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Shox2 is Required for Chondrocyte Proliferation and Maturation in Proximal Limb Skeleton

Ling Yu[1], **Hongbing Liu**[1,2], **Mingquan Yan**[1], **Jing Yang**[3], **Fanxin Long**[4], **Ken Muneoka**[1], and **YiPing Chen**[1,2,†]

1*Department of Cell and Molecular Biology, Tulane University, New Orleans, LA 70117*

2*Department of Oral Biology, the Ohio State University Health Sciences Center, Columbus, OH 43210*

3*Columbus Children's Research Institute, Columbus, OH 43205*

4*Department of Medicine, Washington University Medical School, St. Louis, MO 63110*

Abstract

Mutations in the short stature homeobox gene *SHOX* lead to growth retardation associated with Turner, Leri-Weill dyschondrosteosis, and Langer mesomelic dysplasia syndromes, with marked shortening of the forearms and lower legs. We report here that in contrast to the *SHOX* mutations in humans, *Shox2* deficiency in mice leads to a virtual elimination of the stylopod in the developing limbs, while the zeugopod and autopod appear relatively normal. This phenotype is consistent with the restriction of the *Shox2* expression to the proximal mesenchyme in the limb bud and later to chondrocytes associated with the forming stylopod. In the *Shox2*$^{-/-}$ embryo, the mesenchymal condensation for the stylopod initiates normally but the cartilaginous element subsequently fails in growth, chondrogenesis and endochondral ossification. A dramatic down-regulation of *Runx2* and *Runx3* could account for the lack of chondrocyte hypertrophy, while a down-regulation of *Ihh* expression may be responsible for a significant reduction in chondrocyte proliferation in the mutant stylopod. We further demonstrate that an enhanced and ectopic *Bmp4* expression in the proximal limb of the *Shox2* embryo may underlie the down-regulation of *Runx2*, as ectopically applied exogenous BMP4 represses *Runx2* expression in the early limb bud. Moreover, we show that mouse Shox2, similar to human SHOX, can perform opposite roles on gene expression: either as a transcription activator or a repressor in different cell types. Our results establish a key role for *Shox2* in regulating the growth of stylopod by controlling chondrocyte maturation via *Runx2* and *Runx3*.

Keywords

Shox2; *Runx2*; BMP4; Mouse; Limb patterning; Chondrogenesis

Introduction

The vertebrate limb contains three distinct compartments along the proximodistal (PD) axis, the stylopod, zeugopod, and autopod, which are defined by the number, size, shape and the

†Corresponding author: Department of Oral Biology, The Ohio State University Health, Sciences Center, 305 W. 12th Ave., Columbus, OH 43210, Tel: 614-247-4288; Fax: 614-247-6945; Email: chen.1278@osu.edu

arrangement of skeletal elements. Limb grows and lengthens in a PD sequence, either with skeletal elements specified progressively, as hypothesized in the progress zone model (Saunders, 1948; Summerbell et al., 1973), or with progressive differentiation of elements that were specified in the early limb bud, as proposed in the early specification model (Dudley et al., 2002). Regardless of the mechanism for early specification, subsequent growth of each element is essential for achieving the eventual pattern of the limb skeleton, but the molecular mechanisms for this later phase of development have only begun to be elucidated. Most notably, members of the fibroblast growth factors (FGFs) expressed in the apical ectodermal ridge (AER), including FGF4, FGF8, FGF9 and FGF17, collectively are essential for the outgrowth of the limb bud (Fallon et al., 1994; Niswander et al., 1994; Niswander, 2003). In particular, removal of *Fgf8* in the AER of developing mouse limbs causes a severe reduction in the stylopod (Lewandowski et al., 2000; Moon and Capecchi, 2000). In addition, the *Hox* genes, particularly the 5′ *Hoxa* and *Hoxd* genes, are critical for the eventual pattern of the limb skeleton, as deletions of *Hoxa10*, *Hoxc10* and *Hoxd10* drastically shorten the femur, whereas loss of *Hoxa11* and *Hoxd11* results in a virtual loss of the radius and the ulna (Davis et al., 1995; Wellik and Capecchi, 2003; Boulet and Capecchi, 2004). Finally, two closely related homeobox genes *Meis1* and *Meis2*, which encode Hox cofactors, exhibit a restricted expression pattern in the proximal limb region and are thought to be the determinants of proximal limb compartments (Capdevila et al., 1999; Mercader et al., 1999, Moens and Selleri, 2006). Ectopic expression of either *Meis1* or *Meis2* in developing chick limbs reduces or truncates distal limb compartments (Capdevila et al., 1999; Mercader et al., 1999). However, targeted disruption of *Meis1* in mice does not result in an obvious PD defect in limbs (Hisa et al., 2004).

The vertebrate limb skeleton is derived from cartilage templates that form from mesenchymal condensations, and are eventually replaced by bone. Whereas growth factors such as BMPs and TGF-βs, Hox and Pax homeodomain transcription factors, and cell adhesion and ECM molecules have all been implicated in mesenchymal condensation and subsequent differentiation of chondrocytes (Hall and Miyake, 2000), the HMG transcription factor Sox9 is essential for cartilage formation (Akiyama et al., 2002).. Upon formation of the cartilage primordium, chondrocytes initially all proliferate but subsequently those residing at the middle of the element exit the cell cycle and undergo hypertrophy. Proper proliferation of chondrocytes requires Indian hedgheog (Ihh), as mouse embryos lacking *Ihh* exhibit a profound defect in proliferation (St-Jacques et al., 1999; Long et al., 2001). In addition, the transition from proliferation to hypertrophy is negatively regulated by IHH, which induces *PthrP* in chondrocytes and perichondrial cells within the periarticular region; PTHrP in turn keeps chondrocytes in the proliferating pool and prevents premature entry to hypertrophy (Lanske et al., 1996; Vortkamp et al., 1996; Chung et al., 1998, Karp et al., 2000). Moreover, Runx2, a Runt-domain transcription factor indispensable for osteoblast differentiation (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997), is important for chondrocyte hypertrophy, as its removal significantly delays hypertrophy in the stylopod although not other skeletal elements (Inada et al., 1999; Kim et al., 1999). Importantly, *Runx2-Runx3* double knockout mice lack chondrocyte hypertrophy in all long bones, underscoring the importance of Runx proteins in chondrocyte maturation (Yoshida et al., 2004). Finally, *Runx2* on the one hand regulates *Ihh* expression in chondrocytes (Yoshida et al., 2004), and on the other hand is regulated positively by BMP and negatively by PTHrP (Lee et al., 2000; Gu et al., 2004; Li et al., 2004; Friedman et al., 2005; Guo et al., 2006).

