The homeobox transcription factor Barx2 regulates chondrogenesis during limb development

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Summary

Among the many factors involved in regulation of chondrogenesis, bone morphogenetic proteins (BMPs) and members of the Sox and homeobox transcription factor families have been shown to have crucial roles. Of these regulators, the homeobox transcription factors that function during chondrogenesis have been the least well defined. We show here that the homeobox transcription factor Barx2 is expressed in primary mesenchymal condensations, digital rays, developing joints and articular cartilage of the developing limb, suggesting that it plays a role in chondrogenesis. Using retroviruses and antisense oligonucleotides to manipulate Barx2 expression in limb bud micromass cultures, we determined that Barx2 is necessary for mesenchymal aggregation and chondrogenic differentiation. In accordance with these findings, Barx2 regulates the expression of several genes encoding cell-adhesion molecules and extracellular matrix proteins, including NCAM and collagen II (*Col2a1*) in the limb bud. Barx2 bound to elements within the cartilage-specific *Col2a1* enhancer, and this binding was reduced by addition of Barx2 or Sox9 antibodies, or by mutation of a HMG box adjacent to the Barx2-binding element, suggesting cooperation between Barx2 and Sox proteins. Moreover, both Barx2 and Sox9 occupy *Col2a1* enhancer during chondrogenesis in vivo. We also found that two members of the BMP family that are crucial for chondrogenesis, GDF5 and BMP4, regulate the pattern of Barx2 expression in developing limbs. Based on these data, we suggest that Barx2 acts downstream of BMP signaling and in concert with Sox proteins to regulate chondrogenesis.

Key words: Barx2, GDF5, Limb development, Adhesion, Chondrogenesis, Joint, BMP, Mouse

Introduction

Development of the vertebrate limb involves a complex cascade of events, including growth factor signaling, regulation of transcription and expression of target genes. These molecular interactions result in the correct positioning and differentiation of skeletal elements (Cohn et al., 1995; Moftah et al., 2002; Sanz-Ezquerro and Tickle, 2003; Shum et al., 2003; Spitz and Duboule, 2001). Chondrogenesis is an essential process during skeletogenesis because primary cartilaginous elements are formed that are ultimately replaced by bone (Erlebacher et al., 1995). Despite the importance of this process, the sequences of events and molecular mechanisms regulating chondrogenesis have not been fully defined.

Several studies indicate that chondrogenesis comprises three main steps: chondrogenic lineage commitment, mesenchymal cell condensation and differentiation into cartilage. During the condensation phase, mesenchymal cells aggregate to form chondrogenic and non-chondrogenic cell populations. This step involves differential regulation and synthesis of specific adhesion molecules that change adhesive properties and mediate cell sorting (Cottrill et al., 1987; Ide et al., 1994; Tavella et al., 1994). After mesenchymal condensation is complete, the prechondrogenic cells differentiate further to produce specific extracellular matrix components, such as collagen II and aggrecan, both of which are classic markers of chondrogenesis.

Many different signaling molecules are involved in regulation of chondrogenesis; however, bone morphogenetic proteins (BMPs) and members of the Sox and homeobox transcription factor families play central roles (Bi et al., 1999; Yi et al., 2000; Zhao et al., 1997). In developing limbs, BMPs and BMP-related molecules, such as the growth and differentiation factors (GDFs), have been implicated in the establishment of limb axes, chondrogenesis, osteogenesis and tissue patterning by apoptosis (Hoffmann and Gross, 2001; Macias et al., 1997; Niswander, 2002; Pizette et al., 2001; Tang et al., 2000; Yi et al., 2000). BMPs induce the expression of specific markers of chondrogenesis (Enomoto-Iwamoto et al., 1998; Shea et al., 2003; Tsumaki et al., 2002) via a transcriptional cascade that often involves other regulators such as Sox and homeobox proteins (Baur et al., 2000; Boulet and Capecchi, 2004; Zhang et al., 2000).

Sox proteins belong to the high-mobility group (HMG) DNA-binding family of transcription factors. Members of this family bind to and activate chondrocyte-specific enhancers in genes encoding the various collagens (Bi et al., 1999; Lefebvre et al., 1996; Zhou et al., 1998). In particular, Sox9, the first transcription factor to specify the chondrogenic lineage, plays

a crucial role in chondrogenesis through activation of the collagen II (*Col2a1*) and collagen alpha2(XI) genes (*Col11a2*) (Bi et al., 1999).

