigated whether BMPs expressed in IB10 ES cells could account for these basal levels of reporter activity. Transcripts for BMP4 and BMP7, but not BMP2, were detected by RT-PCR on cDNA from wild-type IB10 (WT) and transgenic ES lines BRE-lac1, BRE-lac2 and BRE-luc (Fig. 3A). Furthermore, medium conditioned in WT ES cells for 24 hours transactivated BRE-luc in transiently transfected HepG2 cells, while unconditioned medium not exposed to ES cells did not (Fig. 3B). This suggested that the basal levels of reporter activity (Fig. 2B,D,E,F) were not due to nonspecific signals nor to BMPs in basal ES cell culture medium, but to BMPs (most likely BMP4 and BMP7) produced by the ES cells.

Noggin is an extracellular inhibitor of BMP signaling (Zimmerman et al., 1996). Noggin molecules bind directly to BMPs, preventing subsequent binding to their cognate receptors (Groppe et al., 2002). Addition of increasing amounts

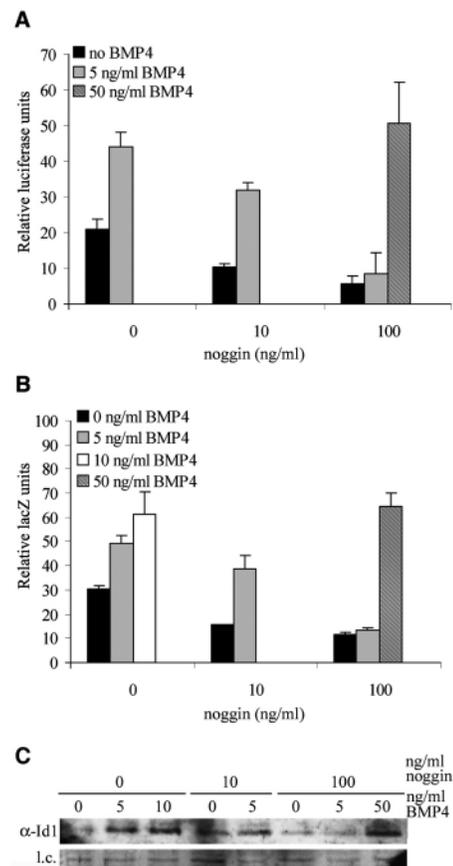


Fig. 4. Autocrine BMP pathway in ES cells. Noggin and BMP were added to reporter ES cell lines (A) BRE-luc and (B) BRE-lac2 at different concentrations to assess the reporter activity in response to activation/inhibition in vitro. The same results were obtained in BRE-lac1 ES cells (results not shown). (C) Id1 protein levels in a western blot correlated with the reporter activity in BRE-lac2 protein extracts. Equal amounts of protein (10 μ g) were loaded in each lane and confirmed with Ponceau S staining; nonspecific bands are shown as loading control (l.c.). Results are presented as the mean of triplicate experiments (mean \pm s.d.).

of noggin to BRE-luc ES cells markedly decreased basal reporter activity (Fig. 4A), which together with the observation that medium conditioned in ES cells activated the BRE-luc reporter in HepG2 cells, showed that active BMPs are present in the conditioned medium. In similar experiments on BRE-lac2 and BRE-lac1 ES cells, noggin blocked both basal and reporter activity induced by 5 ng/ml of BMP4 (Fig. 4B and results not shown). While 10 ng/ml of BMP4 could partially rescue the inhibitory effect of 10 ng/ml of noggin, the highest concentration of noggin, 100 ng/ml, strongly inhibited both the basal and the reporter activity induced by BMP4. Increasing the concentration of BMP4 to 50 ng/ml was sufficient to overcome the inhibitory effect of 100 ng/ml of noggin. This showed that BMP4 can rescue the inhibitory effect of noggin in a dose-dependent fashion. As expected, western blot analysis of Id1 on BRE-lac2 whole cell lysates showed strong correlation between the induction of Id1 by BMP and the reporter activity (Fig. 4C): levels of Id1 decreased in response to increased inhibition of BMP signaling by noggin while

increasing amounts of BMPs also increased the levels of Id1 protein. Similar results were obtained with BRE-lac1 ES cells (results not shown). These results suggested that the reporter genes in the generated transgenic lines reflect activation and inhibition of the BMP signaling pathway similarly to the endogenous target Id1 in ES cells.

