Regulation of BMP-dependent chondrogenesis in early limb mesenchyme by TGFβ signals

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Summary

In the developing axial skeleton, sequential sonic hedgehog (SHH) and bone morphogenetic protein (BMP) signals are required for specification of a chondrogenic fate in presomitic tissue. A similar paradigm is thought to operate in the limb, but the signals involved are unclear. To investigate the nature of these signals, we examined BMP action in mesenchymal populations derived from the early murine limb bud (approximately embryonic day 10.5). These populations exhibited a graded response to BMPs, in which early limb mesenchymal cells (from the distal hind limb) displayed an anti-chondrogenic response, whereas BMPs promoted chondrogenesis in more mature cell populations (from the proximal fore limb). Under these conditions, multiple Gata genes were induced by BMPs and the extent of induction correlated with BMP anti-chondrogenic activity. A screen of limb-bud-expressed ligands revealed that prior short-term exposure to transforming growth factor β1 (TGFβ1) ameliorated the anti-chondrogenic response to BMP. Furthermore, brief activation of the TGFβ pathway was found to be necessary for subsequent induction of chondrogenesis by BMPs. Our findings indicate that, similar to axial skeletogenesis, induction of chondrogenesis in the appendicular skeleton is a two-step process. However, the programs differ in the transient signals driving chondrogenic responsiveness to BMPs, with SHH operating in the former and TGFβ activation in the latter.

Key words: Skeletogenesis, Chondroblast differentiation, Chondrogenic program, GATA factors

Introduction

Cartilage plays a fundamental role in the development of most of the skeleton. In the limb, cells from the lateral plate mesoderm (LPM) contribute to multiple cell lineages, including cartilage, perichondrium, tendon and other non-muscle cell types. Lineage-tracing studies have demonstrated the presence of multipotent mesenchymal progenitors within the early limb bud and, through poorly understood mechanisms, these cells become lineage restricted (Pearse et al., 2007). A number of external signaling cues can influence the fate of progenitor cells; figuring prominently in these activities are members of the transforming growth factor β (TGFβ) superfamily.

Within the TGFβ family, numerous members have been shown to impact limb development and cell-fate specification. Bone morphogenetic proteins (BMPs) regulate formation of the cartilage anlagen and have been shown to promote a chondrogenic fate in non-chondrogenic cells within the limb (Duprez et al., 1996). Further evidence that BMPs play an important role in regulating chondrogenesis comes from recent studies describing the conditional deletion of either BMP genes or their receptors. Several genes encoding BMPs are expressed in the developing limb, and deletion of *Bmp2* and *Bmp4* within the limb mesenchyme leads to the loss of precartilaginous condensations (Bandyopadhyay et al., 2006). Similarly, deletion of BMP receptors *Bmpr1a* and *Bmpr1b* leads to the absence of most limb condensations; in those that do form, the cells fail to differentiate into chondrocytes (Yoon et al., 2005). Consistent with this idea, recent studies have demonstrated that BMP signaling is required for the 'compaction' of chondroprogenitors, whereby mesenchymal-cell aggregates coalesce and form tight interactions that lead to the establishment of cartilages (Barna and Niswander, 2007). The BMPs operate, in part, through regulating the activity and nuclear localization of several SMADs, including SMADs 1, 5 and 8 (Massague et al., 2005). Combined deletion of these SMADs in developing chondrocytes leads to severe chondrodysplasia, indicating that BMP signaling is essential for maintenance of a chondrogenic phenotype (Retting et al., 2009). In the limb, BMPs also regulate apoptosis within the interdigital region. In the early chick limb bud (<HH20), BMPs have been reported to promote apoptosis in the mesenchyme at stages preceding overt chondrogenesis (Ganan et al., 1996). The mechanism(s) underlying this differential responsiveness to BMPs is presently unclear.

In axial skeletal development, local sonic hedgehog (SHH) signals have been shown to be crucial for specifying presomitic tissue to a chondrogenic fate (Murtaugh et al., 1999). This fate is subsequently reinforced by BMP signaling. In this manner, chondrogenic specification in the axial skeleton operates through a two-step procedure, with SHH inducing the expression of *Bapx1* (also known as *Nkx3.2*), which encodes a transcription factor that upregulates the expression of *Sox9*, a central regulator of chondrogenesis (Murtaugh et al., 2001). In the presence of BMPs, a positive feedback loop is established between SOX9 and BAPX1 that drives expression of the chondrogenic program. Animals deficient for *Bapx1* present with axial skeletal defects; however, their limbs appear normal (Tribioli and Lufkin, 1999). *Bapx1* is expressed in the limb, but appears after *Sox9* is expressed, suggesting that an alternative chondrogenic specification program is operating in the limb (Murtaugh et al., 2001).

To better understand the mechanisms regulating BMP responsiveness in the limb, we analyzed BMP action in subpopulations of mesenchymal cells from the early [approximately embryonic day (E) 10.5] murine limb bud. These populations

represent cells at various developmental stages within the chondrogenic program and, interestingly, they were found to exhibit disparate responses to BMPs. BMPs inhibited chondrogenesis in cultures established from the earliest population of limb mesenchymal cells (the distal hind limb). This is associated with the induction of a distinct genetic program that involves induction of Gata genes. Subsequent analysis of these populations has revealed that BMP-mediated chondrogenesis in the limb involves at least a two-step process that is dependent on prior activation of the TGFβ pathway.

Results

BMPs exhibit stage-dependent chondrogenic activities in E10.5 limb buds

In the mouse, fore-limb development precedes hind-limb development by ~0.5 days. Furthermore, limb outgrowth is driven by cell expansion in the distal tip. Concurrent with this, cell differentiation occurs in a proximo-distal direction, with chondrogenic cells first appearing in more proximal regions. BMPs play a fundamental role in the chondrogenic program; however, in the early limb bud, activation of this pathway is associated with non-chondrogenic fates. To better understand the mechanisms regulating BMP responsiveness, three populations of cells representing different stages within the cartilage-formation program were evaluated for their response to BMP. These three populations from the E10.5 limb bud include cells collected from the hind-limb distal tip, which represent the youngest or earliest (EL) limb-bud mesenchymal cells in the E10.5 embryo, an intermediate (IM) population (in age) from the distal region of the fore-limb bud, and an older or later (LT) population from the proximal fore limb (Fig. 1A). BMP action in these populations was assessed using high-density micromass cultures. Microdissection of the limbs of ~12 embryos yielded sufficient cells for 4, 4 and 10 cultures of EL, IM and LT cells, respectively. Consistent with their origin, these cell populations exhibited a graded response to BMP4 (Fig. 1A-D). Addition of BMP4 to EL and to a lesser extent IM cultures significantly reduced in a dose-dependent manner the activity of a chondrogenic-responsive reporter (Col2-LUC). The LT population was not significantly impacted (Fig. 1A). The Col2-LUC reporter gene is based on the binding site for SOX5, SOX6 and SOX9 in *Col2a1* (Lefebvre et al., 1997; Lefebvre et al., 1998). The activity of this reporter has been found to tightly correlate with chondroblast differentiation and cartilage formation (Hoffman et al., 2006; Muramatsu et al., 2007; Weston et al., 2002; Weston et al., 2003). Consistent with the reporter gene findings, all three populations exhibited similar graded responses to BMP4 addition, as assayed by cartilage nodule formation using Alcian blue staining (Fig. 1B) and quantitative PCR (qPCR) analysis of *Sox9* and *Col2a1* expression (Fig. 1C).