The first intron of the rat *Col2a1* gene contains a 620 bp chondrocyte specific regulatory enhancer (Horton et al., 1987) and two shorter overlapping fragments of this enhancer direct chondrocyte-specific expression in transgenic mice (Bell et al., 1997; Zhou et al., 1995). Moreover, a 48 bp sequence within the region of overlap between these two fragments that contains three HMG motifs is sufficient to confer chondrocyte-specific expression in cell lines (Lefebvre et al., 1996; Zhou et al., 1998). HMG motifs bind to members of the Sox family and various Sox proteins, including Sox9, can activate this enhancer (Zhou et al., 1998). However, multiple copies of the 48 bp sequence are required to give the same level and pattern of expression as the larger *Col2a1* enhancer fragment in cell lines and transgenic mice (Lefebvre et al., 1996), suggesting that other regions of the *Col2a1* enhancer or promoter may contribute significantly to the level and pattern of *Col2a1* expression in vivo.

The *Col2a1* gene contains many other potential transcription factor recognition motifs in addition to those for Sox proteins (Ala-Kokko et al., 1995; Ghayor et al., 2000; Huang et al., 2002; Kamachi et al., 1999; Murray et al., 2000). However, the identities most of the factors that bind to these motifs and their possible interactions with Sox proteins have not been elucidated. It is known that homeobox transcription factors, including members of the Hox, Msx and Dlx families, coordinate the expression of genes that are essential for differentiation of skeletal elements (Ferrari et al., 1994; Ferrari et al., 1995; Rogina et al., 1992; Satokata et al., 2000). Several lines of evidence also indicate that homeobox proteins are important transducers of BMP signaling pathways during chondrogenesis (Xu et al., 2001). For example, activation of the collagen II gene (*Col2a1*) enhancer in a chondroblast cell line by BMP2 is eliminated by antisense oligonucleotides against the mRNA encoding the homeobox factor Dlx2, suggesting that Dlx2 acts downstream of BMP signals (Xu et al., 2001). Whether homeobox transcription factors act cooperatively or in parallel pathways with Sox proteins remains unknown.

The homeobox transcription factor Barx2 regulates the expression of cell adhesion molecules (CAMs) including NCAM (Edelman et al., 2000a; Meech et al., 1999; Meech et al., 2003) and cadherin 6 (Sellar et al., 2001), suggesting that it can influence processes such as cell aggregation, formation of intercellular contacts and cell fusion. Our previous work also indicates that Barx2 is involved in limb development. For example, we found that Barx2 is required for myotube formation in limb bud cultures and that overexpression of Barx2 accelerates the fusion of both C2C12 and embryonic limb myoblasts (Meech et al., 2003).

In this study, we report that Barx2 is expressed during limb development in patterns that suggest a role in chondrogenesis. Barx2 is necessary for the formation of primary mesenchymal aggregations and for cartilage differentiation in limb bud cultures, and it regulates the expression of several genes encoding CAMs and extracellular matrix proteins, including NCAM, tenascin C and collagen II. We identify two conserved binding sites for Barx2 within the *Col2a1* intronic enhancer and show that addition of Sox9 antibodies, or disruption of an adjacent HMG-box, reduces Barx2 binding, suggesting cooperation between Barx2 and Sox9. In addition, we show that the BMP family members BMP4 and GDF5 regulate Barx2 expression in the developing limb. Overall, these data suggest that Barx2 provides a crucial link between BMP signaling and mesenchymal condensation and differentiation, and that it acts concert with other BMP targets such as Sox9 to directly regulate the expression of chondrogenic genes.

Materials and methods

Limb bud micromass cultures

Embryos were obtained from timed pregnant CD1 mice (Harlan laboratories). Micromass cultures were prepared from limbs at E10-11.0 as described (Vogel and Tickle, 1993). Cells were plated as micromass cultures at a density of 2×10^7 cells/ml and maintained for 2-5 days in CMRL-1066 medium (Invitrogen) supplemented with 2% FCS and glutamine.

Cell adhesion assays

D1 cells (ATCC, CRL-12424) were transfected with pcDNA3 or with pcDNA3 containing a mouse full-length Barx2 cDNA and cell aggregation assays were performed (Kawano et al., 2002). Briefly, for the Ca^{+2}-dependent assays, cells were incubated for 20 minutes at 37°C in Ca^{+2}, Mg^{+2}-free HEPES-buffered saline (HCMF) containing 0.01% trypsin (type XI, Sigma) and 10 mM $CaCl_2$. Cells were washed and resuspended at a density of 2×10^5 cells/ml in DMEM-Hanks solution with 1 mM $CaCl_2$. Cells were transferred to 24-well plates coated with 1% bovine serum albumin (Sigma) and incubated for 15-30 minutes at 37°C with constant rotation at 40 rpm. To evaluate cell aggregation, cells were compared at 0 and 30 minutes incubation. For the Ca^{+2}-independent assay cells were treated for 20 minutes with 2mM EDTA in HCMF, then dissociated into a single cell suspension in Hanks solution with 1 mM EDTA and 2% FBS. Cell aggregation assay was performed as described above. The extent of aggregation was determined by the measuring the appearance of aggregates larger than 30 μm using a Beckman Coulter Counter (Fullerton, CA).