The short stature homeobox (*SHOX*) gene is associated with idiopathic growth retardation, Turner syndrome, and Lẹri-Weill dyschondrosteosis in humans (Ellison et al., 1997; Rao et al., 1997; Belin et al., 1998; Shears et al., 1998). Loss-of-function mutations in one allele of *SHOX* result in a phenotype of short stature with shortening of the forearms and lower legs, while homozygous mutations in *SHOX* cause Langer mesomelic dysplasia syndrome, a severe dwarfism with marked shortening of the limbs and almost complete elimination of the

zeugopod (Zinn et al., 2002). SHOX2, a second member of the *SHOX* family, has an overall 83% homology at the amino acid level and an identical homeodomain as SHOX (Blaschke et al., 1998; Semina et al., 1998). In the developing limbs of human embryos, *SHOX* and *SHOX2* exhibit a partially overlapping but complementary expression pattern, with *SHOX* expressed in a domain that is more distal relative to *SHOX2*. The distal SHOX expression domain is coincident with the skeletal defects observed in patients with Leri-Weill dyschondrosteosis and Langer mesomelic dysplasia (Clement-Jones et al., 2000). A zooblot analysis of *SHOX* and *SHOX2* demonstrates that they are only present in all vertebrates studies but not in the invertebrates examined, suggesting that these two genes have a central role in the development of the internal skeleton and its related structures (Clement-Jones et al., 2000). The mouse *SHOX* ortholog does not exist, but a true *Shox2* ortholog does, which shares 99% identity at the amino acid level with its human counterpart (Rovescalli et al., 1996; Blaschke et al., 1998; Semina et al., 1998; Clement-Jones et al., 2000). Since *SHOX2* has not yet been linked to any known syndromes in humans so far, its role in skeletogenesis, particularly in limb development, remains unknown.

We have previously generated *Shox2* mutant mice via gene targeting (Yu et al., 2005). Here we report detailed analyses of the *Shox2* mutant limb phenotype at the morphological, cellular, and molecular levels.

Materials and Methods

Mice and embryos

Shox2 mutant mice were generated via gene targeting and have been maintained on C57BL/6 background, as reported previously (Yu et al., 2005). Mice and embryos were genotyped by a PCR-based method using genomic DNA extracted from tail or extra-embryonic membranes, as described previously (Yu et al., 2005). Embryonic age was determined by the day when the vagina plug was discovered and designated as embryonic day 0.5 (E0.5).

Skeletal preparation, histology, and BrdU labeling

Skeletal staining was performed using Alcian Blue for non-mineralized cartilage and Alizarin Red for bone as described previously (Zhang et al., 2000b). For histological analysis, tissues were fixed in 4% paraformaldehyde (PFA) in PBS. Standard paraffin sectioning at 10 μm and Hematoxylin and Eosin staining were carried out. For in vivo BrdU labeling, timed pregnant *Shox2*$^{+/-}$ female mice (crossed with *Shox2*$^{+/-}$ male) were injected intraperitoneally with BrdU labeling reagent (3 mg/100 g body weight). Embryos were harvested two hours after injection and processed for fixation, embedding, sectioning, and immunodetection stained using a BrdU labeling and detection kit (Boehringer Mannheim), as described previously (Zhang et al., 2002).

In situ hybridization

Section in situ hybridization was performed to examine gene expression, as describe previously (Zhang et al., 1999). Samples were fixed in 4% PFA, paraffin embedded, and sectioned at 10 μm. Non-radioactive riboprobes were generated by in vitro transcription labeling with digoxigenin-UTP according to the manufacturer's instructions (Boehringer Mannheim). The following cDNA fragments were used to generate antisense riboprobes: *Bmp4* (1.0-kb), *Col II*)500-bp), *Col X* (650-bp), *Fgf8* (800-bp), *Hoxa10* (1.3-kb), *Hoxc10* (579-bp), *Hoxd10* (697-bp), *Ihh* (1.8-kb), *Meis1* (810-bp), *Meis2* (720-bp), *Pthrp* (1.5-kb), *Runx2* (550-bp), *Runx3* (456-bp), *Shox2* (550-bp), *Sox9* (586-bp).

Bead implantation and organ culture

Limb buds from E11.5 wild type embryos were microdissected in PBS and placed on filter in the Trowell type organ culture. Affi-Gel blue agarose beads (100 - 200 mesh, 75 - 150 μm in diameter, from Bio-Rad) that were soaked with BMP4 protein (from R&D Systems) at a concentration of 100 ng/μl or BSA (1 mg.ml) were implanted into the proximal region of the explanted limb buds. Samples were cultured in α-MEM media containing 10% knockout serum replacement, 3,500 mg/l glucose, 0.55 mM glycine, 0.056 mM ascorbic acid for 24 hours prior to harvest for section in situ hybridization.

In vitro transfection and CAT assay

To generate constructs expressing fusion proteins with the GAL4 DNA-binding domain, the following DNA fragments were subcloned, respectively, into the pBXG1 vector that harbors the GAL4 DNA-binding domain under the control of SV40 enhancer/promoter (Lillie and Green, 1989): DNA fragments encoding the N-terminus (amino acids 1-140) and the C-terminus (amino acids 201-331) of the mouse Shox2a, the C-terminus (amino acids 71-190) of the mouse Shox2b, the N-terminus (amino acids 1-117) and the C-terminus (amino acids 178-292) of the human SHOXa (Blaschke et al., 1998). To test potential transactivating activities, the resultant expression vectors were co-trasnfected, respectively, with the pG_5ECAT reporter plasmid that containing five GAL4-binding sites upstream of the adenovirus E1b minimal promoter and the chloramphenicol acetyltransferase (CAT) reporter gene. To determine potential repressive activities, the resultant expression vectors were co-transfected with the pG5tkCAT reporter vector in which the CAT reporter gene is directed by the herpe simplex virus thymidine kinase (TK) promoter with five GAL4 binding sites upstream of the TATA-box. A CMV-β-gal plasmid was included in transfection as internal control for transfection efficiency. P19 cell line and the human osteosarcoma cell line U20s were used for trasnfection and reporter gene expression assays. Transfection and CAT assays were performed as previously described (Yu et al., 2001; Wang et al., 2004). Transfected cells were cultured for 36 hours and the CAT activities were determined by scintillation counting. Each experiment was repeated three times to ensure consistent results.

Results

Shox2 expression is restricted to the proximal region of the developing limb

In order to reveal the expression pattern and course of *Shox2* in developing mouse limb, we carried out in situ hybridization on limbs at different developing stages. At E9.5 when the mouse limb development initiates, *Shox2* expression was found in the limb bud mesenchyme with stronger expression in the dorsal region (Fig. 1A). At E10.5 as limb bud elongates, *Shox2* expression was confined to the mesenchyme of the proximal limb, with stronger expression remaining in the dorsal region (Fig. 1B). At E11.5, *Shox2* transcripts were detected strongly in the proximal limb mesenchyme associated with the stylopodial cartilaginous condensation and relatively weakly in the mesenchymal tissue surrounding the condensations of the future zeugopodial skeletons (Fig. 1C). The dorsal-ventral asymmetrical expression pattern of *Shox2* was not seen at this stage. The expression pattern remained basically the same as that seen at E12.5 (Fig. 1D). However, at this stage chondrocytes in the both ends of the stylopod appear to exhibit weak *Shox2* expression. At E13.5 and E14.5 when differentiated hypertrophic chondrocytes have appeared in the central portion, *Shox2* expression was restricted in the proliferating chondrocytes in the stylopodial element (Fig. 1E, F). Meanwhile, *Shox2* expression remained in the connective tissues surrounding the cartilaginous element, but appeared down-regulated. Strong *Shox2* expression was also detected in the perichondrium (Fig. 1E, F). The unique expression pattern of *Shox2* in the developing limb suggests a role for *Shox2* in the formation of the proximal limb skeleton. Since *Shox2* expression and the knockout

phenotypes (described below) are identical in both forelimb and hindlimb, to make it consistent, we are reporting results only from the forelimb.