To further evaluate the contribution of BMP signaling to the responses of the various cell populations, the effect of the BMP inhibitor NOGGIN was assessed. NOGGIN interferes with the ability of multiple BMPs, including BMP4, to bind their receptors. For the most part, NOGGIN attenuated the response to BMP4 (Fig. 1D). Interestingly, in the EL cultures, NOGGIN alone was found to significantly stimulate Col2-LUC activity, indicating that inhibition of endogenous BMP signaling at this early stage might promote chondrogenesis. NOGGIN has been reported by us and other groups to inhibit chondrogenesis and its ability to stimulate chondrogenesis in this setting probably reflects the nature of the EL population. For comparative purposes, BMP4 activity was assessed in cells from E11.5 limbs, a stage exhibiting overt chondrogenesis. Consistent with previous reports, BMP4 induced a robust pro-chondrogenic response in mesenchymal cells from either the distal mesenchyme (DM) or the whole limb (WL) of E11.5 embryos (Fig. 1E,F). Together, these results suggest that the limb mesenchyme exhibits stage-dependent responses to BMP signaling, with BMPs inhibiting expression of the chondroblastic

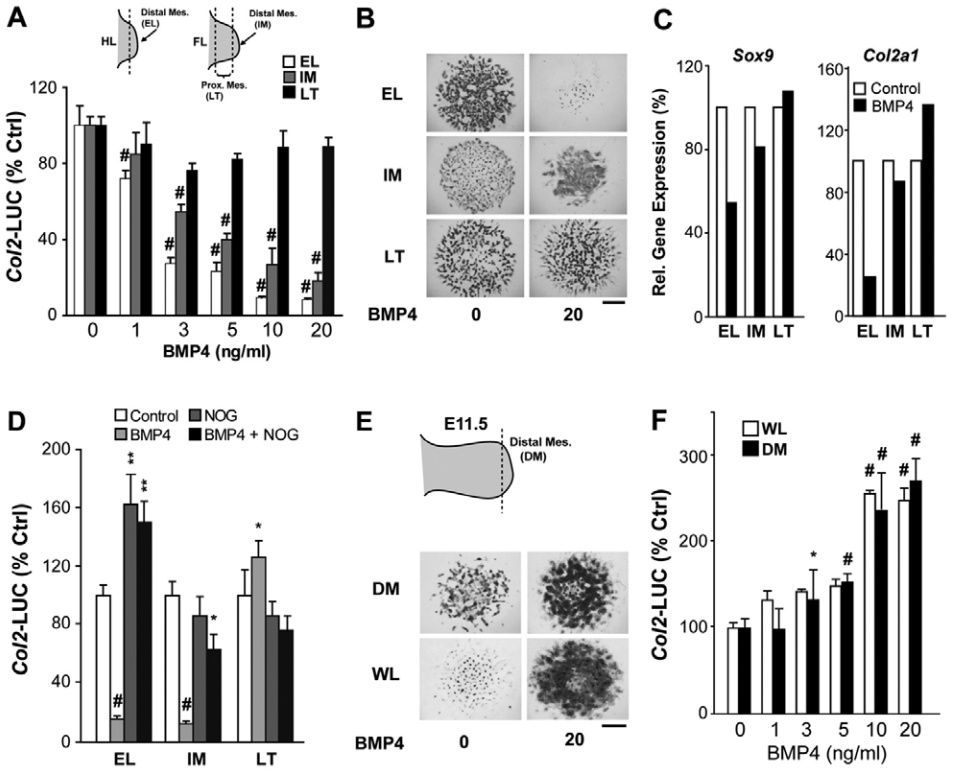

Fig. 1. BMP4 exhibits stage-dependent activities in cultures derived from early limb mesenchyme. (**A**) Graphical depiction of sections from E10.5 murine limbs used to generate EL, IM and LT cell populations. HL, hind limb; FL, fore limb. A *Col2a1*-derived reporter gene (Col2-LUC) was used to follow SOX5, SOX6 and SOX9 activity in the three cell populations. (**B**) BMP4 exhibits substantially different activities in EL, IM and LT cultures, as determined by Alcian blue staining of 4-day cultures. (**C**) qRT-PCR of *Sox9* and *Col2a1* expression in EL, IM and LT cultures. qPCR analysis was performed on single samples and then repeated with similar results. (**D**) Col2-LUC reporter activity was followed in EL, IM and LT cultures treated with NOGGIN (100 ng/ml) and/or BMP4. (**E**) Schematic representation of the regions of E11.5 murine limbs used to generate whole-limb (WL) and distal mesenchyme (DM) cultures. BMP4 stimulates cartilage formation in both WL and DM cultures, as determined by Alcian blue staining of 4-day cultures. (**F**) The activity of the Col2-LUC reporter also increases in a dose-dependent fashion with increasing concentrations of BMP4 (0-20 ng/ml) in WL and DM cultures. Error bars represent 1 s.d. $^{**}P<0.01$; $^{\#}P<0.001$. Scale bar: 1 mm.

phenotype in the more immature (younger) populations (EL and to a lesser extent IM) in E10.5 limb buds.

To investigate the molecular basis of the differential responsiveness to BMPs in the E10.5 limb mesenchyme, genome-wide expression profiling was employed. EL, IM and LT cell populations were isolated and cultured in the presence or absence of BMP4 for 0, 24 and 48 hours. Transcriptional profiles were generated through hybridization of processed RNA to Affymetrix MOE430 2.0 chips. Consistent with the qPCR described earlier, expression of chondroblast-associated genes (*Col2a1*, *Sox6*) decreased the most in EL samples following addition of BMP4, whereas the IM population exhibited a response intermediate to that of EL and LT (Fig. 2A). Analysis of differentially expressed genes in each of the samples using condition tree hierarchical clustering further showed that the freshly isolated (day 0, D0) EL population was distinctly different from the IM and LT populations (Fig. 2B). Furthermore, in the absence of BMP4, the EL cultures were more closely related to the IM cells than the LT cells. Moreover, in all conditions with the exception of the EL cells, BMP4-treated and untreated cells exhibited closely related gene expression profiles, suggesting that BMP4 reinforces existing gene expression patterns. In the EL populations, BMP4 treatment induces a distinct expression pattern; note the distance between the EL BMP4-treated and untreated samples in Fig. 2B (EL-D1-C, EL-D2-C versus EL-D1-B, EL-D2-B). Together, these results show that BMP4 addition to the EL cultures induces a gene expression program distinct from that of the untreated cultures.

