Smad4 is required to induce digit ray primordia and to initiate the aggregation and differentiation of chondrogenic progenitors in mouse limb buds

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SUMMARY
SMAD4 is an essential mediator of canonical TGFβ/BMP signal transduction and we inactivated Smad4 in mouse limb buds from early stages onward to study its functions in the mesenchyme. While this Smad4 inactivation did not alter the early Sox9 distribution, prefiguring the chondrogenic primordia of the stylopod and zeugopod, it disrupted formation of all Sox9-positive digit ray primordia. Specific inactivation of Smad4 during handplate development pointed to its differential requirement for posterior and anterior digit ray primordia. At the cellular level, Smad4 deficiency blocked the aggregation of Sox9-positive progenitors, thereby preventing chondrogenic differentiation as revealed by absence of collagen type II. The progressive loss of Sox9 due to disrupting digit ray primordia and chondrogenesis was paralleled by alterations in genes marking other lineages. This pointed to a general loss of tissue organization and diversion of mutant cells toward non-specific connective tissue. Conditional inactivation of Bmp2 and Bmp4 indicated that the loss of digit ray primordia and increase in connective tissue were predominantly a consequence of disrupting SMAD4-mediated BMP signal transduction. In summary, our analysis reveals that SMAD4 is required to initiate: (1) formation of the Sox9-positive digit ray primordia; and (2) aggregation and chondrogenic differentiation of all limb skeletal elements.

KEY WORDS: BMP signaling, Limb bud, Mouse

INTRODUCTION
Bone morphogenetic proteins (BMPs) are ligands belonging to the transforming growth factor beta (TGFβ) superfamily, which control inductive processes in early embryos and during organogenesis (reviewed by Zakin and De Robertis, 2010) and are required for chondrogenesis, growth and ossification of bones in vertebrates (reviewed by Wu et al., 2007). BMP ligands interact with two types of BMP receptors (BMPR1 and BMPR2), which in turn trigger intracellular signal transduction by phosphorylation of the receptor-associated SMAD1, -5 and -8 proteins. These in turn form a complex with SMAD4, an essential mediator of both canonical BMP and TGFβ signal transduction (Yang et al., 2002). These SMAD complexes translocate to the nucleus and regulate the expression of target genes in concert with other transcriptional regulators (Feng and Derynck, 2005). Genetic and functional analysis of mouse and chicken limb bud development has uncovered important morphoregulatory functions of the BMP pathway in the establishment and functioning of the two limb bud signaling centers, namely sonic hedgehog (SHH) signaling by the posterior mesenchymal organizer and fibroblast growth factor (FGF) signaling by the apical ectodermal ridge (AER).

During limb bud outgrowth, BMPs and the BMP antagonist gremlin 1 (GREM1) control the epitheial-mesenchymal signaling interactions that coordinate outgrowth with patterning and determination of digit identities (reviewed by Zeller et al., 2009). During the onset of limb bud development, BMP4 is first required in the mesenchyme and BMPR1A in the ectoderm for formation of the AER, as inactivation of these molecules disrupts establishment of the AER-FGF signaling centre, which results in limb truncations and loss of dorsoventral polarity (Ahn et al., 2001; Pizette et al., 2001; Pajni-Underwood et al., 2007; Bénazet et al., 2009). During initiation of limb bud outgrowth, the SHH signaling centre is established and restricted to the posterior mesenchyme under the influence of BMP signaling (Bastida et al., 2004). Concurrently, BMP4 triggers expression of the BMP antagonist Grem1 in the posterior limb bud mesenchyme, which causes rapid lowering of mesenchymal BMP activity (Nissim et al., 2006; Bénazet et al., 2009). The subsequent distal-anterior expansion of the Grem1 expression domain depends largely on its positive regulation by AER-FGF and SHH signaling. This self-regulatory SHH/GREM1/AER-FGF feedback loop coordinates anteroposterior (AP) with proximodistal (PD) limb bud outgrowth and patterning (Züñiga et al., 1999; Michos et al., 2004; Panman et al., 2006; Bénazet et al., 2009; Probst et al., 2011).

During limb bud outgrowth, low mesenchymal BMP activity is required to restrict the length of the AER, as inactivation of Bmp4 during this developmental period results in extended AER-FGF signaling and digit polydactyly (Selever et al., 2004; Bénazet et al., 2009). As inactivation of Bmp1a in the mesenchyme also disrupts limb bud outgrowth (Ovchinnikov et al., 2006), tight control of BMP activity is required for normal progression of limb development (reviewed by Zeller et al., 2009).