Differentiation potential of the ES reporter lines

Because the Smad1/5 ES reporter lines generated expressed the stem cell marker Oct4 (Fig. 3A), they were likely to be pluripotent and able to participate in the development of an embryo. However, to determine whether they had retained pluripotency, BRE-lac1, BRE-lac2 and BRE-luc ES cells were injected in the testis capsule of syngenic mice (129 OLA). All three ES reporter lines gave rise to teratomas, which are tumors composed of well-differentiated tissues from endodermal, mesodermal and ectodermal origin (Stevens, 1967). Morphological analysis of 4-week-old teratomas derived from line BRE-lac1 showed differentiated tissues, such as epithelium, bone, striated muscle, adipose tissue and cartilage (Fig. 5B-D), similarly to teratomas derived from BRE-lac2 and BRE-luc reporter lines (results not shown). When stained for β -galactosidase activity, areas of cartilage in the teratomas showed strong blue staining (Fig. 5D), which correlated with the expression of Smads 1 and 5 in chondrocytes of E15 embryos (Flanders et al., 2001) and indicated that BMP signaling is active in developing cartilage within the teratoma. These experiments showed that the reporter ES cells retained pluripotency and that differentiated derivatives of the BRE-lac1 ES cell line responded to endogenous signaling.

Transient chimeric embryos show reporter expression at sites of BMP expression

To establish whether the reporter lines would be able to respond to the effective levels of Smad-dependent BMP signaling in mouse embryos, we injected BRE-lac1 ES cells into blastocysts expressing GFP ubiquitously (Hadjantonakis et al., 1998). When injected into the inner cell mass, the reporter ES cells contribute to the formation of the embryo proper (Nagy et al., 1993). Transient chimeric embryos were isolated at E9.5 and stained for β -galactosidase activity. Virtually uniform green fluorescence suggested that the degree of chimerism was low and mosaic (results not shown). However, 3 of 10 embryos showed prominent β -galactosidase staining in the heart, the optic vesicle, somewhat weaker staining in the ventral limb bud and scattered cells in the first and second branchial arches (Fig. 6A). At E9.5, BMP2, 4, 5, 6, 7 and 10 are expressed in the heart (Schneider et al., 2003) and BMP4, BMP2 and BMP7 in the optic vesicle (Dudley et al., 1995; Furuta and Hogan, 1998; Lyons et al., 1995; Wawersik et al., 1999). In the forelimb bud, both BMP2 and BMP7 are expressed in the apical ectodermal ridge (AER) and in the limb mesenchyme (Lyons et al., 1995). Interestingly, in the forelimb bud, reporter activity appears restricted to the ventral mesenchyme (Fig. 6A). Transverse sections through the heart of chimeric embryos showed β -galactosidase staining in the myocardial layer of the outflow tract (OT), bulbus cordis (BC) and ventricle, as well as in the myocardium surrounding the atrio-ventricular canal (AVC, Fig. 6D,E). Low β -