Antisense inhibition of Barx2 expression in limb bud micromass cultures

To examine the role of Barx2 in mesenchymal condensation and chondrogenesis, we used morpholino oligodeoxynucleotides (ODNs) (Heasman, 2002; Summerton, 1999; Summerton and Weller, 1997). Antisense and control Barx2 ODNs were synthesized by Gene Tools (Corvalis, OR). ODNs (2-5 μM) were added to micromass cultures using the osmotic delivery system recommended by the manufacturer or using Lipofectamine 2000 (Invitrogen). Cultures were maintained for 48-96 hours and ODNs were replaced every 6-8 hours to increase the efficiency of antisense treatment. After 48 hours, the number of mesenchymal aggregates was assessed and 24-48 hours later, cultures were fixed in 2% PFA in PBS with 0.05% Triton X-100, and stained with Alcian Blue to assess the extent of chondrogenesis.

Retroviral construction and packaging

Retroviral vectors were constructed that contained the enhanced green fluorescent protein (EGFP) gene, full-length mouse Barx2 cDNA, or cDNA fragments that expressed the following combinations of Barx2 protein domains: the homeodomain and Barx basic region (HD-BBR); or the homeodomain, Barx basic region and C-terminal activation domain (HD-BBR-C) (Edelman et al., 2000a). The retroviral vector was based on the murine embryonic stem cell (MESV) virus with modifications (Owens et al., 2002). Retroviral particles were packaged in COS1 cells (Edelman et al., 2000b). Supernatant containing retroviral particles was collected after 48 hours, filtered and used to infect primary cells in suspension.

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Retroviral transduction in micromass cultures

Retroviral infection of micromass cultures was carried out as described (Stott and Chuong, 1997). Infected cells were cultured for an additional 2-4 days, fixed in 4% paraformaldehyde (PFA) in PBT (PBS with 0.05% Triton) and processed for immunostaining or stained with Alcian Blue to quantify levels of chondrogenesis and to examine nodule formation. At least five micromass cultures infected with each retroviral construct were analyzed for each experiment. Each experiment was repeated three or four times, yielding similar results.

Alcian blue staining and quantitation of chondrogenesis

Micromass cultures were fixed with 2% PFA, washed in PBT and stained with 1% Alcian Blue 8GX (Sigma) in 0.1 N HCl, pH 1 for 5 hours (Lev and Spicer, 1964). Cultures were then de-stained with 70% ethanol. Alcian Blue incorporated into the cell matrix was extracted with 0.5 ml of 4 M guanidine HCl (pH 5.8), and quantified by measuring absorbance at $OD_{600\ nm}$ (Lev and Spicer, 1964). The statistical significance of the difference in Alcian Blue staining between control and experimental micromass cultures after antisense treatment or retroviral delivery of Barx2 constructs was assessed using the nonparametric Wilcoxon signed rank test (Ostle, 1975). A value of $P<0.01$ was considered to reflect a statistically significant difference.

Whole limb cultures

Limbs were dissected from E11 and E12.5 embryos, placed on a 0.8 µm Millipore filter supported by a metal grid, and cultured in Fitton-Jackson modified BGJb medium (Invitrogen) supplemented with glutamax, human transferin 40 µg/ml, 1× insulin/transferin selenium, and albumax I 50 µg/ml (Invitrogen). The limbs were incubated at 37°C, in 5% CO_2 and the culture medium was changed daily.

Bead implantation

Heparin acrylic beads (150-200 µm, Sigma) were washed in PBS and soaked in recombinant human BMP4 (Genetics Institute) or recombinant mouse GDF5 (#853-G5, R&D Systems) at concentrations of either 200 or 500 µg/ml for 3 hours at 4°C. Control beads were soaked in bovine serum albumin (BSA) in PBS. BMP4-, GDF5- or BSA-loaded beads were implanted in the distal interdigital mesenchyme of mouse fore- and hindlimbs at E12.5. Limbs with implanted beads were cultured for 48 hours, fixed in 4% PFA and processed for in situ hybridization or immunohistochemistry.

Histology and immunohistochemistry

Frozen sections and micromass cultures were stained as described previously (Makarenkova et al., 1997) using the following antibodies: mouse monoclonal antibody to collagen II (Abcam, Clone 5B2.5, AB 3092); rabbit polyclonal antibody to Barx2 (Santa Cruz Biotechnologies, M-186, sc-9128); and rabbit polyclonal antibody to collagen type II (Chemicon, ab-2031). Alexa- or rhodamine-conjugated antibodies (Molecular Probes) were used as secondary antibody. Nuclei were stained with Oli-Green. ProLong anti-fade reagent was used to reduce sample fading (Molecular Probes).