Shox2-deficiency leads to chondrodysplasia of the stylopod

We have previously reported the generation of *Shox2* null mutant mice that exhibit a unique cleft palate phenotype (Yu et al., 2005). *Shox2* mutants also exhibit shortened limb reminiscent of the short stature syndrome in humans. Skeletal preparations of the mutants revealed a marked reduction in size of the stylopod, that was first noticeable at E12.5 (data not shown) and became prominent by E13.5 (Fig. 2A). Subsequently, the mutant stylopod exhibited very little growth and failed to undergo endochondral ossification (Fig. 2B), so that at E17.5, the latest viable stage for the mutants (Yu et al., 2005), the mutant stylopodial skeletal elements remained extremely undergrown and completely unossified (Fig. 2C). The zeugopod elements of the mutant limbs appeared normal at E13.5 (Fig. 2A), although they too were slightly shortened, and delayed in ossification at E15.5 (Fig. 2B). At E17.5, the bone collars of the mutant zeugopod appeared slightly shorter than normal but otherwise similar to their wild type counterparts (Fig. 2D). Histological examination revealed a severe defect in chondrocyte maturation in the mutant stylopod (Fig. 2F), as no hypertrophy occurred at E16.5, a stage when hypertrophy and ossification are normally well underway (Fig. 2E). Thus, a failure in chondrocyte maturation underlies the dysplasia phenotype in the stylopod of *Shox2* mutants.

Failure of chondrocyte differentiation in the Shox2 mutant stylopod

To confirm the defect in chondrocyte maturation in the *Shox2*$^{-/-}$ stylopod, we examined by in situ hybridization the expression of *Sox9* and collagen type II (*Col II*), both markers for immatured chondrocytes, and collagen type X (*Col X*), a marker for hypertrophic chondrocytes. At E11.5, *Sox9* expression in all skeletal anlagen including the stylopod is similar between wild type and *Shox2*$^{-/-}$ limbs (Fig. 3A, B), indicating normal initiation of chondrogenesis in the *Shox2*$^{-/-}$ stylopod. However, all chondrocytes of the *Shox2*$^{-/-}$ stylopod remained immature at E14.5, as evidenced by the persistent expression of *Sox9* and *Col II* (Fig. 3D, F), and the absence of *Col X* expression (Fig. 3H). In contrast, in the wild type littermate, *Sox9* and *Col II* were now restricted to chondrocytes at both ends but absent in the central portion where *Col X*-expressing hypertrohpic chondrocytes resided (Fig. 3C, E, G). This modification of *Sox9, Col II* and *Col X* expression is spatially restricted to the stylopodial region since gene expression appears similar to wild type controls in the forming zeugopodial skeletal elements. We conclude that the deletion of *Shox2* leads to a complete absence of chondrocyte hypertrophy in the embryonic stylopod.

Expression of PD early patterning genes is not altered in Shox2 mutant limbs

Since *Shox2* exhibits a unique restricted expression pattern in the proximal region of the developing limb, and loss of *Shox2* leads to a virtual absence of the stylopod, it is possible that *Shox2* controls early PD patterning. We therefore analyzed the expression of a number of genes known to participate in early PD patterning of the limb, including *Hoxa10, Hoxc10, Hoxd10, Meis1, Meis2*, and *Fgf8*. Our results indicate that none of these genes altered its expression pattern in the mutant limb bud (supplementary Fig. 1, Fig. 2). Thus, the assayed genes do not appear to be downstream targets of *Shox2*, but may function upstream of or in parallel to *Shox2* in normal limb development.

Shox2 controls the expression of Ihh, Runx2 and Runx3 in the stylopodial

Since chondrocytes of the *Shox2*$^{-/-}$ stylopod failed to undergo hypertrophy, we examined the expression of several genes known to play crucial roles in chondrocyte maturation, including *Ihh, Pthrp, Runx2*, and *Runx3*. In the normal limb, *Ihh* expression initially appears in the chondrogenic mesenchymal condensations at E11.5 (Fig. 4A), and then in nascent cartilage at

E12.5 (Fig. 4C). Subsequently *Ihh* expression is restricted to the prehypertrophic chondrocytes of developing long bones (Bitgood and McMahon, 1995;St-Jacques et al., 1999; and data not shown). In the *Shox2*$^{-/-}$ limbs, at E11.5 *Ihh* was expressed in a wild-type pattern in all skeletal condensations including the presumptive stylopodial element (Fig. 4B). However, by E12.5 *Ihh* expression was almost completely lost in the *Shox2*$^{-/-}$ stylopod (Fig. 4). Interestingly, *Ihh* expression in the mutant zeugopod remained normal, suggesting a specific role of *Shox2* in maintaining *Ihh* expression in the stylopod. Consistent with the drastic down-regulation of *Ihh* expression, and the known proliferative role of Ihh (St-Jacques et al., 1999;Long et al., 2001), chondrocyte proliferation in the *Shox2*$^{-/-}$ stylopod was significantly reduced at E12.5 (Fig. 4E, F). Thus, the failure to maintain *Ihh* in the *Shox2*$^{-/-}$ stylopod may account for the decrease in chondrocyte proliferation, and in part the reduction in stylopod growth.

We next examined expression of PTHrP, known to critically regulate chondrocyte hypertrophy. Interestingly, at E12.5 when *Ihh* is down-regulated in the *Shox2*$^{-/-}$ stylopod, *Pthrp* expression appeared normal in this element (supplementary Fig. 3), suggesting that the residual Ihh in the mutant stylopod is sufficient to maintain *PthrP* expression.

Finally, we examined the expression of *Runx2* and *Runx3* in the developing stylopod of *Shox2*$^{-/-}$ versus wild-type littermate embryos. In wild-type embryos, *Runx2* and *Runx3* are detected in the central portions of chondrogenic condensations at E11.5 (Fig. 5, A, C), and of nascent cartilage at E12.5 (Fig. 5, E, G). From E13.5 onward, *Runx2* and *Runx3* are more restricted in pre- and early hypertrophic chondrocytes, in addition to their expression in the perichondrium (Fig. 5I, K, and data not shown). In contrast, in *Shox2*$^{-/-}$ embryos, *Runx2* and *Runx3* were either dramatically down-regulated (Fig. 5B) or undetectable (Fig. 5C) in the presumptive stylopod at E11.5. *Runx2* expression was further reduced at E12.5 (Fig. 5F) and became undetectable by E13.5 (Fig. 5J), whereas *Runx3* remained undetectable in the stylopod at all stages examined (Fig. 5, H, L). Thus the loss of *Runx2* and *Runx3* expression at early stages of cartilage development may be responsible for the failure of chondrocyte maturation in the *Shox2*$^{-/-}$ stylopod.

Regulation of Runx2 expression by Shox2 is mediated by BMP4

Since *Shox2* expression in the E11.5 and E12.5 proximal limb mesenchyme normally exhibits a complementary, rather than overlapping pattern to the stylopodial condensation that expression *Runx2* and *Runx3* (Fig. 1 and Fig. 5), it appears unlikely that *Runx2* and *Runx3* are direct targets of Shox2. Instead, we hypothesized that a diffusible factor(s) regulated by *Shox2* in the surrounding limb mesenchyme may regulate *Runx* genes in the cartilage. In an expression survey by in situ hybridization, *Bmp4* was identified as such a candidate. In the proximal region of a wild-type limb primordium, *Bmp4* was initially expressed in the condensations of stylopod and zeugopod at E11.5 (Fig. 6A), but became restricted to the perichondrium and the surrounding mesenchyme, with the highest level at the ends of the stylopod by E12.5 (Fig. 6C). Strikingly, in *Shox2*$^{-/-}$ limbs, *Bmp4* expression was broadly activated in the mesenchyme surrounding the stylopod at E11.5 (Fig. 6B), and markedly up-regulated in both the perichondrium and the surrounding mesenchyme at E12.5 (Fig. 6D). These observations raised the possibility that elevated *Bmp4* expression may be responsible for the repression of *Runx2* and *Runx3* in the mutant stylopod.