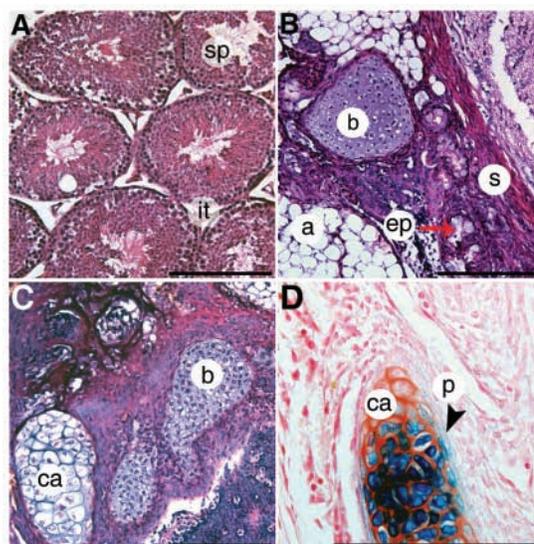


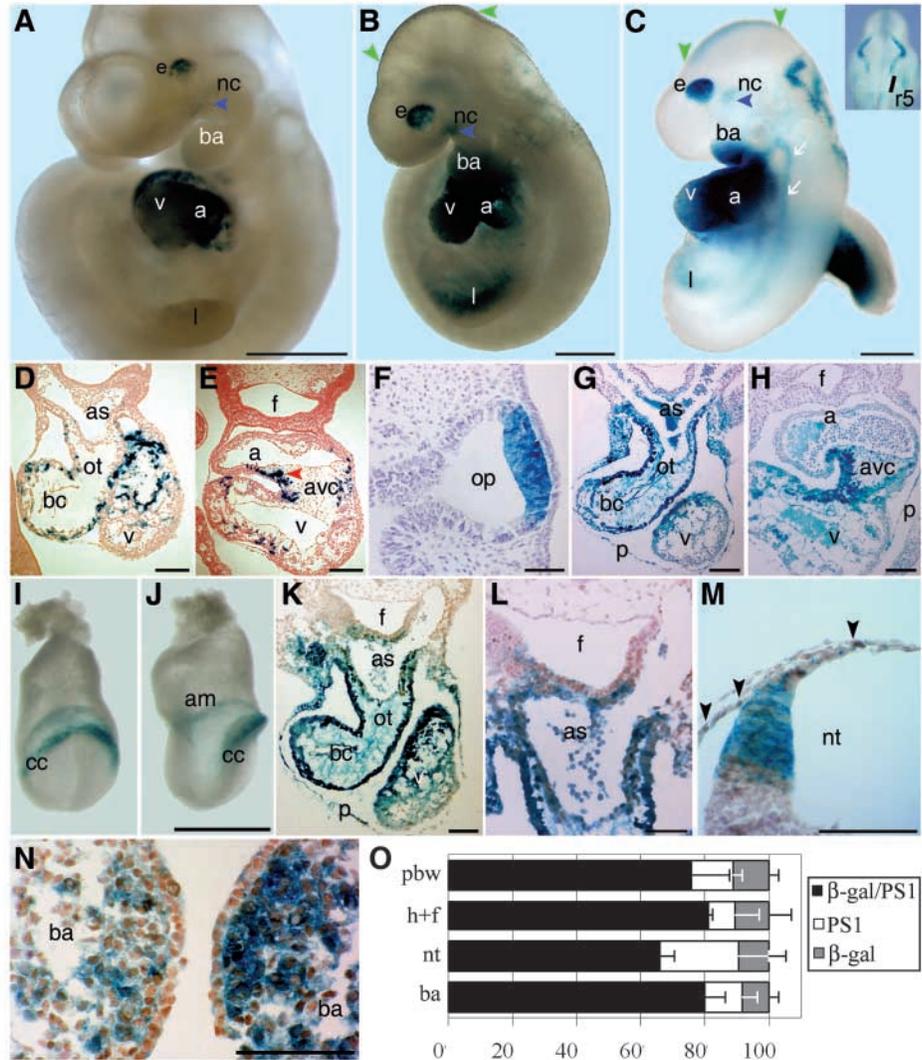
Fig. 5. Teratomas derived from reporter ES cells in adult male mice. At 4 weeks, ES cells injected in the testis capsule of syngenic mice (129 OLA) give rise to different tissues. Compare the normal testis tissue in A with a representative sample of ES-derived tissues in B, C and D. Bone (b), cartilage (ca), striated muscle (s), adipose tissue (a) and epithelial tissue (ep, red arrow) were found (B,C). β -Galactosidase staining of BRE-lac1-derived teratomas showed staining in areas of cartilage and perichondrium (D, black arrowhead). Note that not all the chondrocytes show β -galactosidase staining, suggesting regional differences in activation of Smad1/5 signaling in chondrocytes. Sections (7 μ m) were stained either with Eosin/Hematoxylin (A-C) or Neutral Red and β -galactosidase (D). Abbreviations: it, interstitial tissue; sp, spermatidia. Bar, 100 μ m.

galactosidase staining was found in the endocardium. Thus, the sites of expression of the transgene correlated clearly with sites where BMPs are known to be expressed.