In situ hybridization

For in situ hybridization, embryos were fixed overnight in 4% PFA at 4°C. Antisense RNA probes were labeled with digoxigenin and whole-mount in situ hybridization was performed as described (Nieto et al., 1996). The mouse GDF5 probe was generated from a 493 bp RT-PCR product (bases 437-930 of the mouse GDF5 coding region) and was cloned into pCR™II-TOPO (Invitrogen). The mouse Barx2 probe contained a 431 bp *Eco*RI-*Pst*I fragment from within the Barx2 coding region (Jones et al., 1997).

Limb bud nuclear extract preparation and gel mobility-shift assays

Double-stranded oligonucleotide probes were prepared that correspond to the HBS and HMG motifs within the *Col2a1* intronic enhancer (Lefebvre et al., 1996). Probes containing point mutations were also generated (see Fig. 7A,B). Limb nuclear extract was prepared from E12.5 embryonic limbs as described previously (Schreiber et al., 1989). The gel mobility-shift assays were performed as described (Edelman et al., 2000a). DNA/protein complexes were then resolved by electrophoresis on an 8% native polyacrylamide gel at 4°C. Gels were dried and visualized using a PhosphorImager (Molecular Dynamics).

Chromatin immunoprecipitation (ChIP)

Limb mesenchymal cells, D1 and C3H10T1/2 cell lines were used for ChIP. Limb mesenchymal micromass cultures were prepared and maintained as described above. Cell lines were grown in DMEM/10% FBS, transfected with Myc-tagged Barx2 and/or Flag-tagged Sox9 pcDNA3 plasmids using Lipofectamine-2000 (Invitrogen) and then grown for additional 48 hours. ChIP was performed as previously described (Stevens et al., 2004). Twenty-five percent of the precleared chromatin was set aside (input control) and the reminder was split into two tubes one of which was precipitated with specific antibody and another with an irrelevant antibody or with preimmune serum (negative control). Both samples were treated identically in every other respect. Barx2 (Santa Cruz Biotechnology) or Phospho-Sox9 (Abcam) antibodies were used to precipitate endogenous Barx2 and Sox9; Flag (Sigma, clone M2) or Myc-tag (Santa Cruz, Biotechnology) antibodies were used to precipitate transfected Myc- and flag-tagged proteins. Enrichment of genomic DNA corresponding to the *Col2a1* intronic enhancer was determined by PCR amplification of equal aliquots of ChIP-derived DNA using specific primers (forward, 5′-GCTTTTTAATTGGCCGGCCACAAGGAATCAC-3′; reverse, 5′- GGTGCGGTCCGAAGAAGCCAGCAC-3′).

RT-PCR analysis of micromass cultures

Micromass cultures infected with EGFP- and HDBBRC-expressing retroviruses were harvested after 4 days and total RNA was prepared using Trizol (Gibco). Each RNA (5 µg) sample was DNAse treated with DNA-free (Ambion), and reverse transcribed using Superscript reverse transcriptase (Invitrogen) and random hexamer primers. PCR amplification was performed using a Lightcycler (Roche) and the Roche HotStart Master SYBR-green kit following the manufacturer's instructions. Primer sequences are as follows: mouse *Col2a1*, forward 5′-GAACCCAGAAACAACACAATCC-3′ and reverse 5′-GTTC-GGACTTTTCTCCCCTC-3′; mouse cyclophillin, forward 5′-CCAAAGACCACATGCTTGCCATCC-3′ and reverse 5′-TGG-TCAACCCCACCGTGTTCTTCG-3′.

The relative abundance of the cDNAs representing the *Col2a1* and cyclophillin transcripts were determined using a standard curve for each primer set as described (Stevens, 2004). The concentration of *Col2a1* cDNA was normalized to the concentration of the housekeeping gene cyclophillin cDNA in each same. To determine the influence of retroviral treatment on the expression of *Col2a1*, the relative expression of *Col2a1* in the HDBBRC-retrovirus treated sample was divided by the relative expression of *Col2a1* in the control-retrovirus treated samples. Mean and s.e.m. values were determined from three independent retroviral-transduction experiments. A melting curve analysis was performed for each sample after PCR amplification to ensure that a single amplification product was obtained.

Results

Barx2 is expressed in developing mouse limbs

Between E11.0 and 11.5, however, it is expressed in the conjunctival epithelium around the eye, submandibular gland primordium, external ear and developing limbs (Fig. 1A). In the limbs at E11.5 Barx2 is expressed in chondrogenic