These signaling interactions are terminated as the SHH/GREM1/AER-FGF feedback signaling system breaks down owing to progressive inhibition of Grem1 expression by high AER-FGF levels and the increasing separation of the Shh and Grem1

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expression domains (Scherz et al., 2004; Verheyden and Sun, 2008). This termination results in a renewed increase in BMP activity during development of the digit ray primordia (giving rise to metacarpals and phalanges) (Bénazet et al., 2009), which probably serves two purposes: first, to control digit identity by signaling from the interdigital mesenchyme to the distal phalanx-forming region (PFR) (Dahn and Fallon, 2000; Suzuki et al., 2008; Witte et al., 2010), and second, to induce apoptosis of the interdigit mesenchyme (Bandyopadhyay et al., 2006; Pajni-Underwood et al., 2007; Maatouk et al., 2009; Wong et al., 2012). In agreement with these dynamic changes in BMP activity, BMPs inhibit chondrogenic differentiation of early limb bud mesenchymal progenitors while promoting chondrogenesis at late stages (Karamboulas et al., 2010). Noggin-mediated BMP antagonism under experimental conditions showed that BMPs are required to initiate compaction and chondrogenic differentiation of Sox9-positive mesenchymal progenitors (Brunet et al., 1998; Pizzette and Niswander, 2000; Barna and Niswander, 2007). Genetic inactivation of both Bmp2 and Bmp4 in the limb bud mesenchyme disrupted formation of the two posterior-most digit primordia and the ulna (Bandyopadhyay et al., 2006). Experimental evidence indicated that BMPs participate in activating Sox9 expression in prechondrogenic progenitors, and Sox9 not only marks the chondrogenic lineage but is essential to initiate chondrogenesis during formation of skeletal elements (Bi et al., 1999; Akiyama et al., 2005; Pan et al., 2008). Genetic analysis of the BMP pathway during chondrogenesis established that BMP signaling and antagonism are required recurrently for the proliferation of prechondrogenic progenitors, chondrocyte differentiation, shaping the skeletal primordia and formation of the endochondral growth plate (Brunet et al., 1998; Kobayashi et al., 2005; Yoon et al., 2005). In parallel, mesenchymal cells receiving different signals are specified as tendons, ligaments and connective tissues. For example, chondrogenic differentiation is inhibited and mesenchymal cells remain in a proliferative, undifferentiated state when they are exposed to both ectodermal WNT and AER-FGF signaling, whereas WNT signaling alone promotes the development of non-specific connective tissue (ten Berge et al., 2008; Gros et al., 2010). TGFβ ligands are expressed from early limb bud stages onward, and inhibition of TGFβ signal transduction in cultured mesenchymal cells provided evidence that TGFβ signaling functions in early limb buds to alleviate the inhibitory effects of BMPs on initiation of chondrogenic differentiation (Karamboulas et al., 2010). Therefore, brief exposure of mesenchymal progenitors to TGFβ signaling seems necessary to allow subsequent induction of chondrogenesis by BMPs (Roark and Greer, 1994; Karamboulas et al., 2010). Furthermore, implantation of TGFβ1-loaded beads into the interdigital mesenchyme of chicken limb buds is able to induce formation of ectopic digit phalanges, i.e. trigger ectopic chondrogenesis (Merino et al., 1999; Lorda-Diez et al., 2011). Genetic analysis of TGFβ ligands and receptors in mouse embryos has revealed their essential roles during limb long bone, joint and tendon morphogenesis (Sanford et al., 1997; Seo and Serra, 2007). Although this genetic analysis revealed essential functions during limb skeletal development, it is possible that earlier functions in initiating chondrogenesis were masked by functional complementation among TGFβ and/or BMP ligands and receptors (see e.g. Karamboulas et al., 2010).

As Smad4-deficient (Smad4Δ/Δ) embryos die during gastrulation (Chu et al., 2004), we used a conditional loss-of-function allele (Yang et al., 2002) to study its requirement in the limb bud mesenchyme. Prxl-Cre-mediated conditional inactivation of Smad4 in limb bud mesenchyme resulted in the clearance of SMAD4 protein during early limb bud outgrowth, and the distribution of Sox9 transcript and SOX9 protein was normal until at least embryonic day (E) 11.0 in the proximal mesenchyme. By contrast, Sox9 expression was not activated in the distal Smad4-deficient limb bud mesenchyme that would normally give rise to the digit ray primordia. The analysis of SOX9 also indicated that with the exception of a small proximal aggregate, the aggregation and condensation of all mesenchymal progenitors to form the cartilage elements was disrupted in Smad4-deficient limb buds. Mutant cells failed to deposit extracellular collagen type II (COL type II) fibres, a hallmark of chondrogenesis, and progressively lost SOX9, i.e. chondrogenic lineage. In parallel, the expression of markers for other mesenchymal lineages was upregulated in Smad4-deficient limb buds without their normal spatial restriction, which provided evidence for a diversion of Smad4-deficient progenitors toward non-specific connective tissue.