Smad 1/5 transcriptional activity in transgenic E9.5 embryos

The Smad 1/5 ES reporter lines were injected into C57BL/6 blastocysts and the resulting male chimeras were crossed with wild-type C57BL/6 or C57BL/6xCBA females for germline transmission of the reporter. First generation transgenic males were crossed with wild-type C57BL/6xCBA females and their progeny analyzed at E9.5. For simplicity, transgenic embryos derived from the BRE-lac1 or BRE-lac2 ES reporter lines are referred to as BRE-lac1 and BRE-lac2 embryos, respectively. While the pattern of β -galactosidase expression in heterozygous BRE-lac1 or BRE-lac2 embryos was similar to that in chimeric embryos, other sites also stained. β -Galactosidase reporter expression was found in the roof of the neuroepithelium of the midbrain (Fig. 6B,C, green arrowheads). In the hindbrain, expression is absent in the region of rhombomere 5 (Fig. 6C; see inset). Reporter expression was further detected in the first and second branchial arches, in the ventral forelimb bud and in the ventral posterior mesenchyme (Fig. 6B,C and results not shown). β -Galactosidase was observed in the dorsal optic vesicle, while low expression was detected in the overlying ectoderm (Fig. 6F). Phosphorylated Smad1/5/8 immunostaining in the dorsal

Fig. 6. Analysis of reporter expression in transgenic E9.5 embryos. (A) Chimeric embryos were generated with the BRE-lac1 ES cell line by injection into blastocysts of β -actin-GFP mice and analyzed at E9.5. β -galactosidase staining was present in the heart, the optic vesicle (op), the ventral forelimb (l), neural crest (nc, blue arrowhead) and in the branchial arches (ba). Adult male chimeras generated with BRE-lac1 and BRE-lac2 ES lines were crossed with wild-type females and the progeny analyzed at E9.5. BRE-lac1 (B) and BRE-lac2 (C) transgenic embryos showed reporter expression pattern similar to that in transient chimeras. β -Galactosidase staining was also detectable in the roof of the midbrain neuroepithelium (green arrowheads), in the hindbrain, where the region around rhombomere 5 showed very low β -Galactosidase staining, in the posterior mesenchyme and in the aorta (white arrows). (D-E) Transverse sections through the heart of the embryo shown in A. β -Galactosidase staining is found in the outflow tract (ot), the bulbus cordis (bc) and the ventricle (v), as well as in the atrioventricular canal (avc, red arrowhead). (F) In the optic vesicle, reporter expression is restricted to the dorsal side, while low expression was observed in the overlying ectoderm. (G,H) Transverse sections through the heart of BRE-lac2 transgenic embryos showed reporter expression in endocardium, pericardium and myocardium, with stronger expression in the outflow tract and the atrioventricular canal. Frontal (I) and lateral (J) views of a transgenic embryo showing β -galactosidase expression in the cardiac crescent and in the amnion at E7.5. (K-N) Co-staining for phosphorylated Smad1/5/8 (PS1 antibody) and β -galactosidase in E9.5 transgenic embryos. β -Galactosidase expression coincides with PS1 staining in the heart and in the foregut (K,L), while in the neural tube and neural crest some cells are stained for PS1 and not β -galactosidase (M, black arrowheads). High correlation between phosphorylated Smad1/5/8 and β -galactosidase was observed in the branchial arches (N). (O) Quantification of the incidence of β -galactosidase and PS1 staining. Sections containing posterior body wall (pbw), heart and foregut (h+f), neur ectoderm (nt) and branchial arches (ba) were scored for double or single β -galactosidase and PS1 staining. Results are presented as the mean of the percentage of single or double stained versus total of stained cells within three sections per count. Bars, 500 μ m (A-C,I-J) and 100 μ m (D-H,K-N). Abbreviations: a, atrium; am, amnion; as, aortic sac; cc, cardiac crescent; f, foregut; l, forelimb bud; p, pericardium; r5, rhombomere 5.



optic vesicle, dorsal neural tube and ventral limb bud mesenchyme in E9.75 embryos has been reported (Ahn et al., 2001), which is consistent with the β -galactosidase staining found in the BRE-lac1 and BRE-lac2 embryos.

Although expressed essentially at the same sites, BRE-lac2 embryos at E9.5 showed an overall stronger expression of the β -galactosidase reporter (Fig. 6C) compared with BRE-lac1 embryos (Fig. 6B). Strong β -galactosidase staining was observed in the aorta of BRE-lac2 but not in BRE-lac1 embryos. In embryos of both transgenic lines, strong β -galactosidase staining was found in the OT, bulbus cordis, AVC and in the common atrial chamber and ventricle, although not uniformly (Fig. 6G,H). Expression of β -galactosidase was found in endocardium and in the pericardium (Fig. 6G,H). In BRE-lac1 embryos, the reporter expression in the endocardium and pericardium is sparser,

suggesting that the sensitivity to Smad1/5 transcriptional activity in these embryos was less than in BRE-lac2 embryos (results not shown). The results obtained indicate that activation of target genes by Smad1/5 proteins is important for heart development. In agreement, we found expression of the β -galactosidase reporter in the cardiac crescent in E7.5 and overall in the heart in E8.5 embryos (Fig. 6I,J and results not shown).