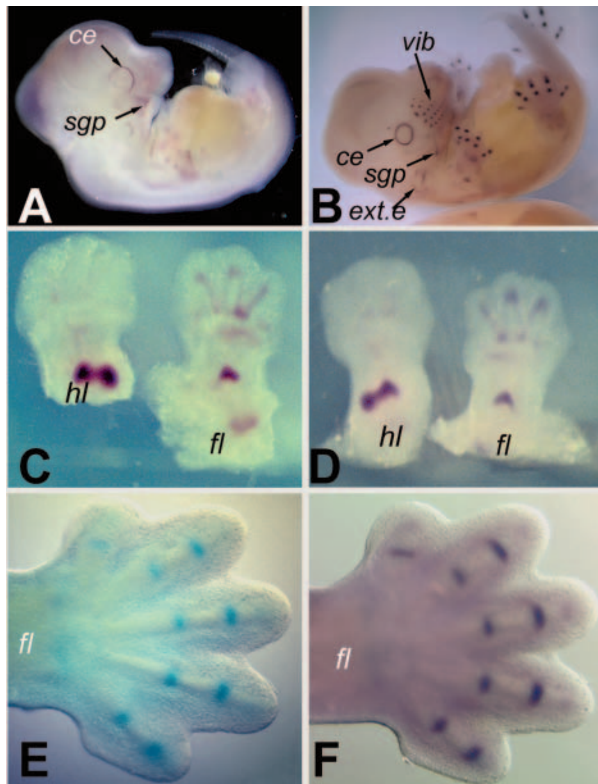

Fig. 1. Expression of Barx2 and GDF5 during mouse embryogenesis and limb development. Barx2 mRNA expression at E11.5 (A) and E13.5 (B). Barx2 (C,E) and GDF5 (D,F) are expressed in a similar pattern in the prechondrogenic condensations and in the digital rays at E11.5 (C,D) and in phalangeal joints at E13.5 (E,F). ce, conjunctival epithelium; sgp, submandibular gland primordium; vib, vibrissa; ext.e, external ear; fl, forelimb; hl, hindlimb.

condensations and in the digital rays (Fig. 1C; Fig. 2A,D) and during the development of digits (E12.5 to 16.0) the mRNA is highly expressed in the joint interzone and articular cartilage of the carpal and phalangeal joints (Fig. 1B,E; Fig. 2B,C,E,F). At E12.5 Barx2 continues to be expressed in the conjunctival epithelium around the eye, submandibular gland primordium, developing vibrissa and the external ear (Fig. 1B). The expression patterns of Barx2 and GDF5 in the limb between E11.5 and E13.5 are very similar (compare Fig. 1C,D with 1E,F) and they genes are co-expressed between E12.5 and 13.5 (Fig. 1E,F). This finding suggests a possible functional connection between these two molecules.

At E11.5 Barx2 protein is expressed in the nuclei of condensing pre-chondrogenic mesenchyme expressing collagen II (Fig. 2A,D). At E12.5 and E14.5 (Fig. 2B,E), chondrogenic cells of digital ray condense to form future joint interzone. Barx2 expression is strongly upregulated in the forming interzone (Fig. 2B,E); at the same time Barx2 continues to be expressed throughout the digital ray. Collagen II expression is almost absent in the joint interzone at E12.0-E13.4; however, collagen II continues to be expressed in the digital ray and in the flattened cell layers of the interzone (Fig. 2E). Later in development at E20-21 and in newborn mice (Fig. 2C,F), Barx2 and collagen II are expressed in the perichordium, developing joint capsula and joint surfaces. Overall, the expression patterns of Barx2 strongly suggest that it has role in the regulation of chondrogenesis and articular cartilage formation.

Inhibition of Barx2 expression by morpholino antisense oligonucleotides blocks mesenchymal aggregation and chondrogenic differentiation of limb bud micromass cultures

To determine whether Barx2 functions during primary mesenchymal condensation and chondrogenic differentiation, its expression was inhibited in limb bud micromass cultures using morpholino-modified antisense Barx2 oligonucleotides (ODNs). Treatment of micromass cultures with 5 μM Barx2 antisense ODNs (Fig. 3A) significantly reduced Alcian Blue staining of cartilaginous matrix, whereas similar treatment with sense and control ODNs showed little or no change. To quantify the inhibition of chondrogenesis by antisense Barx2 ODNs, the Alcian Blue that had incorporated into micromass cultures was extracted and quantified in five separate experiments. As shown in Fig. 3B, Barx2 antisense ODNs reduced Alcian Blue staining by 90% relative to control cultures. These results indicate that expression of Barx2 is required for chondrogenic differentiation of primary limb bud micromass cultures.