MATERIALS AND METHODS

Ethics statement concerning animal experiments and mouse strains

Animal experiments were approved by the legally required regional commission in strict accordance with Swiss law. All studies were classified as grade zero, which implies minimal suffering. The 3R and Basel Declaration principles were implemented. The Prxl-Cre was used to conditionally inactivate Smad4 (Smad4lox/lox) (Yang et al., 2002) in the mesenchyme from early limb bud stages onward (Logan et al., 2002), and the Hoxa13CreERT knock-in allele was used for autopod specific inactivation (Lopez-Rios et al., 2012; Scotti and Kmita, 2012). Prxl-Cre-mediated inactivation of Bmp2 and Bmp4 was done as described (Bandyopadhyay et al., 2006).

In situ hybridization and skeletal preparations

Whole-mount in situ hybridization was performed using standard protocols. Control and experimental embryos were age-matched by counting somites. To assess the clearance of Sox9 transcript in Smad4-deficient limb buds, a probe against the deleted region, coding exon 8 (Yang et al., 2002) was used for hybridization. Depending on the analysis, Smad4lox/lox, Smad4lox/lox, Smad4lox/lox or Smad4lox/lox or Smad4lox/lox or Smad4lox/lox or Hoxa13CreERT heterozygous embryos were used as controls and collectively referred to as ‘wild-type controls’. Alcian Blue and Alizarin Red were used to reveal cartilage and bone.

Optical projection tomography

Optical projection tomography (OPT) imaging (Sharpe et al., 2002) was used to acquire 3D images and prepare optical sections. RNA hybrids were detected using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBTH/BCIP, Roche). Whole-mount immunofluorescence analysis was done as follows: embryos were permeabilized in cold acetone (15-30 minutes) and then incubated in blocking solution (PBT with 1% bovine serum albumin, 1% dimethyl sulfoxide and 5% inactivated bovine serum) for 20 minutes. Smad4 was detected using rabbit monoclonal anti-Smad4 antibodies, 1:50 in blocking solution (Abcam; 12 hour incubation at 4°C). Samples were washed extensively and incubated with secondary antibodies Alexa Fluor 594-conjugated anti-rabbit antibody, 1:250 (Invitrogen; overnight at 4°C). Subsequently, samples were embedded in 1% low melting point agarose (Sigma), dehydrated in 100% methanol and cleared in BABB. Samples were scanned either at high (1024×1024 pixels) or intermediate resolution (512×512 pixels) in the Biotronics OPT scanner using SkyScan software (Biotronics, MRC Technology). The GPF1 filter (425/40 nm, 475 nm LP) was used to detect sample anatomy. No filter (bright field) or the TXR filter (560/40 nm, 610 nm LP) was used to image NBT/BCIP signals or fluorescently labeled samples, respectively. OPT scans were reconstructed using NRecon software (SkyScan) and analysed using the Biotronics Viewer. Rendering images were taken using the maximum intensity projection function. For generation of iso-surfaces, the iso-surface editor of the Biotronics viewer program was used at 25%
iso-surface quality and 50% Gaussian smoothing parameters. Iso-surface values were determined and compared with the strength and distribution of the original signal to exclude aberrant iso-surfaces.

Quantification of transcript levels by quantitative real-time PCR
Forelimbs were isolated at E10.5 (35-39 somites) and E11.75 (51-53 somites) and analysed (Bénazet et al., 2009) using the primers listed in supplementary material Table S1. At least eight samples per stage and genotype were analysed. All results shown are mean±SD; each dot represents one sample, and the significance of all differences was verified using the two-tailed non-parametric Mann-Whitney U-test.

Immunofluorescence analysis
Embryos or forelimb buds were isolated and fixed in 4% PFA overnight at 4°C. Samples were dehydrated stepwise into 100% ethanol, then transferred to xylene. Subsequently, samples were embedded in Paraplast wax (Sigma) and sectioned following standard procedures. For most antibodies, antigen retrieval was achieved by heating the dewaxed sections at 121°C in 1 atm for 6 minutes in a desktop autoclave. The COL type II antigen was retrieved by digesting sections with pepsin (1 mg/ml in 10 mM HCl, pH 2.0) for 15 minutes (37°C). Sections were blocked using PBS containing 0.3% Triton X-100 and 1% BSA (blocking solution; 1 hour at room temperature) before permeabilization and antigen retrieval. The COL type II antigen was detected using the following secondary antibodies (1 hour at room temperature): goat anti-rabbit Alexa Fluor 594 1:500 (Invitrogen), goat anti-mouse Alexa Fluor 488 1:200 (Invitrogen). Apoptotic cells were detected by whole-mount Lysotracker analysis (Invitrogen) as previously described (Benazet et al., 2009).