To examine the potential for BMP4 to regulate *Runx2* expression, we performed bead implantation experiments on limb buds in vitro. As shown in Fig. 6, *Runx2* expression was detected in both chondrocytes and the perichondrium of the control humerus implanted with BSA control bead (Fig. 6E; 5/5), but was completely inhibited in the samples treated with BMP4 (Fig. 6F; 10/10). Our previous studies have clearly shown that similar application of exogenous BMP4 to the mouse developing limb buds at the same concentration (100 ng/μl) does not enhance cell death in the limb bud mesenchyme (Zhang et al., 2000a). These results

demonstrate that high levels of BMP4 can inhibit *Runx2* expression. It is therefore possible that BMP4 up-regulation in the *Shox2*$^{-/-}$ limb underlies the down-regulation of *Runx2*.

Shox2 protein can act as either a transcriptional repressor or an activator

Previous studies have demonstrated that the human SHOX protein functions as a potential transcription activator, with its transactivation domain residing within the C-terminus (Rao et al., 2001). To determine if the mouse *Shox2* gene encodes a transcription activator or a repressor and to map the functional domain within the protein, we carried out in vitro reporter gene assays. The mouse *Shox2* gene encodes two isoforms *Shox2a* and *Shox2b* (Blaschke et al., 1998). The *Shox2b* isoform has an extremely short N-terminal amino acid sequence with only 6 amino acid residues prior to the homeodomain and a C-terminus missing a sequence of 12 amino acid residues present in the *Shox2a* isoform. We thus tested the transcriptional activities of the N- and C- terminal domains from Shox2a and the C-terminus of Shox2b. We generated constructs in which the N-terminus (the first amino acid to the amino acid prior to the homeodomain) of Shox2a and the C-terminal regions (from the amino acid immediately after the homeodomain to the last amino acid) of Shox2a and Shox2b were fused in frame to the 147 amino acid DNA-binding domain of yeast GAL4. The N- and C-termini of the human SHOXa were used as controls. These constructs were co-transfected, respectively, with the pG_5ECAT reporter plasmid to determine transactivation activity, and with the pG5tkCAT reporter vector for transcriptional repressive activity, as previously reported (Yu et al., 2001; Wang et al., 2004). The results demonstrate that the transcription activity of Shox2 protein resides within the C-terminus of either isoforms, with no significant activity detected in the N-terminus of Shox2a (Fig. 7).). Interestingly, the C terminal sequences acted as a transcriptional activator in the osteogenic cell line U20s (17-fold for Shox2a, 14-fold for Shox2b), but as a repressor in P19 cells (about 4-fold repression for both C-termini) (Fig. 7). Similarly, we found that the C-terminus of human SHOXa also activated in U20s cells but repressed transcription in P19 cells (data not shown). Thus, both mouse Shox2 and human SHOX can either activate or repress transcription in different cell types.

Discussion

Mice lacking *Shox2* die prenatally and exhibit severe abnormalities in several developing organs (Yu et al., 2005). Here we report a unique limb phenotype in the mutant mice: limbs form but develop chondrodysplasia specifically in the stylopod, resulting in the virtual absence of the humerus and femur. Our studies showed that a complete absence of chondrocyte maturation and a marked decrease in proliferation in the stylopod underlies the chondrodysplasia phenotype. Our results are consistent with that reported in a recent study in which *Shox2* was conditionally removed in developing limbs (Cobb et al., 2006). However, we have further demonstrated that *Shox2* regulates the expression of *Runx* genes, via control of BMP4, to control chondrocyte maturation. Shox2 protein acts either as a transcription activator or a repressor in different cell types.

Shox2 is essential for patterning the proximal limbs in mice

Consistent with the restricted expression of *Shox2* in the proximal region of developing limbs, the absence of *Shox2* essentially eliminates of the stylopod, while the more distal structures, including the zeugopod and the autopod, appear relatively normal. The defect was unlikely due to early patterning defects, as the stylopod primordia appeared normal at early stages, and expression of known patterning genes including *Hoxa10, Hoxc10, Hoxd10, Meis1, Meis2,* and *Fgf8* was not altered in *Shox2* mutants (data not shown). Similarly, expression of *Hoxd9, Hoxd11, Hoxd13* and *Hoxa11* was reported normal in the limbs of *Shox2* limb-specific knockout embryos (Cobb et al., 2006). Moreover, a recent study reported that over-expression of the chick *Shox* gene led to consistent elongation of skeletal elements of chick limbs but a

normal PD patterning with unaltered expression of *Hoxa13, Hoxd11*, and *Meis1* (Tiecke et al., 2006).

Shox2 regulates chondrocyte maturation via controlling Runx gene expression

Chondrocyte maturation in long bones is regulated by an integration of multiple signaling pathways, including BMP, FGF, Wnt, and IHH/PTHrP pathways (Akiyama et al., 2004; Minina et al., 2001; 2002). Members of the *Runx* gene family, particularly *Runx2*, play an essential role in chondrocyte maturation, most likely by mediating the functions of these signaling pathways. Our results demonstrate that *Shox2* functions genetically upstream of *Runx2* and *Runx3* in the stylopd, as expression of *Runx2* and *Runx3* was significantly down-regulated in the *Shox2* mutant. Loss of *Runx2* and *Runx3* could in turn accounts for the lack of hypertrophic chondrocytes in the *Shox2*$^{-/-}$ stylopod. The lack of hypertrophy in our mutant mice appears to represent an extreme delay in maturation, as hypertrophic chondrocytes were reported to eventually occur in the limb-specific *Shox2* knockout animals (Cobb et al 2006).

Shox2 apparently does not regulate the expression of *Runx* genes directly, at least not at the early stage of limb development. This is supported by the observations that at E11.5, *Shox2* expression does not overlap with that of *Runx2* and *Runx3* in the proximal limbs, even though a down-regulation of *Runx2* and *Runx3* occurs in the *Shox2* mutants at this stage. We have provided evidence that BMP4 may mediate the indirect regulation of *Runx2* by *Shox2*. First, *Bmp4* expression was markedly up-regulated both in the normal domains as well as ectopically in the proximal limbs of *Shox2* mutants. Second, exogenous BMP4 is able to repress *Runx2* expression in the cartilaginous elements of wild-type limbs. It is therefore conceivable that in the normal mouse limb development, *Shox2* functions to repress *Bmp4* expression in the proximal limbs, allowing the activation of *Runx* genes. In this scenario, Shox2 may act as a transcriptional repressor. Our in vitro reporter gene expression assays indicate that this can indeed be the case in certain cell types. Roles of BMPs in chondrogenesis have been previously reported (Capdevila and Johnson, 1998; Minina et al., 2001; 2002; Pathi et al., 1999; Pizette and Niswander, 2000; Yoon et al., 2005). However, BMP signals were previously shown to activate *Runx2* expression in micromass culture of embryonic limb mesenchyme as well as C2C12 mouse myoblast precursor cells (Lee et al., 2000; Gu et al., 2004; Wang et al., 2005). The seemingly contradictory results on the regulation of *Runx2* by BMP could be explained by differences in experimental systems and/or levels of BMP signaling, as BMP4 was previously shown to exert opposite roles on the regulation of *Shh* expression in developing teeth (Zhang et al., 2000a). The regulation of *Runx2* by BMP may not be direct, since neither BMP nor Smad affected the activities of a mouse *Runx2* promoter (Xiao et al., 2001; Lee et al., 2002).