Barx2 promotes aggregation of mesenchymal cells

To study the role of Barx2 in regulation of cell aggregation we

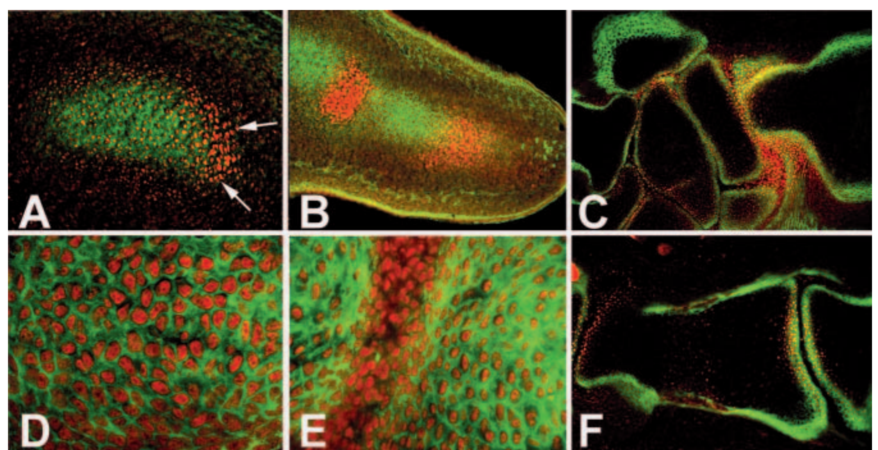

Fig. 2. Barx2 (red) and collagen II (green) protein expression during limb development. At E11.0, Barx2 and collagen II are co-expressed in mesenchymal condensations (A,D). Barx2 expression precedes collagen II expression in the chondrogenic front (A, arrows). At E12.5, Barx2 and collagen II continue to be expressed along the developing digit. Barx2 expression is upregulated in the cell layer marking the joint interzone (B,E), whereas collagen II expression is downregulated in the developing joint. Barx2 and collagen II are co-expressed in the developing articular cartilage of carpal (C) and phalangeal (F) joints in newborn mice. (D) A higher magnification of A. (E) A higher magnification of B.

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performed Ca^{+2}-dependent and Ca^{+2}-independent aggregation assays in D1 cells after transfection of a Barx2 expression construct. D1 cells are bone marrow derived and can differentiate into chondrocytes under appropriate conditions. The extent of aggregation was determined at 0 or 30 minute time points by appearance of aggregates greater than 30 μm measured using a Coulter counter. The aggregation of Barx2 transfected cells in both assays was significantly accelerated compared with control pcDNA3 transfected cells (Fig. 4A). However, the effect of Barx2 was greater in Ca^{+2}-dependent assays (Fig. 4B). Overall, these data indicate that Barx2 is involved in regulation of Ca^{+2}-independent and Ca^{+2}-dependant adhesion.

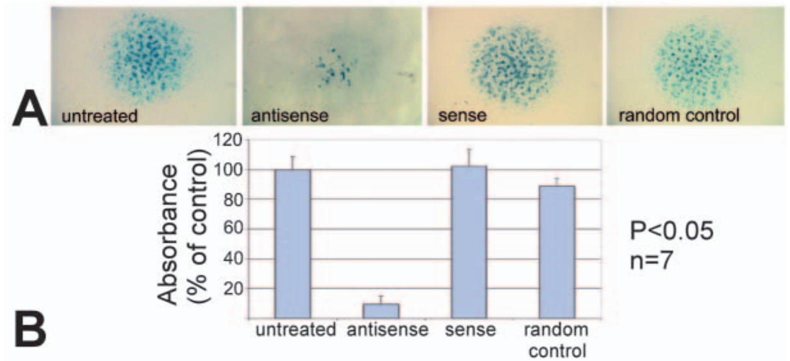

Fig. 3. (A) Morpholino Barx2 antisense ODNs dramatically reduces nodule formation compare to untreated cultures or cultures treated with sense or random control ODNs. (B) Quantitation of Alcian Blue staining in micromass cultures shows that Barx2 antisense ODNs result in a 90% reduction of Alcian Blue staining; *n*, number of experiments.

Barx2 promotes chondrogenic differentiation in micromass cultures of distal limb bud mesenchyme

To determine which regions of Barx2 protein are required for chondrogenesis, three retroviral constructs (Fig. 5A) containing different regions of the Barx2 protein were tested for their ability to promote chondrogenesis in limb bud micromass cultures: Barx2 encoded the full-length protein, HD-BBR encoded the DNA-binding region (the homeodomain and the BBR), HD-BBR-C encoded the C-terminal activation domain as well as the DNA-binding domain (Edelman et al., 2000a), and a control vector was made that expressed EGFP. Micromass cultures prepared from E10.0-11.0 mouse limb buds were infected with each of these constructs and the extent of chondrogenesis was measured after 4 days using quantitative Alcian Blue staining.

As shown in Fig. 5B, expression of Barx2 or HD-BBR-C proteins promoted chondrogenic condensation and increased Alcian Blue staining in micromass cultures by five- and sixfold, respectively (Fig. 5C), whereas the EGFP and HD-BBR constructs had little effect. These findings indicate that both the DNA-binding region and the C-terminal activation domain are required to stimulate chondrogenesis.