RESULTS
Requirement of mesenchymal Smad4 for propagation of the SHH/GREM1/AER-FGF feedback loop during mouse limb bud development

Prx1-Cre-mediated conditional inactivation of Smad4 in the mesenchyme (Smad4ΔΔm) resulted in fore- and hindlimbs lacking skeletal elements (Fig. 1A). Stunted paddles replaced the autopod with digits at embryonic day E14.5 (Fig. 1B, arrowheads). As this initial analysis pointed to a late onset phenotype, the clearance of Smad4 gene products was assessed (Fig. 1C,D; supplementary material Fig. S5). Inhibition of p38, which is involved in non-canonical BMP signal transduction, had no effect on cell aggregation and the onset of chondrogenic differentiation (data not shown).

Limb mesenchymal cell culture and immunofluorescence
Forelimbs were dissected in ice-cold PBS, and 2% trypsin (minimize at 4°C) in combination with gently pipetting resulted in single cell preparations of mesenchymal cells that were plated in DMEM/F12 medium (supplemented with 0.5% penicillin/streptomycin and 10% FBS, Gibco-BRL) at 7.5×10³ cells/300 µl in eight-well chamber slides (Ibidi). After 48 hours’ culturing, samples were washed in PBS and fixed in 4% PFA (30 minutes at room temperature) before permeabilization and antigen detection as described above. BMP signaling was specifically inhibited by adding 10 µM dorsomorphin (Yu et al., 2008) and TGFβ signaling was inhibited by adding 10 µM SB431542 (Inman et al., 2002) in 0.1% DMSO (final concentration in medium). Control cells were cultured in medium containing 0.1% DMSO. Media were refreshed every 24 hours. The results shown are representative of eight independent experiments (supplementary material Fig. S5). Inhibition of p38, which is involved in non-canonical BMP signal transduction, had no effect on cell aggregation and the onset of chondrogenic differentiation (data not shown).

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In agreement with these inactivation kinetics, the expression of the BMP target Msx2 was reduced from ~E9.75 onward (Fig. 1E; supplementary material Fig. S1B). By contrast, the three main Bmp ligands were either unchanged or slightly increased in mutant limb buds (supplementary material Fig. S2).

Previous analysis provided evidence that activation and initial upregulation of Grem1 expression in the limb bud mesenchyme depended on BMP4 signaling (Nissim et al., 2006; Bénazet et al., 2009). In Smad4<sup>ΔMA</sup> forelimb buds, Grem1 expression was reduced, but its posterior domain was maintained at E10.25 (Fig. 2C and data not shown), whereas the AER-Fgf8 expression domain was broadened and levels increased from ~E10.75 onward (supplementary material Fig. S2). However, apoptosis of core mesenchymal cells was increased up to ~E11.5, whereas apoptosis in mutant handplates was suppressed because of the absence of the interdigital mesenchyme (supplementary material Fig. S3B; Fig. 3).