To gain additional insights into the mechanisms underlying BMP action, Ingenuity pathway analysis (IPA) was used to identify over-represented biological functions and canonical pathways in treated versus untreated samples. For these analyses, a list of genes was generated using a twofold cut-off between corresponding BMP4-treated and untreated samples (e.g. EL-D1-C versus EL-D1-B4 gives rise to EL-D1). For all samples, biological functions associated with tissue, organ, organism, cellular and embryonic development, organ morphology, and cardiovascular and skeletal and muscular system development and function were over-represented, in addition to other functions (supplementary material Fig. S1). Canonical pathway analysis in IPA revealed enrichment of genes involved in a variety of pathways, including calcium signaling and axonal guidance, with the early populations typically showing greater enrichment than later populations (Fig. 2C). Not surprisingly, both analyses identified pathways or functions associated with the skeleton. Interestingly, a subset of the samples (EL-D1 and IM-D1) also showed significant enrichment of genes involved in cardiogenesis. These analyses further underscore the unique response of the EL populations (especially EL-D1) to BMP4 treatment in comparison to the IM and LT populations.

To further characterize the molecular differences underlying the differential responsiveness of the populations to BMP4, hierarchical clustering was used to identify genes associated with the response in EL cultures. For these analyses, a list of 288 genes (twofold cut-

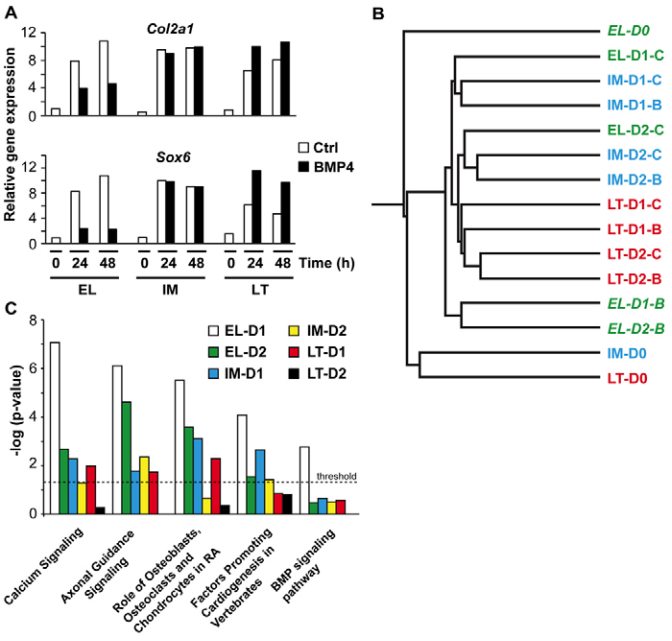

Fig. 2. Microarray analysis of BMP4 action reveals that the three region-specific cell populations exhibit markedly different responses. (**A**) *Col2a1* and *Sox6* expression was analyzed in microarray data sets generated from RNA collected 24 and 48 hours following BMP4 (20 ng/ml) addition to EL, IM and LT cultures. (**B**) Condition tree hierarchical clustering in GeneSpring was used to assess sample similarity. Individual samples are shown with the following nomenclature: cell population (EL, IM or LT) – time point (number of days) – treatment (C, control; B, BMP4). (**C**) Canonical pathways enriched following BMP4 treatment were identified using IPA. RA, rheumatoid arthritis.

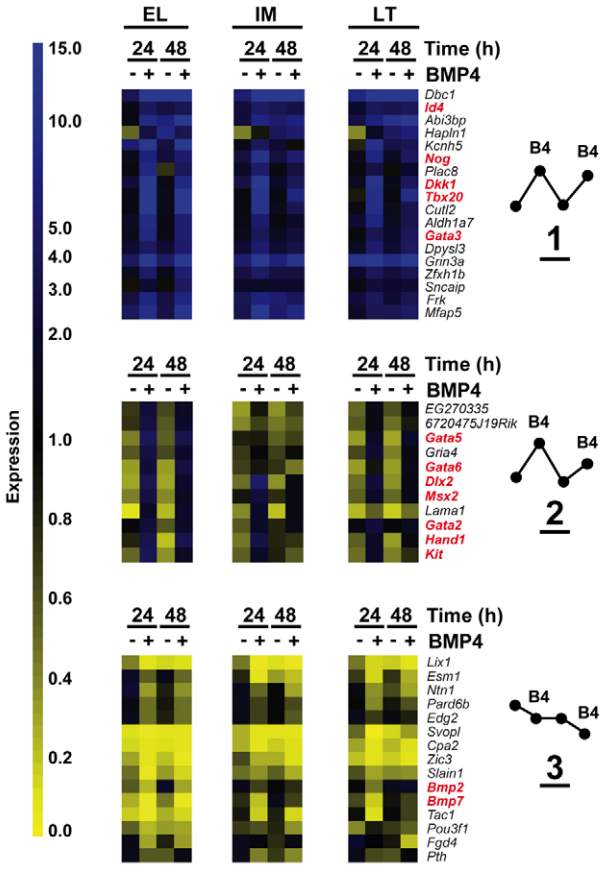

Fig. 3. Elucidation of the genetic programs underlying BMP action in early primary limb mesenchymal cultures. Hierarchical clustering of filtered genes following BMP4 (20 ng/ml) addition to EL, IM and LT cultures. The general gene expression profile of each cluster is shown above the cluster number (1-3). B4, BMP4. Genes highlighted in red represent potential BMP-regulated genes. Blue indicates upregulation of genes, whereas yellow indicates downregulation (the expression key is shown on the left).

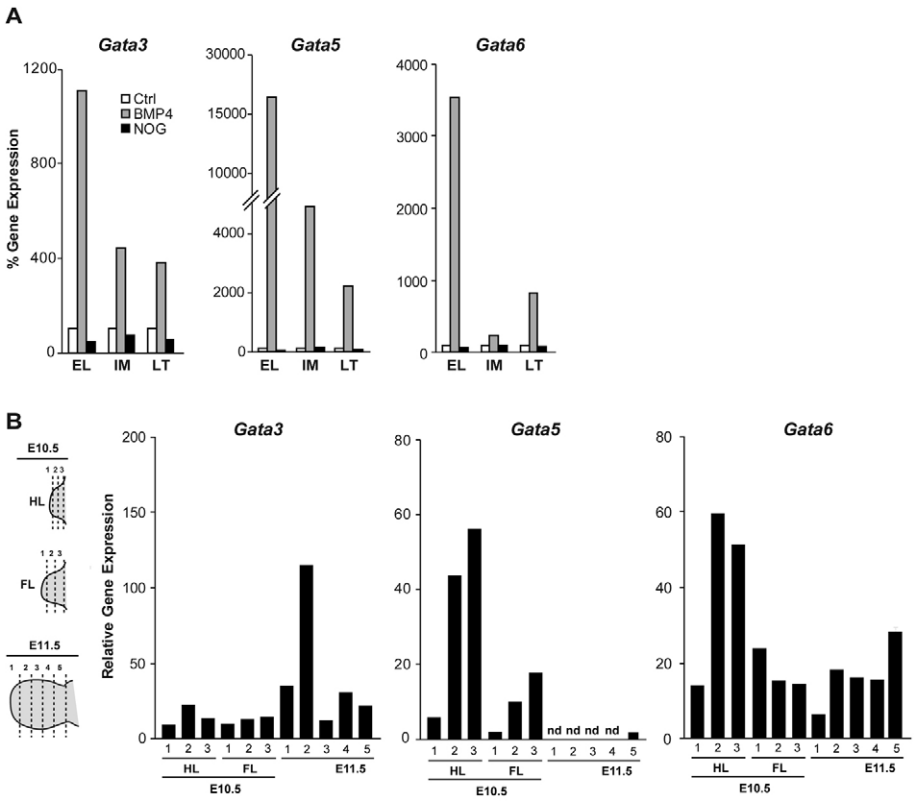

Fig. 4. Multiple Gata genes are expressed in the early limb and are differentially regulated in EL, IM and LT populations. (**A**) Gata gene expression was followed using qPCR of E10.5-derived cultures treated with BMP4 and NOGGIN (NOG). Factors were added at the time of culture initiation (day 0) and gene expression was analyzed at day 2. (**B**) The expression of *Gata3*, *Gata5* and *Gata6* in E10.5 and E11.5 limb buds was determined using qPCR. The regions corresponding to the samples analyzed by qPCR are shown on the left. HL, hind limb; FL, fore limb. n.d., 40 cycles of qPCR and *Gata5* transcript not detected. qPCR analysis was performed on single samples and then repeated, with similar results.