Contribution of Ihh down-regulation to the decrease in growth of mutant stylopods

Previous studies have revealed a crucial role of IHH in chondrocyte proliferation (St. Jacques et al., 1999) in part by modulating the expression of *cyclin D1* (Long et al., 2001). In *Shox2* mutants, *Ihh* expression initiated normally in the developing limb at E11.5 but greatly diminished in the stylopod by E12.5. Also at E12.5, chondrocyte proliferation in the stylopod was significantly reduced, consistent with the role of IHH in chondrocyte proliferation. We therefore conclude that *Ihh* down-regulation is at least partially responsible for the proliferation defect in the *Shox2* mutant stylopod. However, *Shox2* is unlikely to regulate *Ihh* expression directly, since expression *Shox2* does not overlap with that of *Ihh* in the early limbs. Instead, the down-regulation of *Runx2* and *Runx3* may be responsible for the loss of *Ihh* expression in the *Shox2* mutant stylopod, as *Ihh* expression was dramatically down-regulated in the *Runx2-Runx3* double mutant limbs and *Runx2* strongly induced *Ihh* expression by directly binding to the promoter region of *Ihh* gene (Yoshida et al., 2004).

Shox2 can function as a transcriptional repressor or activator

Members of *SHOX* gene family (*SHOX* and *SHOX2*) encode the homeodomain-containing transcriptional regulators. The human SHOX proteins were shown to act as transcriptional activator, with its transactivation domain mapped to the C-terminal region (Rao et al., 2001). However, the transactivating activity of SHOX was observed only in cells of osteogenic origin, indicating that specific co-factors are required for the transcriptional activation function of SHOX (Rao et al., 2001). In this study, we show that the C-terminal region of mouse Shox2 possesses strong transactivating activities in the osteogenic cell line U20s. To our surprise, the same domain of Shox2 exerted an opposite effect on the expression of reporter gene in P19 cell line. Similar dual activities were observed with the C-terminus of human SHOX, indicating that both SHOX and Shox2 can act as either a transcriptional activator or a repressor, depending on the cell-type specific co-factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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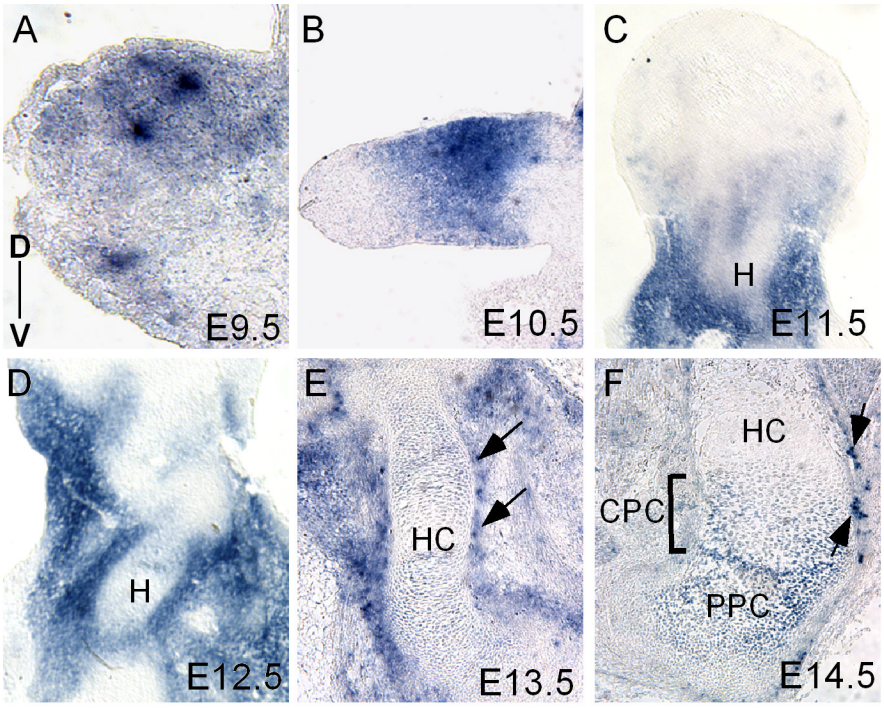

Figure 1.

Expression of *Shox2* in the developing mouse limb. (A,B) *Shox2* transcripts are detected in the limb mesenchyme at E9.5 (A) and are restricted to the proximal limb mesenchyme at E10.5 (B). (C,D) *Shox2* expression is seen in the mesenchyme of the proximal limbs surrounding the cartilaginous elements at E11.5 (C) and at E12.5 (D). (E,F) *Shox2* expression is detected in the immature chondrocytes (proliferating chondrocytes) and the perichondrium (arrows) of the stylopodial elements at E13.5 (E) and E14.5 (F). The expression in the surrounding connective tissues remains but is down-regulated. All sections are longitudinal. H, humerus; HC, hypertrophic chondrocytes; CPC, columnar proliferating chondrocytes; PPC, periarticular proliferating chondrocytes.

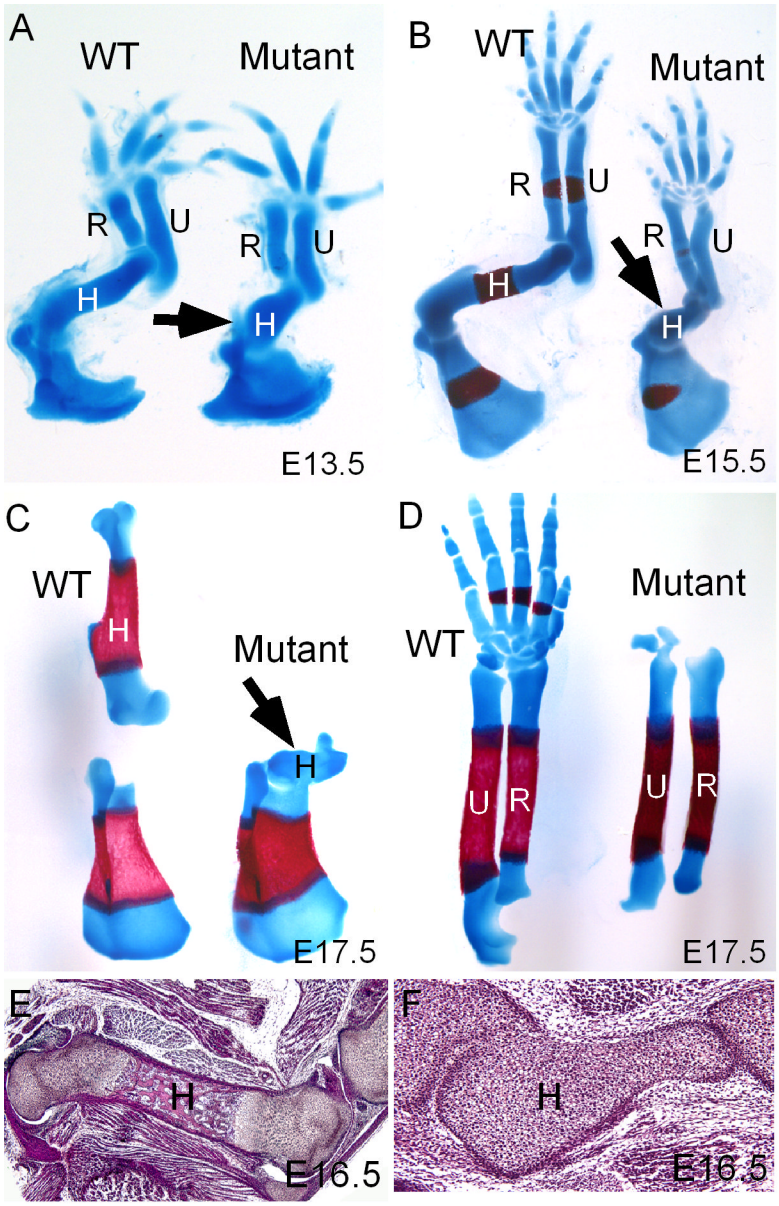

Figure 2.