The spatiotemporal expression of the 5'-most HoxD and HoxA genes, which regulate limb bud patterning and outgrowth (reviewed by Zakany and Duboule, 2007) was not significantly altered up to E11.5 (data not shown). Fig. 3 shows the expression of select markers for the autopod, digit and interdigit territories. Hoxa13 correctly delineated the presumptive autopod territory in mutant limb buds, but none of the putative digit primordia were outlined (Fig. 3A). Cyp26b1 is normally expressed in the distal part of all digit primordia including the PFRs (Fig. 3B) (Yashiro et al., 2004). Smad4<sup>ΔMA</sup> forelimb buds lacked these PFR-like expression domains and Cyp26b1 was largely confined to the anterior and posterior margins (Fig. 3B). Dlx5 is normally expressed by the AER and interdigital mesenchyme in wild-type limb buds (Robledo et al., 2002), but its expression was confined largely to the AER in Smad4<sup>ΔMA</sup> forelimb buds (Fig. 3C). These results indicated that
developmental structures. (interdigit mesenchyme was disrupted in Fig. 3. Mesenchymal Smad4 in wild type, whereas no differential expression is apparent in its expression was assessed (Fig. 4). Despite the early rise to the skeletal primordia (Bi et al., 1999; Akiyama et al., 2005), mesenchymal condensations that initiate chondrogenesis and give stages onward. As Sox9-positive cells (Fig. 4B, brackets), whereas the proximal domain was enlarged in Smad4ΔΔM forelimb buds (Fig. 4B, arrowheads; better seen in supplementary material Movie 1). The requirement of Smad4 for formation of the Sox9-positive digit rays could be transient, as evidenced by the kinetics of Hoxa13Cre+/ mice aged 13 or both Smad4 alleles (Fig. 4C, Smad4ΔΔA13/ΔA13, supplementary material Fig. S4). Inactivation of Smad4 using the Hoxa13Cre+ knock-in allele (Scotti and Kmita, 2012) resulted in the progressive clearance of Smad4 transcripts during autopod development and specific agenesis of the autopod skeleton (supplementary material Fig. S4B,C). The functional loss of Smad4 should be faster in Smad4ΔΔA13 than Smad4ΔΔA13ΔA13 forelimb buds, as two conditional alleles have to be inactivated and twice as much SMAD4 proteins cleared in the latter (Bénazet et al., 2009). As only one Hoxa13 allele is functional in mouse embryos carrying the Hoxa13Cre+ knock-in, we first established that formation of digit ray primordia was not altered in Hoxa13Cre+/ and Hoxa13Cre+/Smad4ΔΔA13 forelimb buds (Fig. 4C). However, only the more proximal digit ray primordia were apparent in Smad4ΔΔA13 forelimb buds, whereas the primordia of digit rays 2-5 formed in all Smad4ΔΔA13ΔA13 forelimb buds (Fig. 4D). These results indicated that morphogenesis of anterior digit ray primordia could require Smad4 for longer or at a later stage than posterior primordia.

**SOX9-positive mesenchymal progenitors fail to aggregate and initiate chondrogenic differentiation of all cartilage elements in Smad4ΔΔM limb buds**

As whole-mount RNA in situ hybridization analysis did not provide cellular resolution, the SOX9 protein distribution was analysed on serial sections of forelimb buds (Fig. 5, red fluorescence). At E10.5, scattered SOX9-positive mesenchymal cells were detected mainly in the core of both wild-type and Smad4ΔΔM forelimb buds (Fig. 5A,B). By E11.75, SOX9-positive progenitors condensed to initiate formation of the cartilage elements of the humerus and the ulna/radius and the posterior digit 4 in wild-type limb buds (Fig. 5C) (Zhu et al., 2008). In Smad4ΔΔM forelimb buds, a small proximal condensation was apparent (Fig. 5D, arrowhead), but most SOX9-positive cells remained loose and in a pattern reminiscent of the Sox9-positive distal arch (compare Fig. 5D and Fig. 4A). During subsequent development, no organized mesenchymal condensations and cartilage elements formed in Smad4ΔΔM forelimb buds (Fig. 5E-H). Rather, the small proximal condensation disappeared and SOX9-positive cells dispersed throughout the distal mesenchyme (Fig. 5F,H). Taken together, this analysis indicated that aggregation of the SOX9-positive progenitors to initiate chondrogenic differentiation and separation of the mutant mesenchyme into digit ray primordia and interdigit mesenchyme was disrupted in Smad4ΔΔM autops.

**Smad4 is required to induce formation of the Sox9-positive digit ray primordia**

Sox9 marks prechondrogenic progenitors from early limb bud stages onward. As Sox9 presages the formation of all mesenchymal condensations that initiate chondrogenesis and give rise to the skeletal primordia (Bi et al., 1999; Akiyama et al., 2005), its expression was assessed (Fig. 4). Despite the early mesenchymal loss of Smad4 and disruption of BMP signal transduction (Fig. 1B-D), the Sox9 transcript distribution remained comparable to wild-type controls until at least E11.0 (Fig. 4A, left panels). These results indicated that Smad4 is not required for activation and patterning of Sox9 during the developmental period characterized by low BMP activity (Bénazet et al., 2009). The Sox9-positive progenitors in the proximal mesenchyme normally give rise to the cartilage elements of the scapula, stylopod (humerus) and zeugopod (radius and ulna). In wild-type forelimb buds, the Sox9-positive progenitors giving rise to the carpals and digit rays (metacarpals and phalanges) were apparent by ~E11.5, and the Sox9-positive digit ray primordia of all except digit 1 had formed by ~E12.25 (Fig. 4A, upper right panels) (Zhu et al., 2008). In Smad4ΔΔM limb buds, the distalmost zone of Sox9-negative mesenchyme expanded (Fig. 4A, brackets) and Sox9 expression was progressively lowered (Fig. 4A, right-most lower panel). This downregulation was paralleled by a complete loss of the Sox9-positive primordia corresponding to carpals and digit rays (Fig. 4A,B). By E12.25, the characteristic Sox9-positive domains prefiguring all of the chondrogenic limb skeletal elements were largely lost from Smad4ΔΔM limb buds (Fig. 4A, right-most panels). By E13.5, only very low Sox9 expression remained in Smad4ΔΔM limb buds, whereas Sox9 was abundant in the PFRs and phalanges of all wild-type digits (supplementary material Fig. S4A). To gain further insight, OPT (Sharpe et al., 2002) was used in combination with iso-surface rendering for comparative analysis of the Sox9 transcript distribution in wild-type and Smad4ΔΔM forelimb buds. This analysis revealed thinning of the distal arch of Sox9-positive cells (Fig. 4B, brackets), whereas the proximal domain was enlarged in Smad4ΔΔM forelimb buds (Fig. 4B, arrowheads; better seen in supplementary material Movie 1).