off) was generated and Pearson correlation analysis was used to cluster genes based on their expression patterns (Fig. 3). Not surprisingly, numerous genes involved in chondrogenesis were differentially expressed. Some known BMP-inducible genes, such as *Noggin*, were upregulated in all BMP4-treated populations (Fig. 3, cluster 1, genes highlighted in red). In contrast to *Noggin*, the expression of *Bmp2* and *Bmp7* was downregulated across all BMP-treated samples (Fig. 3, cluster 3).

To find genes that might underlie BMP responsiveness, clusters that exhibited differential gene induction across samples were identified. Cluster 2 generally contains genes that are induced to a greater extent in the EL population in comparison to either the IM or LT samples. Several genes in this cluster have been reported to be induced by BMPs (Fig. 3, cluster 2, highlighted in red) and some of these genes, particularly *Dlx2* and *Msx2*, have been shown to influence chondrogenesis (Semba et al., 2000; Xu et al., 2001). Interestingly, within this cluster, multiple Gata genes (*Gata2*, *Gata5* and *Gata6*; *Gata3* is within cluster 1) were appreciably induced in the EL population following BMP addition (Fig. 3, clusters 1 and 2). Gata gene expression in these samples was confirmed using qPCR. Consistent with observations of the microarrays, genes encoding GATA proteins were induced to a much greater extent in the EL cultures compared with IM and LT (Fig. 4A). Following 24-hours treatment with BMP4, *Gata3*, *Gata5* and *Gata6* were induced ~11-, 180- and 36-fold, respectively, in EL cultures. By contrast, treatment with NOGGIN appreciably reduced (<50%) Gata gene expression in EL cultures (Fig. 4A). Gata genes are involved in multiple developmental programs, including cardiogenesis (Peterkin et al., 2005); however, their role in the limb is unclear.

Gata gene expression was assessed in the developing limb using qPCR of limb sections encompassing regions containing the EL, IM and LT populations. *Gata5* was expressed in a distal-proximal gradient in the E10.5 hind and fore limbs, with ninefold increased expression in the proximal regions (Fig. 4B). *Gata6* exhibited a similar pattern of expression in the hind limb, but changed little in the fore limb, whereas *Gata3* exhibited little change within the E10.5 limb. Conversely, in the E11.5 limb, *Gata3* was abundantly expressed, and *Gata5* and *Gata6* exhibited reduced expression in comparison to the E10.5 hind limb, with *Gata5* only being detected in the most proximal region (Fig. 4B). In the limb, all three Gata genes are expressed; however, *Gata5* is preferentially expressed in the E10.5 limb and to a higher level in the hind limb than in the fore limb.

Manipulation of Gata gene expression inhibits chondroblast differentiation

To investigate the function of Gata genes in chondrogenesis, we overexpressed *Gata5* in the presence and absence of BMP4 in EL, IM and LT cultures, and followed Col2-LUC activity (Fig. 5A). As shown above, treatment of these cultures with BMP4 on day 0 reduced reporter gene activity to varying extents in the different cell populations, with the EL cultures exhibiting the greatest decline in activity. Expression of *Gata5* alone significantly reduced reporter gene activity by 50-60% (Fig. 5A, left panel). By contrast, addition of BMP4 on day 1, with lysis on day 2, was either neutral or stimulated reporter gene activity in the various cell populations (Fig. 5A, right panel). Under these conditions, expression of *Gata5* significantly reduced reporter gene activity in all cell populations to ~20% of controls and also inhibited BMP4 induction of reporter activity (Fig. 5A). Consistent with observations of cultures derived from the E10.5 limb buds, heterologous expression of *Gata1*, *Gata2*, *Gata3*, *Gata5* and *Gata6* also reduced Col2-LUC reporter activity in cultures derived from the E11.5 limb and attenuated

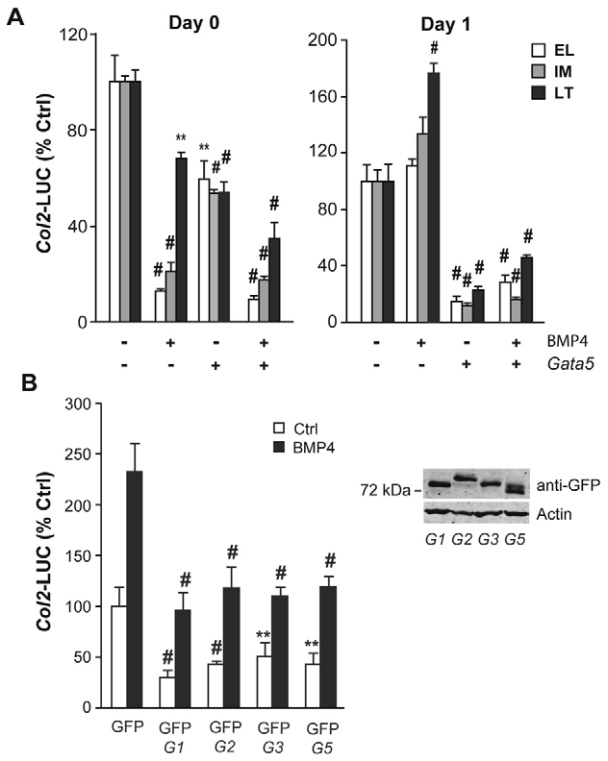

Fig. 5. Heterologous expression of various Gata genes interferes with chondrogenesis. (**A**) Left panel. E10.5 cultures were co-transfected with a Col2-LUC reporter gene and an expression plasmid encoding *Gata5*, treated with BMP4 on day 0 and luciferase activity measured on day 1. Right panel. E10.5 cultures were transfected on day 0, treated with BMP4 on day 1 and assayed on day 2. (**B**) Left panel. GFP-Gata genes were transfected into E11.5 whole-limb cultures, BMP4 was added on day 1 and luciferase activity was measured on day 2. Right panel. Western blot analysis of GFP-Gata (anti-GFP) expression 24 hours following transfection. β-actin is shown as a loading control. Error bars represent 1 s.d. Significance is in comparison to control cultures and is represented as follows: **P*<0.05; ***P*<0.01; #*P*<0.001.