Chondrodysplasia of the stylopodial elements in *Shox2*$^{-/-}$ mice. (A-D) Skeletal preparations of the wild type and *Shox2*$^{-/-}$ forelimbs at E13.5 (A), 15.5 (B), and E17.5 (C,D) exhibit remarkable shortening of the humerus of *Shox2* mutants. The distal elements of both wild type and mutant embryonic limbs appear comparable at the stages examined. (E,F) Longitudinal histology section through the stylopod from E16.5 *Shox2* mutant forelimb (F) shows a complete absence of chondrocyte maturation and ossification in the humerus, as compared to the wild type control (E). Arrows point to the humerus. H, humerus.

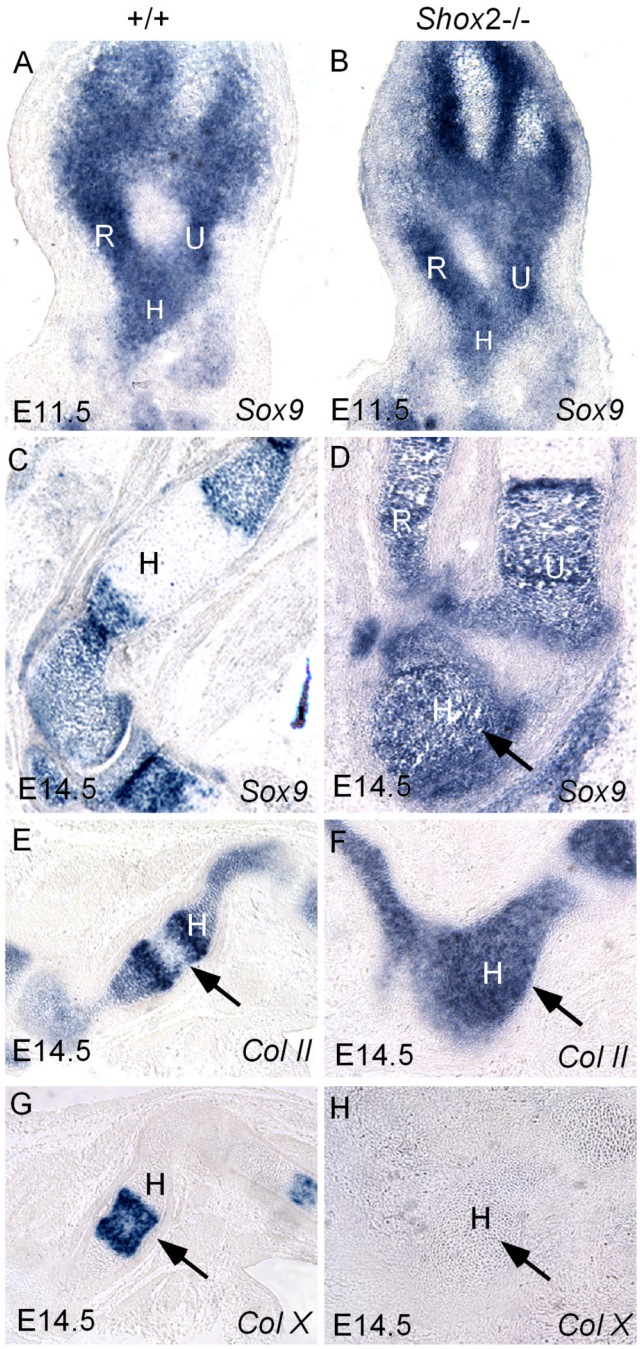

Figure 3.

Failure of chondrocyte maturation in the *Shox2*$^{-/-}$ stylopod. (A,B) *Sox9* expression, which marks the precartilaginous condensations, is unaltered in the E11.5 *Shox2*$^{-/-}$ forelimb (B), as compared to the wild type control (A). (C,D) *Sox9* expression is restricted to the proliferating chondrocytes in the both ends of the humerus of E14.5 wild type embryo (C), and persists in the entire humerus element of E14.5 *Shox2* mutant (D). (E,F) At E14.5, *Col II* expression begins to recess from the middle sector of the wild type humerus (E), but remains in the entire *Shox2*$^{-/-}$ humerus (F). (G) *Col X* expression in the E14.5 wild type humerus indicates the presence of hypertrophic chondrocytes. (H) An E14.5 *Shox2*$^{-/-}$ forelimb shows the lack of *Col X* expression in the humerus. Arrows point to the humerus. H, humerus; R, radius; U, ulna.

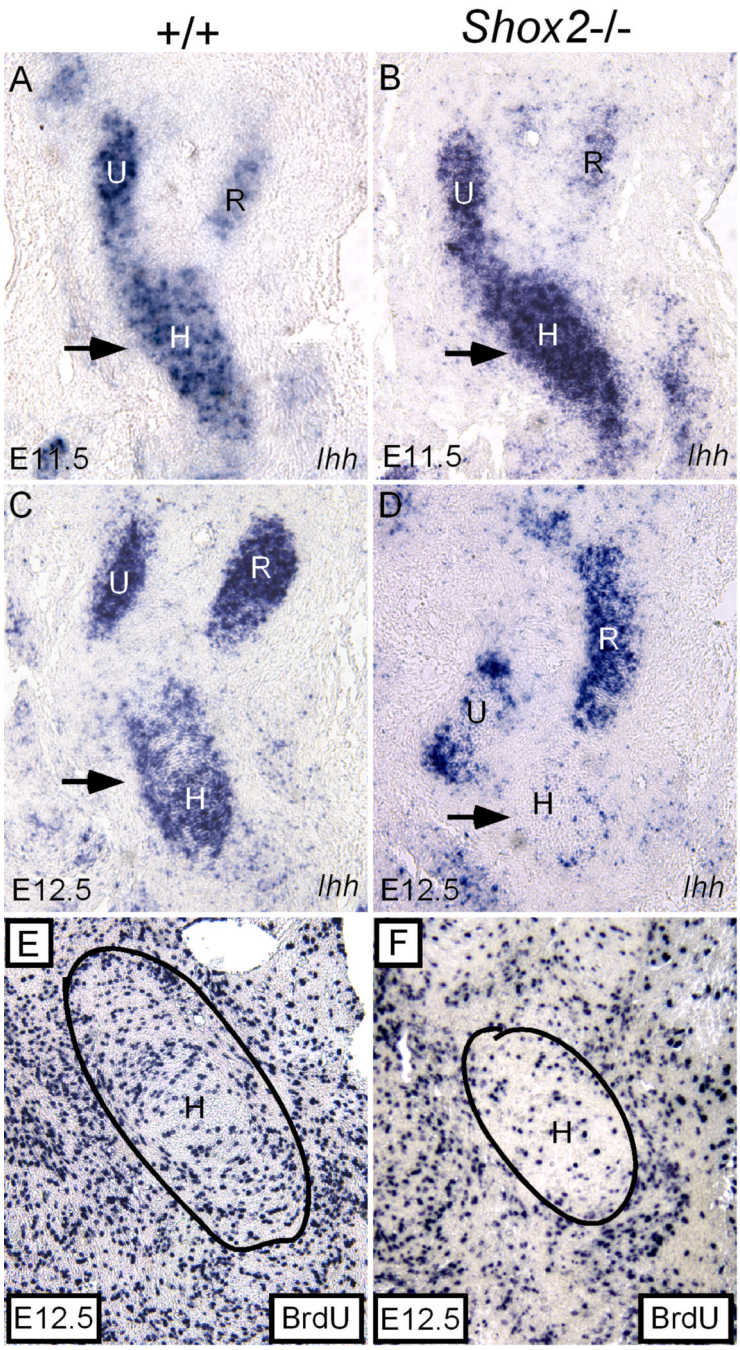

Figure 4.