**Fig. 3. Mesenchymal Smad4 inactivation disrupts the spatial distribution of genes that mark specific autopod territories.**

(A) Hoxa13 expression at E12.5 (60 somites). In wild-type forelimb buds, the distal parts of the developing digits and perichondria express the highest levels of Hoxa13 transcript. In Smad4ΔΔM forelimb buds, Hoxa13 expression is distally restricted but not localized to specific structures. (B) Cyp26b1 marks the PFRs of all developing digit primordia in wild type, whereas no differential expression is apparent in Smad4ΔΔM forelimb buds. (C) Dlx5 is expressed by the AER, sub- ectodermal mesenchyme, and the interdigit mesenchyme in wild type, which is lacking in Smad4ΔΔM forelimb buds. The wild-type digit ray primordia are labelled 1 (thumb) to 5 (little finger).
formation of cartilage elements was disrupted in Smad4-deficient forelimb buds.

To gain further insight into the underlying cellular defects, wild-type and mutant mesenchymal cells were dissociated from limb buds at E11.5 and high-density cultures were used to assess their chondrogenic differential potential (Barna and Niswander, 2007). A hallmark of chondrogenic differentiation is the transcriptional activation of Col2a1, a direct transcriptional target of SOX9 that encodes the pro-alpha1(II) chain of COL type II, which is cartilage-specific (Bell et al., 1997; Lefebvre et al., 1997). In wild-type mesenchymal cultures, cells aggregated and compacted to form the typical cell-dense condensations often paralleled by reduced SOX9 expression, and the onset of chondrogenesis was revealed by abundant COL type II (Fig. 6A). By contrast, no COL type II was detected in areas of less densely packed cells (Fig. 6A, enlargements), which revealed that chondrogenesis was initiated in regions with high mesenchymal cell density, i.e. after cells had aggregated. By contrast, slightly lower levels of SOX9 were consistently detected in mesenchymal cells isolated from Smad4/H9004/H9004M forelimb buds, which failed to aggregate and produce COL type II fibres (Fig. 6B). This failure of Smad4-deficient mesenchymal cells to aggregate appeared more severe than the effects of Noggin-mediated inhibition of BMP activity in limb bud mesenchymal cells, which aggregate but fail to undergo subsequent compaction and initiation of chondrogenesis (Barna and Niswander, 2007). To gain further insight, we selectively inhibited BMP type I receptors and thereby BMP signal transduction using Dorosomorphin (Yu et al., 2008), which disrupted condensation and COL type II expression of wild-type cells similar to Smad4/H9004/H9004M cells (compare supplementary material Fig. S5B and Fig. 6B). Inhibiting TGFβ receptors with the selective antagonist SB431542 (Inman et al., 2002) blocked cell aggregation and COL type II deposition to a similar extent (supplementary material Fig. S5B) (Karamboulas et al., 2010). These results indicated that BMP and TGFβ signal transduction were both required for initiating chondrogenesis of mesenchymal progenitors in culture.

As these experiments revealed striking effects on deposition of extracellular COL type II fibres, the extent to which Col2a1 expression might be altered in Smad4/H9004/H9004M forelimb buds was determined (Fig. 7A). At E11.5, the Col2a1 transcript distributions in wild-type and mutant forelimb buds were similar and comparable to Sox9 at this stage (compare Fig. 7A, left panels and Fig. 4A). As autopod development progressed, Col2a1 was expressed abundantly by all skeletal elements including digit rays in wild-type limb buds (Fig. 7A, upper panels). By contrast, Col2a1 expression remained diffuse and was progressively lost...
from Smad4ΔΔM forelimb buds (Fig. 7A, lower panels). Indeed, high levels of COL type II protein were detected in all chondrogenic primordia of the skeletal elements in wild-type limb buds, whereas only few scattered positive clusters were detected in Smad4ΔΔM limb buds (Fig. 7B). Taken together, this analysis (Figs 5-7) revealed the disruption of chondrogenesis and absence of all cartilage elements prefiguring the limb skeleton in Smad4ΔΔM forelimb buds.