BMP chondrogenic activity (Fig. 5B; supplementary material Fig. S2). These experiments were carried out with constructs containing an N-terminal GFP fusion, such that Gata gene expression could be followed. Un-fused GATA constructs yielded similar results to GFP-GATA constructs (Fig. 5B; supplementary material Fig. S2). Together, these results demonstrate that increased expression of *Gata5* and other Gata genes can phenocopy BMP activity in early limb mesenchyme and that continued expression of Gata genes inhibits chondroblast differentiation.

The E10.5-derived populations exhibit differential responsiveness to BMPs, whereas cultures from E11.5 limbs consistently show a chondrogenic response. Furthermore, the anti-chondrogenic response of the E10.5-derived cultures appears to be linked to the developmental stage, with cartilage formation in the earliest limb-cell population (the EL population) being inhibited to a greater extent than in either the IM or LT populations. To further assess whether the developmental stage or differentiation status of the limb-bud-derived cells impacts BMP responsiveness, cultures were maintained for a period of 24 hours before the addition of BMP4. Under these conditions, BMP4 stimulated cartilage formation in the EL, IM and LT cultures, as determined by Alcian blue staining and qPCR of *Sox6*, *Sox9* and *Col2a1* (Fig. 6). Furthermore, BMP induction of Gata genes was markedly reduced in comparison to addition of BMP4 at culture initiation (Fig. 4A; supplementary material Fig. S3); in EL cultures, *Gata3*, *Gata5* and *Gata6* expression was reduced from ~11- to 3-fold, ~180- to 20-fold and 36- to 4-fold, respectively. Collectively, these results further illustrate that the cellular response of early limb mesenchymal cells to BMPs can be separated into two distinct phases: an early 'anti-chondrogenic' stage associated with strong Gata gene expression, followed by a pro-chondrogenic period with low or absent Gata expression.

TGFβ signals modify cellular response to BMPs

As shown herein, appendicular skeletogenesis involves at least a two-step process akin to that described for presomitic tissues. In presomitic tissues, a chondrogenic response to BMP is dependent on their history, in particular, prior exposure to SHH (Murtaugh et al., 1999). To determine whether a similar scheme operates in the early limb bud, the ability of various signaling molecules to influence subsequent BMP responsiveness in EL populations was tested. The EL population was chosen for this analysis, as it exhibits a robust anti-chondrogenic response to BMP4 (and other BMPs, and growth and differentiation factors; data not shown). Factors and molecules were selected based on their expression in the E10 limb. For this screen, EL cells were exposed to various factors or molecules for 4 hours, followed by BMP addition. Chondrogenesis was assessed using the Col2-LUC reporter gene. The only factor that significantly inhibited the 'anti-chondrogenic' activity of BMP4 and increased reporter activity was TGFβ1 (Fig. 7A). This was further validated by Alcian blue staining of treated cultures; as seen with the reporter gene, short-term (4 hours) treatment with TGFβ1 was sufficient to markedly modify the response to BMP4 (Fig. 7B). Interestingly, simultaneous addition of TGFβ1 and BMP4 greatly inhibited cartilage formation, suggesting a requirement for their sequential exposure to initiate

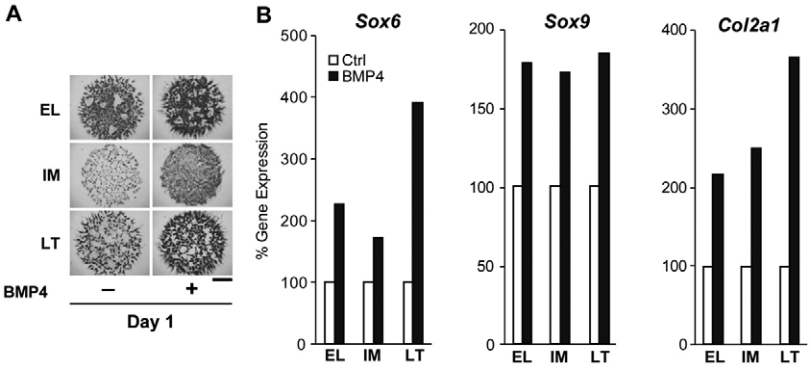

Fig. 6. EL, IM and LT cultures display stage-dependent chondrogenic responsiveness to BMP4. (**A**) BMP4 exhibits similar activities in the three E10.5 cultures, as determined by Alcian blue staining of 4-day cultures. BMP4 (20 ng/ml) was added 24 hours following culture establishment (day 1). Scale bar: 1 mm. (**B**) qPCR analysis of *Sox6*, *Sox9* and *Col2a1* expression in cultures treated with BMP4 24 hours after establishment. RNA was collected on day 2. qPCR analysis was performed on single samples and then repeated, with similar results.

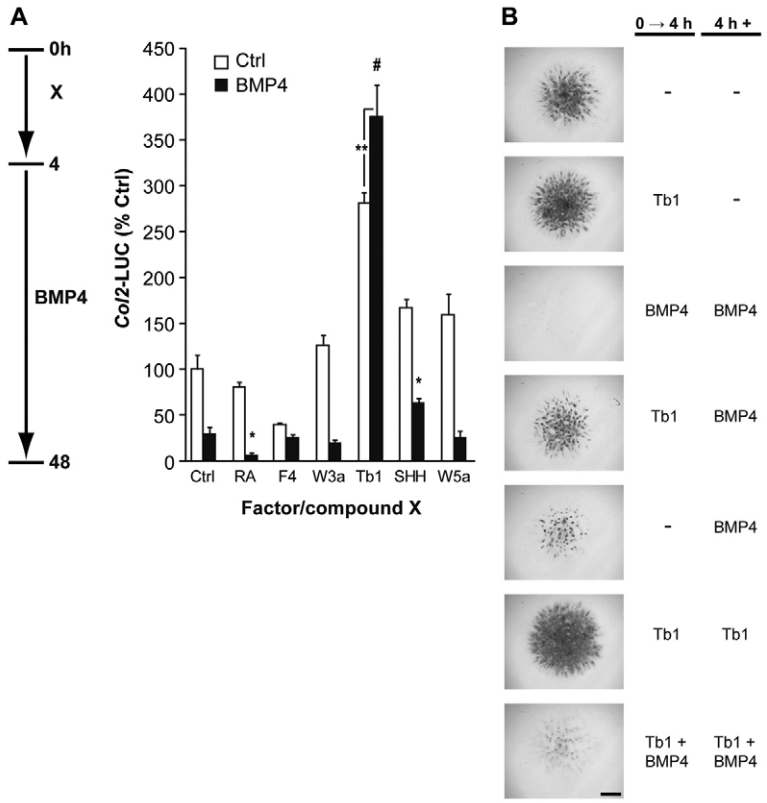

Fig. 7. TGFβ1 regulates chondrogenic responsiveness in early limb mesenchymal cells. (**A**) Early limb cultures were transfected with a Col2-LUC reporter gene and various factors (X) were added for 4 hours, after which BMP4 was added and luciferase activity was determined 20 hours later. Error bars represent 1 s.d. Significance was evaluated between BMP-treated samples, unless otherwise indicated, and is represented as follows: **P*<0.05; ***P*<0.01; #*P*<0.001. (**B**) Modulation of BMP pro-chondrogenic competency by TGFβ1 was evaluated in Alcian-blue-stained cultures. Early limb cultures were established and treated as indicated, and stained after 4 days. Scale bar: 1 mm. RA, retinoic acid (100 nM); F4, FGF4 (20 ng/ml); W3a, Wnt3a (50 ng/ml); Tb1, TGFβ1 (2 ng/ml); SHH (250 ng/ml); W5a, Wnt5a (transfected).

chondrogenesis (Fig. 7B). Consistent with a potential role in early skeletogenesis, all three genes encoding TGFβ are expressed in the E10.5 limb and TGFβ1 is distributed throughout the E10.5 limb bud (supplementary material Fig. S4).