We next asked whether the β -galactosidase reporter expression coincided with phosphorylated Smad1/5/8 in the nucleus. Immunostaining with an antibody against PSmad1/5/8 (PS1) was performed in E9.5 embryos, following β -galactosidase staining (Fig. 6K-N). The results showed that in the tissues examined, most of the PSmad1/5/8 positive cells also stained for β -galactosidase (approximately 80%) with 8-12% of cells only staining for β -galactosidase and 8-13% stained only

with PS1 (Fig. 6O). Exceptionally, in the neural tube and in migrating neural crest cells an enhanced proportion of cells stained with PS1 only (approximately 66%, see Fig. 6M, black arrowhead and 6O, nt). This probably reflects the kinetic differences in each readout of BMP. Phosphorylation of Smad1/5/8 is rapid and transient while β -galactosidase detection is relatively delayed and sustained, indicated the cells that have activated a transcriptional response earlier but may no longer be receiving an activating signal. Furthermore, the presence of co-repressors in the nucleus might prevent transcription of target genes by activated Smad1/5/8 (Zwijnen et al., 2003).

Discussion

BMPs are known to play roles in many aspects of embryonic patterning and development (Hogan, 1996). Several components of the BMP pathway members have been deleted in mice (reviewed in Chang et al., 2002; Goumans and Mummery, 2000) and this has yielded important information on the functions of the BMP signaling early in development. BMPs are secreted molecules and can therefore reach sites distant from the source (Dosch et al., 1997). In addition, BMPs are sensitive to the presence of specific secreted inhibitors, such as noggin and chordin, that contribute to establish gradients of BMP signaling in the developing embryo (Balemans and Van Hul, 2002). Therefore, the question of where and when active BMP signaling takes place during development is very important to understanding the functional relevance of this signaling pathway. Development of a phospho-specific antibody capable of recognizing the activated forms of Smad1/5/8, but not Smad2 or Smad3 (Persson et al., 1998) has been pivotal towards understanding BMP signaling activation in vitro and in vivo. However, the presence of phosphorylated Smads (1, 5 or 8) in the nucleus is not necessarily equivalent to BMP-induced transcriptional activity. Recent studies have shown that Smad6 can recruit CtBP in the nucleus and repress Smad1-induced transcription (Lin et al., 2003). An alternative approach to study Smad1/5 transcriptional activity is to isolate enhancer elements from the promoters of BMP target genes and use these to drive reporter gene expression in cells in vitro and in embryos in vivo. We have generated transgenic ES cell lines that express a reporter gene (β -galactosidase or luciferase) under the control of a BMP-responsive element (Korchynskiy and ten Dijke, 2002), which we showed is specifically activated by Smad1/4 and Smad5/4 (and to a lesser extent, by Smad8/4) complexes and to respond to BMPs. In BRE-lac1 and BRE-lac2 ES cells in vitro, we have observed that β -galactosidase was not expressed in all the cells in culture upon stimulation with high concentration of BMP4. Upon single cell re-cloning, approximately 20 subclones of the BRE-lac1 and BRE-lac2 ES reporter lines were evaluated for responsiveness to BMP4 (100 ng/ml). No significant increase in the proportion of cells expressing β -galactosidase was observed (results not shown), suggesting that transgene inactivation occurs in vitro. Random inactivation of transgenic β -galactosidase-containing constructs by methylation in P19 embryonal carcinoma (EC) cells has been reported (McBurney et al., 2002). We do not know whether this is involved in the in vitro observations described, but germline transmission in transgenic mice generated from the ES reporter lines (BRE-lac1, BRE-lac2 and BRE-luc) ensures that the whole animal is derived from a single ES cell

genome. In addition, we have shown that β -galactosidase expression was found in the same tissues in transgenic embryos derived from two independent integration events in ES cells (BRE-lac1 and BRE-lac2 ES reporter lines). Moreover, β -galactosidase expression generally coincided with phosphorylated Smad1/5/8 and with known sites of BMP expression in vivo, indicating the transgenic Smad1/5 mice are efficiently reporting in vivo transcriptional activity of Smad1/4 and Smad5/4 complexes, making it unlikely that silencing of the β -galactosidase transgene takes place in vivo.