To determine the extent to which Smad4-deficient mesenchymal cells might acquire non-chondrogenic fates, genes marking other mesenchymal lineages were analysed. Collagen type I (COL type I) is expressed by diverse connective tissues such as skin, tendons, ligaments and bones, and muscle-associated connective tissues (see e.g. ten Berge et al., 2008). In wild-type limb buds, Col1a2 was detected first in the proximal mesenchyme (Fig. 7C, E11.5). At later stages, Col1a2 outlined the forming perichondria and tendons (Fig. 7C, arrowheads). In Smad4ΔΔM forelimb buds, Col1a2 transcript and protein were detected throughout the mesenchyme (Fig. 7C,D, lower panels). COL type I localized to the perichondrium and developing dermis in wild type (Fig. 7D, upper panels), whereas its expression appeared more widespread and disorganized in Smad4ΔΔM forelimb buds (Fig. 7D, lower panels). As decorin (Dcn) regulates the assembly of collagen fibres (Danielson et al., 1997), its expression was analysed. In wild-type limb buds, the initially rather diffuse Dcn expression became excluded from the chondrogenic cores of all developing cartilage elements including digit rays (Fig. 8A, upper panels and data not shown). By contrast, Dcn expression was enhanced and persisted throughout the mesenchyme of Smad4ΔΔM forelimb buds (Fig. 8A, lower panels; supplementary material Fig. S6A). Possible effects on Dcn expression were also assessed in forelimb buds lacking both mesenchymal Bmp2 and Bmp4, as the two posteriormost digit ray primordia are not formed (Fig. 8B) (Bandyopadhyay et al., 2006). Analogous to the Smad4 deficiency (Fig. 8A), the absence of these two digit ray primordia results in uniformly increased expression of Dcn in the posterior mesenchyme of limb buds lacking both Bmps (Fig. 8B, posterior region indicated by a bracket). The striking similarity of these alterations indicates that formation of the posterior Sox9-positive digit ray primordia and restriction of Dcn expression depends on BMP signal transduction that is mediated by SMAD4.

In addition, the expression of the transcriptional regulator scleraxis (Scx, marking tendon and ligament progenitors) (Schweitzer et al., 2001) was also increased in Smad4ΔΔM forelimb buds (supplementary material Fig. S6B). While Scx marked both dorsal and ventral tendons in wild-type limb buds (Fig. 8C, upper panels), its expression was upregulated and diffuse in Smad4ΔΔM forelimb buds (Fig. 8C, lower panels). This was paralleled by widespread and diffuse expression of Fjx1, which normally marks the attachment points of the forming tendons and the developing joints (Rock et al., 2005), in Smad4-deficient forelimb buds (supplementary material Fig. S6C). In addition to tendons and ligaments, limb skeletal muscles form in concert with the limb skeletal elements. Analysis of the MyoD distribution (marking all limb muscle primordia) (Francis-West et al., 2003) showed that dysplastic skeletal muscles were present in the flank and proximal part of Smad4-deficient forelimb buds, whereas the distal mesenchyme was devoid of MyoD-expressing cells (supplementary material Fig. S6D).
Our genetic analysis of mesenchymal Smad4 during mouse limb bud development uncovers its dual requirement for initiating: (1) formation of the Sox9-positive digit ray primordia; and (2) aggregation and chondrogenic differentiation of all Sox9-positive prechondrogenic progenitors that give rise to the cartilage elements of the future limb skeleton (Fig. 8D). SMAD4 is essential to induce chondrogenesis because cell aggregation, which is the first step toward chondrogenic differentiation, is disrupted and mutant progenitors remain scattered in Smad4<sup>ΔΔM</sup> forelimb buds (Fig. 8D) (see also Pizette and Niswander, 2000; Barna and Niswander, 2007). By contrast, the expression of Sox9, which prefigures the primordia of the stylopod and zeugopod, is normal up to at least E11.0 in Smad4<sup>ΔΔM</sup> forelimb buds. A role of SMAD4 in activation of Sox9 expression in early limb buds cannot be formally excluded, as the protein is only cleared around the time Sox9 is activated in Smad4<sup>ΔΔM</sup> forelimb buds. Nevertheless, the Smad4-independent phase of Sox9 expression coincides well with the period of low BMP activity in mouse limb buds (Bénazet et al., 2009), which corroborates the proposal that the spatiotemporal regulation of Sox9 expression does not require SMAD4-mediated signal transduction before handplate formation (this study). Indeed, it has been proposed that BMPs inhibit Sox9 expression and initiation of chondrogenic differentiation in the mesenchyme of early limb buds; an effect that can be alleviated by short-term exposure to TGFβ signaling in culture (Karamboulas et al., 2010). However, inhibition of BMP or TGFβ signal transduction in vitro disrupted chondrogenesis in a similar way to the genetic Smad4 deficiency, but did not cause complete loss of SOX9 (this study).