Altered *Ihh* expression and cell proliferation in the cartilaginous elements of the *Shox2*$^{-/-}$ stylopod. (A,B) *Ihh* expression remains the same in the mesenchymal condensations of the wild type (A) and *Shox2*$^{-/-}$ (B) forelimbs at E11.5. (C,D) At E12.5, *Ihh* expression is significantly down-regulated in the *Shox2*$^{-/-}$ humerus (D), as compared to that in the wild type control (C). Note the expression of *Ihh* is unaltered in the elements of mutant zeugopod. (E,F) Cell proliferation as labeled by BrdU is significantly reduced in the cartilaginous element of *Shox2*$^{-/-}$ humerus (F), as compared to the wild type control at E12.5 (E). Arrows point to the humerus. H, humerus; R. radius; U, ulna.

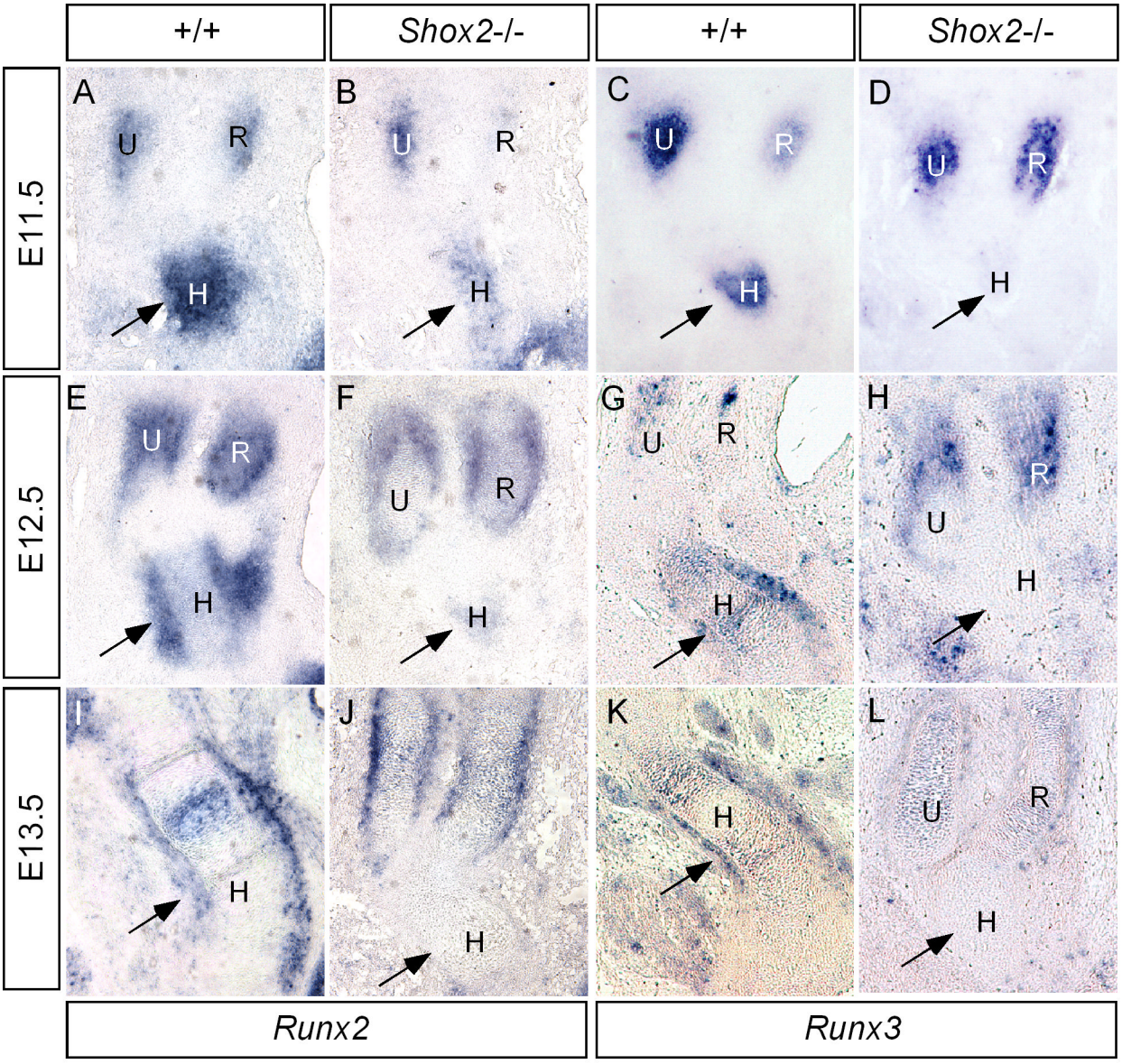

Figure 5.

Down-regulation of *Runx2* and *Runx3* in the cartilaginous elements of the *Shox2*$^{-/-}$ stylopod. (A-D) In E11.5 wild type forelimbs, *Runx2* (A) and *Runx3* (C) exhibit an identical expression pattern restricted to the mesenchymal condensations. In limbs from *Shox2* mutant of the same stage, *Runx2* (B) and *Runx3* (D) expression is dramatically down-regulated in the condensation of the humerus, but remains similar in the condensations of the zeugopod. (E-H) At E12.5, *Runx2* and *Runx3* expression continues in the cartilaginous elements of the wild type limbs (E,G), but is almost completely absent in the humerus of *Shox2* mutant (F,H). Note the expression of *Runx* genes remains in the cartilaginous elements of the *Shox2*$^{-/-}$ zeugopod. (I-L) At E13.5 the expression of *Runx2* and *Runx3* is detected in the perichondrium and hypertrophic chondrocytes of the wild type humerus (I,K), but is completely absent in the *Shox2*$^{-/-}$ humerus (J,K). Arrows point to the humerus. H, humerus; R, radius; U, ulna.