Barx2 does not promote chondrogenesis of mesenchymal cells from proximal regions of the limb bud

During limb development, specification of proximal mesenchyme precedes the specification of distal mesenchyme, which is in contact with the apical ectodermal ridge (AER) (Dudley et al., 2002; Muneoka et al., 1989; Vargesson et al., 1997). To determine whether Barx2 could influence the differentiation of proximal limb bud mesenchyme, we compared the effect of Barx2 expression on chondrogenic differentiation in micromass cultures prepared from the proximal and distal regions of the limb bud at E11.0. As shown in Fig. 5D, none of the Barx2 constructs induced chondrogenesis in micromass cultures prepared from the proximal region of the limb bud, whereas micromass cultures made from the distal mesenchyme of the same limb bud showed a fivefold increase in chondrogenesis after overexpression of Barx2. These results suggest that Barx2 can promote chondrogenesis only in the distal mesenchyme of the limb bud, which contains mostly uncommitted cells (Dudley et al., 2002).

Barx2 regulates expression of Collagen II in limb bud micromass cultures and binds to regulatory sequences of the Col2a1 enhancer

The results described above show that ectopic expression of Barx2 in limb bud micromass cultures accelerates chondrogenic differentiation. To determine whether this effect involves regulation of the *Col2a1* gene, we first examined *Col2a1* mRNA levels. There was a nearly fivefold upregulation of *Col2a1* mRNA levels ($P<0.05$; $n=4$) in Barx2 retrovirus infected cultures, relative to controls (Fig. 6A), suggesting that Barx2 can activate the *Col2a1* gene. In addition, immunostaining of limb bud cultures treated with Barx2 retroviruses also showed an increase in collagen II protein expression compared with control (Fig. 7B).

It has been shown previously that two intronic regulatory

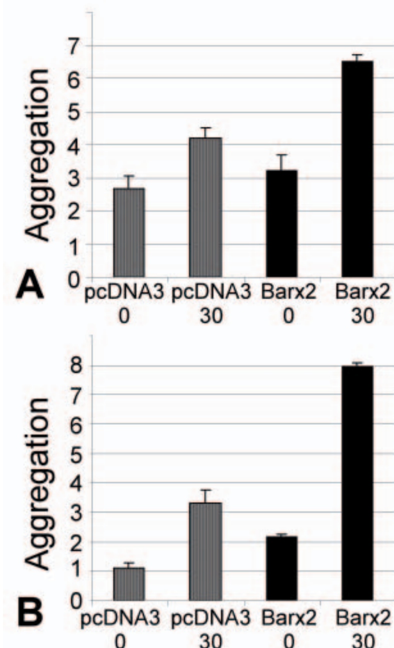

Fig. 4. Barx2 increases cell aggregation in Ca^{+2}-independent (A) and Ca^{+2}-dependant (B) aggregation assays.

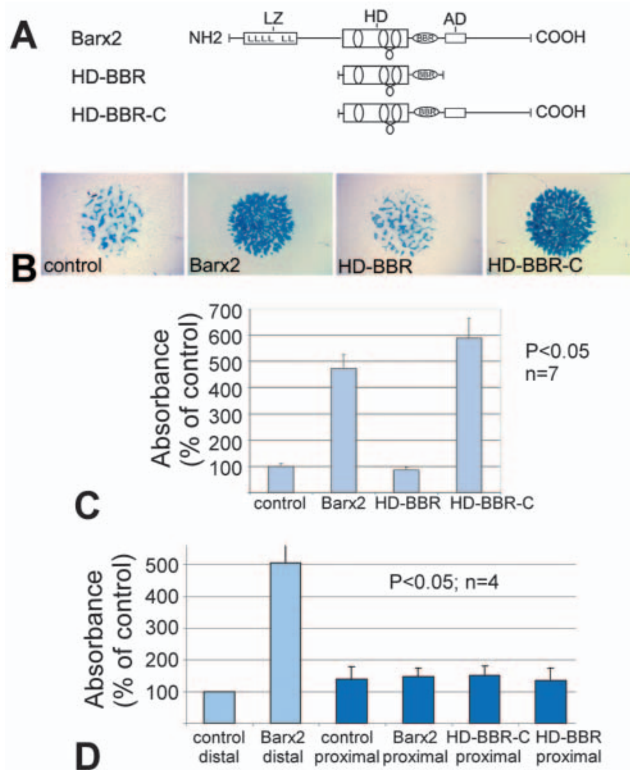

Fig. 5. Retroviral expression of Barx2 and HD-BBR-C proteins in limb bud micromass cultures increases chondrogenic differentiation. (A) Three different Barx2 proteins encoded in retroviral vectors: the full-length Barx2 protein (Barx2) that contains the homeodomain (HD), Barx basic region (BBR) and an acidic activation domain (AD); the HD-BBR protein contains only the DNA-binding region of Barx2; and the HD-BBR-C protein, which contains the DNA-binding region as well as the C-terminal activation domain. (B) Expression of Barx2 or HD-BBR-C proteins, but not HD-BBR protein, induced chondrogenesis in limb bud micromass cultures as indicated by Alcian Blue staining. (C) Quantitative analyses of Alcian Blue incorporation in retroviral infection experiments; *n*, number of experiments. (D) Micromass cultures prepared from proximal limb bud mesenchyme show no response to overexpression of control, Barx2, HD-BBR-C or HD-BBR proteins, whereas distal limb bud cultures show a fivefold increase in Alcian Blue staining upon infection with the Barx2 retroviral construct; *n*, number of experiments.