An autocrine BMP pathway in ES cells

The BMP antagonists chordin and noggin induce differentiation of ES cells into neural lineages (Gratsch and O'Shea, 2002). Accordingly, BMP promotes differentiation of ES cells into non-neural fates (Johansson and Wiles, 1995; Wiles and Johansson, 1999). It was, therefore, intriguing that undifferentiated ES cells expressed BMP4 and BMP7. Interestingly, a recent report has shown that Smad-dependent BMP signaling induces Id1, which together with LIF is sufficient to maintain self-renewal of ES cells in culture (Ying et al., 2003). We have confirmed the existence of this BMP autocrine pathway and the induction of Id1 by studying the response of transgenic Smad1/5 reporter ES cells to BMPs. Endogenous BMP7 can also contribute to the maintenance of the undifferentiated state in these cells.

Smad1/5 ES cell lines give rise to teratomas and respond to endogenous signalling

BMPs play an important role in the differentiation of chondrocytes (Kramer et al., 2000) and further differentiation of hypertrophic chondrocytes into bone (Hogan, 1996; Minina et al., 2001). It was therefore of interest that ES-cell-derived cartilage tissue in teratomas showed β -galactosidase staining. Strikingly, we found that patches of chondrocytes, not the whole cartilage tissue, expressed β -galactosidase in the generated teratomas. This suggested that not all chondrocytes responded equally to BMPs. In agreement, immunohistochemical staining for Smad1 and Smad5 has shown a mix of nuclear and cytoplasmic staining in the chondrocytic population (Flanders et al., 2001), indicating that not all chondrocytes respond to BMP signals simultaneously or that inhibition of BMP signaling takes place in part of the chondrocyte population. It has been shown that osteoblastic differentiation induced by BMP signaling can be inhibited by extracellular-signal-regulated kinases (ERK) by phosphorylation of Smad1 in specific sites in the linker domain (Nakayama et al., 2003).

Endogenous Smad1/5 signaling in transgenic BRE-lac1 and BRE-lac2 mice

In mice, phosphorylated Smad1/5/8 has been shown in the dorsal optic vesicle, dorsal hindbrain and limb bud mesoderm and ectoderm (Ahn et al., 2001). In *Xenopus* as well as in chick, similar studies have shown that phosphorylated Smad1/5/8 protein is found, among other sites, in neural crest, in the dorsal optic vesicle and in the heart, including myocardium and endocardium (Faure et al., 2002; Kurata et al., 2001). β -Galactosidase staining in BRE-lac1 and BRE-lac2 E9.5

embryos showed a high correlation with these findings, supporting the idea that developmental mechanisms are evolutionarily conserved.

β -Galactosidase expression was found to be asymmetrical in the heart of E9.5 Smad1/5 reporter mice. Except for BMP6, which is expressed ubiquitously in the heart, expression of other BMPs, such as BMP4, 5 and 7, is restricted to myocardial cells, while BMP10 is specifically expressed in trabeculae (Chen et al., 2004; Delot, 2003). This suggests that expression of BMP ligands alone is not sufficient to explain regulation of the transcriptional activity of Smad1/5 in the heart and underlines the importance of the Smad1/5 reporter mice for the study of active Smad1/5 induced transcription in development.

Conditional deletion of ALK3 in cardiomyocytes has highlighted the potential of paracrine BMP signaling in heart development (Gaussin et al., 2002). The fact that we observe reporter activity in myocardium, but also in endocardium and pericardium supports the idea that paracrine BMP signaling takes place in the heart. In addition, the staining found both in the OT and in the AVC is consistent with a pivotal role of BMPs in cardiac septation (Delot et al., 2003; Jiao et al., 2003).

In summary, we have generated reporter mice for monitoring active transcription of Smad1/Smad4 and/or Smad5/Smad4-dependent target genes in vivo. This reporter system allows, for the first time, an in vivo readout of the spatio-temporal activation of Smad1/Smad5 target genes in mice. The usefulness of these mice has been recently demonstrated in a study on the role of ALK2-mediated BMP signaling in the allocation of PGCs (Chuva de Sousa Lopes et al., 2004). Further studies describing the pattern of Smad1/5-mediated transcriptional activity at different stages of mouse development will allow us to elucidate their role in other developmental processes in the mouse.

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