The importance of TGFβ signaling in regulating the competency of EL cells to elicit a pro-chondrogenic response to BMPs was further examined in loss-of-function experiments in which TGFβ signaling was inhibited. As shown above, addition of BMP4 24 hours after culture initiation had a pro-chondrogenic effect on EL cultures. To test whether endogenous TGFβs were important in this response, TGFβ signaling was inhibited with SB431542 (RBI, 10 μM). RBI has been shown to be a selective TGFβ type I receptor (TGFBRI) inhibitor (Laping et al., 2002; Lin et al., 2009; Maeda et al., 2004; Watabe et al., 2003). To ensure that the 10 μM concentration employed did not impact BMP signaling, *Noggin* gene expression was measured following BMP4 treatment in the presence and absence of RBI. RBI did not significantly affect BMP4-mediated induction of *Noggin* (supplementary material Fig. S5). EL cells were treated with RBI for 24 hours, followed by wash out and addition of BMP4 (Fig. 8A). Under these conditions, RBI alone decreased chondroblast differentiation, as assessed by the Col2-LUC reporter and Alcian blue staining (Fig. 8A,B). Interestingly, inhibition of TGFβ signaling completely abrogated pro-chondrogenic responsiveness to subsequent BMP4 addition (Fig. 8A,B). Inhibition of TGFβ signaling also reduced the expression of the chondroblast marker *Sox6* and the chondrogenic lineage marker *Sox9* in EL cultures, whereas *Gata5* expression was increased ~6-fold (Fig. 8C). Moreover, consistent with the distinct activities of TGFβ and BMP signals in EL populations, TGFβ did not induce *Gata5*, as did BMPs, and TGFβ1 either increased or had little or no effect on *Sox6* and *Sox9* expression (Fig. 8C). Together, these experiments demonstrate that activation of TGFβ signaling is required early on in the limb chondrogenic program for initiation of chondrogenesis and subsequent BMP responsiveness.

Discussion

BMPs signal through common receptors to regulate highly diverse developmental programs. This diversity of action is achieved through modulating BMP responsiveness, such that appropriate genetic programs can be invoked following pathway activation. In axial skeleton development, brief exposure of the presomitic mesoderm to SHH is necessary to promote chondrogenesis in this tissue. In the absence of SHH, BMP signaling promotes a non-chondrogenic fate. In this manner, SHH and BMPs function sequentially to promote and reinforce a chondrogenic fate in the presomitic mesoderm. Similarly, in the early limb bud, we show that BMPs also induce a non-chondrogenic fate and that a chondrogenic response is dependent upon transient activation of the TGFβ signaling pathway. Together, these observations indicate that initiation of skeletogenesis relies on a two-step process that involves either SHH or TGFβ signals to shape subsequent chondrogenic responses to BMPs.

BMP signaling, Gata and chondrogenesis

Gata5 is transiently expressed during limb development and of the three Gata genes analyzed, *Gata5* exhibits the greatest magnitude of change in response to BMP4. Furthermore, the extent of induction is greatest in the more immature pre-chondrogenic cells within the EL population. Consistent with the transient nature of *Gata5* expression in vivo, BMP4 induction of *Gata5* is also transient. Expression of *Gata5* or other genes encoding GATA reduces the activity of a *Col2a1*-based reporter gene, indicative of impaired chondroblast differentiation. Similarly, Gata genes are

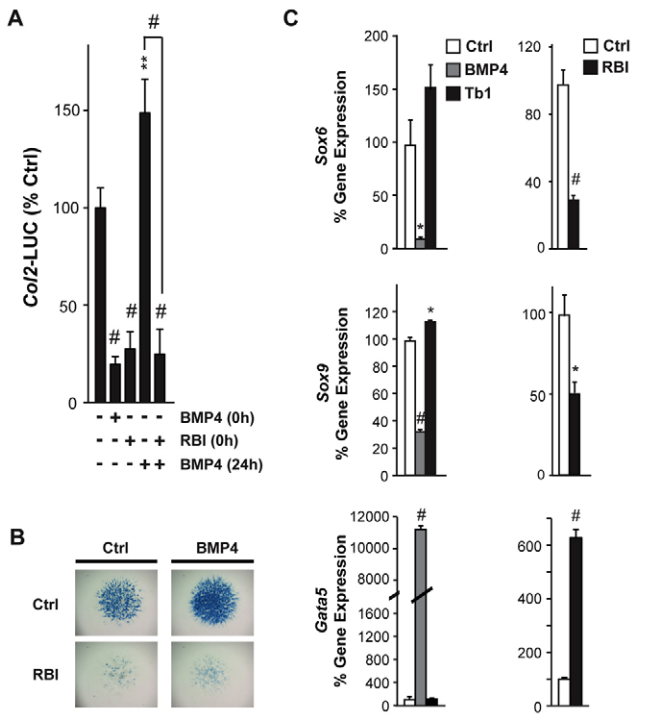

Fig. 8. TGFβ signaling is required in early limb populations for cartilage formation and BMP-dependent chondrogenesis. (**A**) The chondrogenic potential of early limb populations was measured with a Col2-LUC reporter in the presence and absence of SB431542 (RBI; 10 μM) for 24 hours; BMP4 was added at culture initiation (0 hours) or 24 hours later, and luciferase activity was measured 48 hours post-culture initiation. (**B**) Early limb cultures were treated with or without SB431542 (RBI) for 24 hours, treated with BMP4 (20 ng/ml) for 72 hours and stained with Alcian blue. (**C**) qPCR was used to evaluate the expression of *Sox6*, *Sox9* and *Gata5* in early limb cultures following activation (left panel) or inhibition (right panel) of TGFβ (Tb1) signaling for 24 hours (n=2). Error bars represent 1 s.d. Significance is in comparison to control cultures and is represented as follows: *P<0.05; **P<0.01; $^{\#}P$<0.001.

induced to varying extents by BMP4 in the various E10.5 populations; the extent of these induction events is proportional to the 'anti-chondrogenic' activity of BMP4. Together, these various observations suggest that *Gata5* and possibly the other Gata genes at least partly mediate the anti-chondrogenic actions of BMPs in the early limb mesenchyme.