In addition to Sox9, the mesenchymal expression of the early chondrocyte marker Col2a1 is activated normally in Smad4<sup>ΔΔM</sup> forelimb buds. During distal progression of limb bud development, Col2a1 expression is lost concurrent with the failure to refine SOX9 protein distribution, as expected from direct transcriptional regulation of Col2a1 by SOX9 (Bell et al., 1997; Lefebvre et al., 1997). In Smad4<sup>ΔΔM</sup> forelimb buds, the progressive loss of Sox9 and Col2a1 was paralleled by widespread and diffuse expression of markers for other lineages and a general absence of tissue organization and differentiation. Therefore, SMAD4 appears to function in signaling networks that restrict non-chondrogenic progenitors and connective tissue lineages. In agreement with the diffuse Scx expression in Smad4<sup>ΔΔM</sup> forelimb buds, inhibition of BMP signaling results in ectopic Scx expression without the formation of additional tendons (Schweitzer et al., 2001). However,
as both Colla1 and Colla2 are positively regulated by SCX (Cserjesi et al., 1995), the ectopic Scx expression in Smad4<sup>ΔΔM</sup> forelimb buds could contribute to the increased COL type I expression (this study). These molecular changes and the complete lack of tissue organization in Smad4<sup>ΔΔM</sup>-deficient limb buds (Fig. 8D) indicate that Smad4 functions in the morphoregulatory networks that pattern diverse limb bud mesenchymal lineages and/or orchestrate coordinated tissue differentiation. Taken together, these studies uncover the recurrent differential requirement of SMAD4-mediated canonical BMP/TGFβ signaling during specification and aggregation of SOX9-positive prechondrogenic progenitors and initiation of their chondrogenic differentiation (Fig. 8D).

Although formation of the Sox9-positive primordia of the stylopod and zeugopod appears Smad4-independent, the induction of the Sox9-positive digit ray primordia depends critically on Smad4 and increased BMP activity (Bandyopadhyay et al., 2006; Bénazet et al., 2009; Lopez-Rios et al., 2012; this study). As the proximal Sox9-positive domains are enlarged, the mutant prechondrogenic cells may contribute preferentially to proximal primordia as evidenced by thinning of the distal arch in Smad4<sup>ΔΔM</sup> forelimb buds (this study). Such a differential requirement of Smad4 might be of evolutionary relevance as the autopod and digits of tetrapod limbs are so-called neomorphic structures, whose development may have relied on co-opting novel gene-regulatory networks (reviewed by Woltering and Duboule, 2010). Our analysis indicates that limb bud development and formation of Sox9-positive digit ray primordia become Smad4-dependent as BMP activity rises owing to self-termination of the SHH/Grem1/AER-FGF signaling system (Scherz et al., 2004; Verheyden and Sun, 2008; Benazet et al., 2009). During this developmental period, differential BMP signaling appears to determine digit identities in both chicken and mouse (Dahn and Fallon, 2000; Suzuki et al., 2008; Witte et al., 2010). As extensive genetic analysis of TGFβ signaling in mouse embryos has not produced similar phenotypes (see e.g. Sanford et al., 1997; Seo and Serra, 2007), SMAD4-mediated BMP signal transduction is likely to be predominant during these advanced stages of digit ray primordia growth and patterning. It has been proposed that the progenitors giving rise to the digit ray primordia are recruited from the distalmost limb bud mesenchyme (Suzuki et al., 2008), which is defective in Smad4<sup>ΔΔM</sup> forelimb buds as revealed by the increasing gap of Sox9-negative cells in the distal mesenchyme (this study). In addition, temporally controlled Smad4 inactivation in the autopod provided evidence for its possible differential requirement for posterior and anterior digit ray primordia. A region free of the BMP antagonist GREM1 is generated within the posterior limb bud mesenchyme by the population of Shh descendants, which is refractory to Greml expression and increases as limb bud development progresses (Scherz et al., 2004). These results together with the differential requirement observed in this study indicate that BMP activity might first rise in the posterior mesenchyme, where Bmp2 and Bmp4 are co-expressed and required for digit ray formation (Bandyopadhyay et al., 2006). The