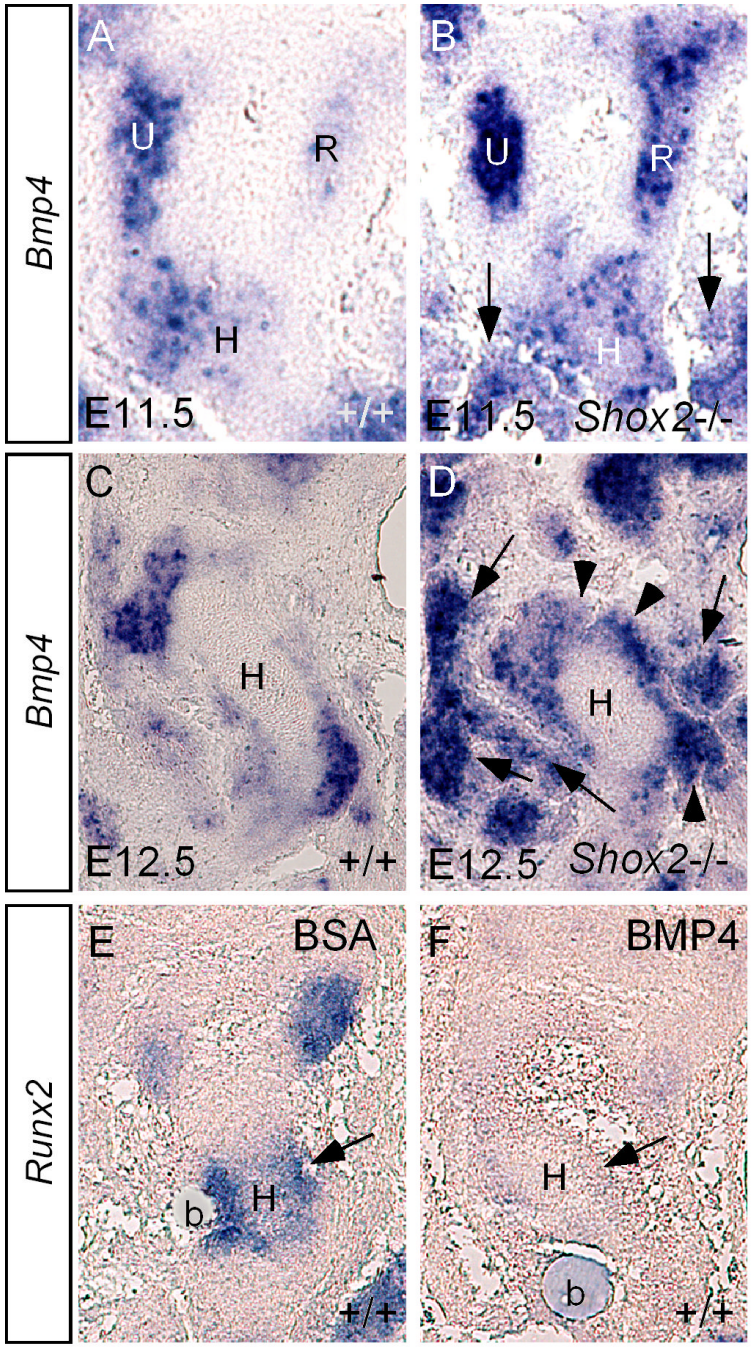

Figure 6.

Up-regulated BMP4 represses *Runx2* expression in the early limbs. (A) *Bmp4* expression is detected in the mesenchymal condensations of an E11.5 wild type forelimb. (B) Ectopic expression of *Bmp4* is detected in the mesenchymal tissues (arrows) surrounding the humerus condensation of E11.5 *Shox2*$^{-/-}$ forelimb. (C) At E12.5, *Bmp4* expression is seen in the perichondral region, with higher level at the both ends of the humerus of the wild type forelimb. (D) Ectopically expressed *Bmp4* is detected in the mesenchymal tissues (arrows) surrounding the humerus of an E12.5 *Shox2* mutant. Arrowheads point to the strong expression of *Bmp4* in the perichondrial region. (E,F) An E11.5 wild type forelimb, after 24 hours in culture with an implanted BMP4-soaked bead, shows completely abolished *Runx2* expression in the humerus

element (arrow) (F), as compared to control which received a BSA-soaked bead (E). H, humerus; R, radius; U, ulna.

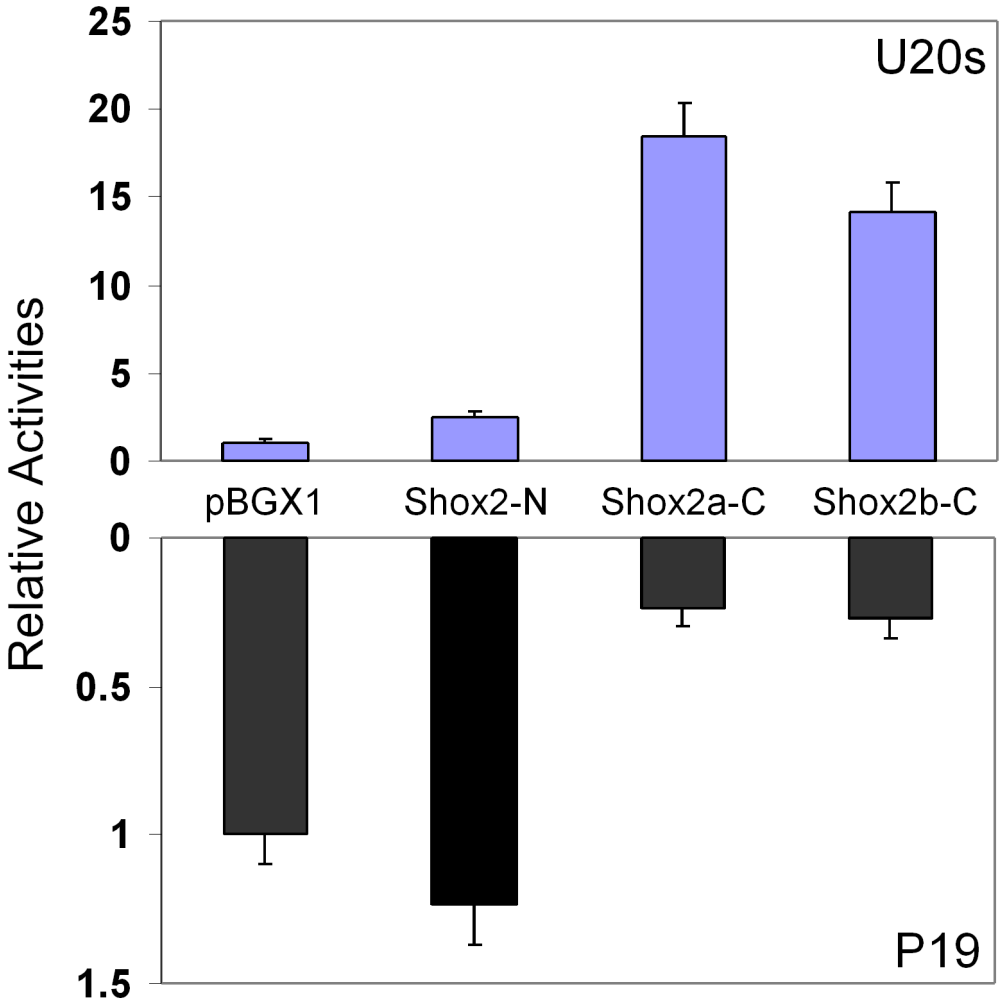

Figure 7.

Mapping of transcriptional regulatory domain of Shox2. In U20s cells, co-trasnfection of the pG_5ECAT reporter plasmid with the indicated constructs demonstrates that the C-termini of Shox2a and Shox2b have strong transactivating potential, while the N-terminus of Shox2a does not affect reporter gene expression, as measured by the CAT activities. pBGX1 is used as control. In contrast, the C-termini of both Shox2a and Shox2b but not the N-terminus, after co-transfection with the pG5tkCAT reporter plasmid, repress reporter gene expression in P19 cells.