In the absence of SHH, BMPs induce the expression of lateral plate markers, such as *Gata4*, in the presomitic mesoderm (Murtaugh et al., 1999). A similar scheme probably operates in the limb. In E10 limb mesenchyme, BMPs induce, to varying extents, Gata and chondrocytic marker expression, whereas by E11.5, BMPs induce only chondrocytic genes. The skeletogenic cells in the limb are derived from the LPM and, in this manner, early exposure to BMPs induces the expression of various Gata genes that are associated with the LPM. Shortly thereafter, BMPs induce the expression of chondrocytic markers and BMP-mediated induction of Gata genes is lost. In the limb mesenchyme, the switch from BMP induction of LPM or alternative LPM fates to a chondrogenic fate is dependent on TGFβ signals. In the absence of TGFβ in the EL and to a lesser extent in the IM populations, BMPs robustly induce Gata gene expression. This is associated with poor expression of the chondroblastic phenotype. Inhibition of TGFβ

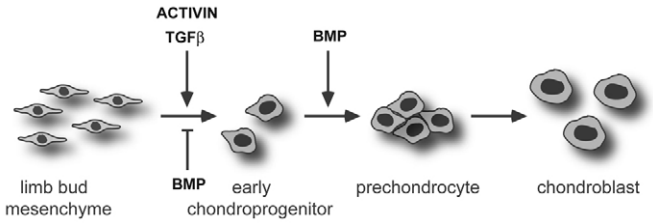

Fig. 9. A two-step model of chondrogenesis in the limb. BMPs inhibit the expression of a chondrogenic fate in naive limb mesenchymal cells in the early limb bud. Transient activation of the TGFβ (and/or possibly activin) signaling pathway in these cells is required to promote a chondrogenic response to BMP signaling. At later stages, BMPs alone are sufficient to promote chondrogenesis in the limb bud.

signaling also leads to increased expression of *Gata5*, and reduced cartilage formation and expression of chondrocytic markers. As chondrogenesis proceeds, manipulation of TGFβ signaling has less of an impact. Consistent with this, only transient exposure to TGFβ was required to alter subsequent responses to BMPs, whereas simultaneous addition of TGFβ and BMP interfered with cartilage formation (Fig. 7C). Together, these findings suggest that the LPM-derived limb mesenchyme is specified to a chondrogenic fate through the sequential actions of TGFβ and BMP signals (Fig. 9).

TGFβ signals in the limb: role in initiation of appendicular chondrogenesis

TGFβ addition has been shown to stimulate cartilage formation in a variety of in vitro models (Kulyk et al., 1989; Schofield and Wolpert, 1990). Studies in high-density chick micromass cultures demonstrated that TGFβ seemed to operate prior to BMPs and regulate the formation of pre-cartilaginous condensations (Leonard et al., 1991; Roark and Greer, 1994). In other reports, TGFβ has been shown to induce chondrocytic markers. Consistent with this, phospho-SMAD3, a downstream effector of TGFβ signaling, can be detected in the pre-chondrogenic mesenchyme (Lorda-Diez et al., 2009). TGFβ has been shown to drive isolated adult mesenchymal cells to a chondrocytic fate and is commonly used to stimulate cartilage formation in 'dedifferentiated chondrocytes' and mesenchymal multipotent progenitor and/or stem cells (Pittenger et al., 1999). In contrast to these in vitro studies, in vivo evidence supporting a crucial role for TGFβ in chondrogenesis is limited. Deletion of *Tgfb2* results in relatively minor limb skeletal defects and this is probably an indirect consequence of defective tendon formation (Pryce et al., 2009; Sanford et al., 1997). The skeletons of *Tgfb1*- and *Tgfb3*-null animals appear normal. Conversely, conditional knockout of the type II TGFβ receptor, *Tgfbr2*, in the limb mesenchyme with a *Prrx1*-Cre driver line did not cause early skeletal defects, but was associated with tendon loss (similar to that observed in the *Tgfb2*-null animals) and growth-plate alterations (Seo and Serra, 2007). Furthermore, addition of TGFβ to established micromass cultures led to decreased cartilage nodule formation and expression of a fibrogenic phenotype. In other studies, deletion of *Tgfbr2* in micromass cultures increased cartilage nodule formation (Lorda-Diez et al., 2009; Seo and Serra, 2007). Consistent with these observations, TGFβ has been shown to induce the expression of a tendogenic program in limb mesenchyme in vitro and in organ culture (Pryce et al., 2009). Interestingly, in E10.5 limb organ culture, implantation of a TGFβ2-soaked bead induced the expression of tendogenic marker *Scleraxis*

(*Scx*), but this was restricted to the mesenchyme proximal to the bead, with little or no expression in the distal mesenchyme (Pryce et al., 2009). As described herein, TGFβ induced the expression of various chondrocytic markers; however, this was stage dependent, with significant induction occurring in more immature populations (EL and IM) (Fig. 8C). Collectively, these findings indicate that TGFβ regulation of chondrogenesis is context dependent and acts early within the chondrogenic program.

Use of both gain- and loss-of-function approaches described herein indicates that TGFβ signaling is required early in the limb chondrogenic program prior to overt chondrogenesis. However, the absence of apparent defects in early limb skeletal development when TGFβ signaling is disrupted in vivo indicates either that TGFβ signaling is not important at this stage or that other signals, such as the activins, might fulfill this role in the absence of TGFβ signaling. From the *Tgfbr2* conditional knockout studies, it is not currently possible to rule out a direct role for transient TGFβ signaling in the early limb, as it is unclear to what extent TGFBR2 was reduced. In these mice, floxed *Tgfbr2* transcripts were present in the E10.5 limb mesenchyme. The very early transient requirement for TGFβ signals would require the complete loss of TGFBR2 by E10.5 in the distal hind limb and ~E10 in the fore limb, which is unlikely with *Prrx1*-Cre-mediated deletion (Seo and Serra, 2007). At present, a role for other signals, such as activins, cannot be excluded. Activins and their receptors are expressed in the early limb, similar to TGFβ they promote precartilaginous condensations, and the TGFBR inhibitors used herein also interfere with activin signaling (Feijen et al., 1994; Jiang et al., 1993; Laping et al., 2002; Merino et al., 1999; Nohno et al., 1993). Furthermore, as activin and TGFβ signals converge on downstream effectors SMAD2 and SMAD3, either signal might be sufficient to initiate chondrogenesis in the limb. Interestingly, SMAD3 and, to a lesser extent, SMAD2 have been shown to directly regulate SOX9 activity through binding to SOX9 in a TGFβ-dependent manner (Furumatsu et al., 2005). A model could be envisaged for early skeletogenesis, in which TGFβ-dependent promotion of nuclear translocation of SMAD2 and SMAD3 would increase SOX9 activity. This enhances chondrogenic potential, which is subsequently reinforced by BMP signaling. Thus, in the limb, TGFβ signaling could be considered to play a role analogous to that of SHH in the presomitic mesoderm, indicating that distinct molecular programs are used in the initiation of axial and appendicular skeletogenesis.

Materials and Methods

Reagents

BMP4, TGFβ1 and NOGGIN were purchased from R&D Systems and resuspended according to the manufacturer's instructions. BMP4 was added to media at a concentration of 20 ng/ml, whereas NOGGIN and TGFβ1 were used at concentrations of 100 ng/ml and 2 ng/ml, respectively. SB431542 (RBI, a TGFBR inhibitor) was purchased from Sigma, resuspended according to the manufacturer's instructions and added to media at a concentration of 10 μM.

Plasmid constructs and western blotting

To assess chondrogenic activity, a SOX-responsive reporter was used. This reporter contains four reiterated binding sites for SOX5, SOX6 and SOX9 (4×48 base pairs) upstream of a minimal type II collagen promoter (–89/+6) coupled to a luciferase gene (pCol2-LUC) (Weston et al., 2002). Full-length cDNA clones for *Gata1*, *Gata2*, *Gata3* and *Gata5* were obtained from the Ultimate ORF clone collection (Invitrogen) and recombineered into a Gateway-compatible destination vector containing a cytomegalovirus (CMV) promoter. A CMV-based expression plasmid for rat *Gata6* was kindly provided by Robert Viger (Université Laval, Canada). For generation of N-terminal EGFP-GATA fusion constructs, the aforementioned GATA constructs were recombineered into the pcDNA6.2/GFP-DEST vector.

For analysis of protein expression using western blots, cells were isolated from E11.5 WL populations, resuspended at a density of 1.2×10^7 cells/ml and transfected as described above. Transfected cells were harvested 24 hours after transfection by adding 200 μl of lysis buffer [10 mM Tris pH 7.4, 5 mM EDTA pH 8.0, 5 mM EGTA pH 8.0, 50 mM NaCl, 1% Triton X-100, with complete protease inhibitor tablet (Roche)], followed by acetone precipitation of proteins. The pellet was resuspended in 25 μl 2× sample buffer (0.125 M Tris pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol and 0.01% bromophenol blue) and heated at 95°C for 5 minutes. Proteins were separated using 10% SDS-PAGE gels and transferred to a nitrocellulose membrane (BioRad Laboratories). Blots were incubated with antibodies against GFP (1:1000, Invitrogen) and β-actin (1:5000, Santa Cruz Biotechnology). Signals were visualized using the Odyssey Infrared System (LI-COR Biosciences).

Establishment and transfection of primary mesenchymal cultures

Limb mesenchymal cultures from CD-1 E11.5 mouse limb buds were established as previously described (Hoffman et al., 2006). E10.5 early limb mesenchyme cultures were established from microdissection of E10.5 fore limb and hind limb, as shown in Fig. 1A. After proteolytic digestion, cells were filtered through a cell strainer (40 μM; Falcon) to obtain a single-cell suspension and resuspended at a density of 2×10^7 cells/ml. For microarray analyses, five 10 μl aliquots of this suspension were plated into a Nunc 35 mm tissue-culture dish and allowed to adhere for 1 hour. Following this period, 2 ml culture medium consisting of 60% F12, 40% DMEM and 10% FBS (Qualified-Invitrogen) was added to each well with or without 20 ng/ml BMP4 – this was considered time 0 (*T*=0). Culture media were replaced on alternate days and cultures were maintained for 1-4 days. Alcian blue staining was carried out as described by Weston et al. (Weston et al., 2002; Weston et al., 2000).

For transfection, a new more efficient protocol was developed because the previous FuGene6-based method exhibited poor transfection efficiency in E10.5 limb-derived cells. The new protocol uses Effectene (Qiagen) supplemented with 0.4 M trehalose – this addition increases transfection efficiency more than threefold (K. Garcha and T.M.U., in preparation). Briefly, 0.25 μg pCol2-LUC reporter, 0.025 μg pRL-SV40 (Promega) and 0.75 μg expression vector were combined and mixed with Effectene according to the manufacturer's instructions. 7.5 μl of this DNA-Effectene transfection mixture was transferred to sterile 1.5 ml microfuge tubes, followed by 40 μl cell suspension. Cells and the transfection mixture were gently triturated, and 10 μl was used to seed a single well of a 24-well culture dish. The cells were allowed to attach for 1 hour, followed by the addition of 1 ml media to each well. Extracts for luciferase analysis were collected on days 1 or 2, as indicated. For experiments involving limb mesenchymal cells alone or cells transfected with reporter genes only, factors or compounds were added at the time of media addition (*T*=0), unless otherwise indicated. For experiments involving co-transfection of cells with expression plasmids, factors and compounds were added up to 24 hours following transfection. Analysis of reporter gene activity was performed using the dual luciferase assay system, according to the manufacturer's instructions (Promega). Firefly luciferase was normalized against *Renilla* luciferase activity to control differences in transfection efficiency and to generate relative light units (RLU).

Transcriptional profiling with microarrays: experimental design and analysis

RNA was harvested from primary cultures using RNAeasy (Qiagen) according to the manufacturer's instructions and as described previously (Hoffman et al., 2006). Following isolation, RNA was diluted to 125 ng/μl, the expression of *Sox9* and *Col2b* was measured using real-time PCR, and RNA quality was examined on a Bioanalyzer 2100 (Agilent).

For each time point, two biological replicates were analyzed. Affymetrix mouse transcriptome arrays (MOE430 2.0) were used to generate transcriptional profiles from RNA derived from E10.5 IM, LT and EL cultures collected at 24 and 48 hours, and treated with BMP4 (20 ng/ml). To eliminate the need for amplification prior to microarray analyses, >1 μg RNA was collected from cultures derived from ~280 embryos. 1 μg RNA was labeled and hybridized to the chips using the manufacturer's recommended protocol (Affymetrix). The microarray data are available from the Gene Expression Omnibus under accession number GSE20398. Data sets were subsequently uploaded into GeneSpring for bioinformatic analysis and analyzed for the expression of individual genes (~39,000 on the chip set, with redundancy closer to 36,000 genes). All data sets were initially filtered to remove genes called absent by GeneSpring. Hierarchical clustering was carried out in GeneSpring with the GeneTree and ConditionTree tool using the Pearson correlation similarity measure. For functional annotation and pathway analysis with IPA, gene lists were initially filtered based on the present, marginal flag (P or M in at least four samples), and significantly regulated genes were identified using ANOVA analysis in GeneSpring (GX 7.3) with the default parameters. Briefly, a parametric test was performed in which the variances were not assumed equal, followed by multiple testing corrections using the Benjamini and Hochberg false discovery rate of 0.05. Differentially expressed genes were selected based on a twofold change in gene expression between BMP4-treated and untreated samples, and these were uploaded into IPA for analysis.

Real-time PCR

To follow the expression of various gene transcripts, quantitative real-time PCR (qRT-PCR) was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems). Some primers and TaqMan minor-groove-binding (MGB) probes were

designed using PrimerDesigner 2.0 (Applied Biosystems). The primer and probe sets used for detection of *Sox9* and *Col2a1* were as described by Weston et al. (Weston et al., 2002). Primer and probe concentrations were optimized according to the manufacturer's instructions. Other primer and probe sets were purchased from the TaqMan gene expression collection (Applied Biosystems). Total RNA was isolated from primary cultures as described above. Quantification was carried out using ~10 ng of total RNA and the expression of all genes relative to endogenous rRNA was determined using TaqMan Ribosomal Control Reagents (Applied Biosystems) and the relative quantitation method.

Statistical analysis

All luciferase assays were performed at least in triplicate and repeated using three distinct preparations of cells. Real-time PCR analysis was carried out in duplicate and repeated a minimum of two times with independent RNA samples. Luciferase reporter data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey post-test for multiple comparisons using GraphPad Prism, version 4.0 (GraphPad Software).

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