regions of the *Col2a1* gene (468 and 309 bp, see Fig. 7A) are necessary and sufficient for chondrocyte-specific expression in cell cultures and transgenic mice (Bell et al., 1997; Zhou et al., 1995). These regions overlap in the *Col2a1* gene and include a 48 bp element containing three HMG-binding sites, one of which specifically binds to Sox9 (Zhou et al., 1998). Moreover, there is an additional Sox9-binding site 50 bp downstream of 48 bp regulatory element (designated HMG4 in Fig. 7A) (Bell et al., 1997). Barx2 is known to bind to DNA elements that contain the sequence TAAT (Edelman et al., 2000a). Alignment of mouse, human and rat intronic *Col2a1* gene sequences revealed two conserved putative homeodomain binding sites (HBS1 and HBS2) downstream of the 48 bp element (Fig. 7A) and an additional conserved putative HMG-binding site (designated HMG5 in Fig. 7A) adjacent to HBS1. To determine

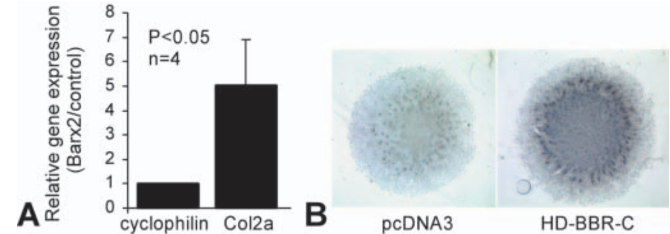

Fig. 6. Retroviral expression of Barx2 in limb bud micromass cultures increases *Col2a1* gene expression. (A) Barx2-retrovirus infection induced approximately fivefold increase in *Col2a1* mRNA relative to control infections as measured by real time PCR. *n*, number of experiments. (B) Expression of HD-BBR-C construct increases collagen II protein expression in micromass cultures.

whether the HBS1 and HBS2 elements bind to Barx2, two probes (designated B1 and B2, respectively, in Fig. 7A,B) were prepared and tested for their binding to in vitro translated Barx2 proteins in gel mobility-shift experiments. In vitro-synthesized Barx2 protein formed complexes with both probes complex formation was completely inhibited by addition of Barx2 antibodies (Fig. 8A). By contrast, Barx2 did not bind to mutated versions of the B1 and B2 probes in which core TAAT motifs were disrupted (Fig. 8A).

Additional binding experiments were performed with two other probes (B1 and B2), which contain mutations in each of the Barx2-binding sites and probes were tested for binding to nuclear extracts prepared from embryonic (E12.5) mouse limbs. E12.5 limbs contain high levels of both Barx2 and Sox9 proteins (Fig. 7C). The B1 and B2 probes both bound to limb nuclear extracts; mutation of the TAAT motifs within the probes abolished this binding (Fig. 8B,C). Addition of 2 μg of Barx2 antisera resulted in an ~50% reduction in the intensity of the B1- and B2-binding complexes (Fig. 8B), suggesting that a significant proportion of the DNA/protein complexes contain Barx2. An equal amount of a pre-immune rabbit serum did not reduce the intensity of the complexes (data not shown), indicating that the effect is specific to Barx2 antisera. Limb nuclear extracts formed much more intense complexes with the B1 probe (which contains both HBS and HMG motifs) than with the B2 probe (which contains only a HBS motif) (Fig. 8B,C). By contrast, complexes of similar intensity were formed with in vitro-translated Barx2 proteins (Fig. 8A). Together, these results suggest that Barx2, and possibly other homeodomain proteins in the limb extract, can cooperate with HMG proteins in binding to the *Col2a1* enhancer. This notion is further supported by experiments (described below) in which the HMG motif within the B1 probe was mutated.

The HMG site is important for binding Col2a enhancer by Barx2 and other homeodomain proteins

The consensus binding sequence for Sox proteins is (A/T)(A/T)-CAA(A/T)G (Lefebvre et al., 2001; Lefebvre et al., 1998; Zhao et al., 1997). The *Col2a* enhancer contains several canonical HMG-box elements that are conserved in the human, mouse and rat *Col2a* sequences (Fig. 7A). The HMG3 (Zhou et al., 1998) and HMG4 (Bell et al., 1997) elements have been shown to interact specifically with Sox9. We identified another conserved element that matched the HMG-box

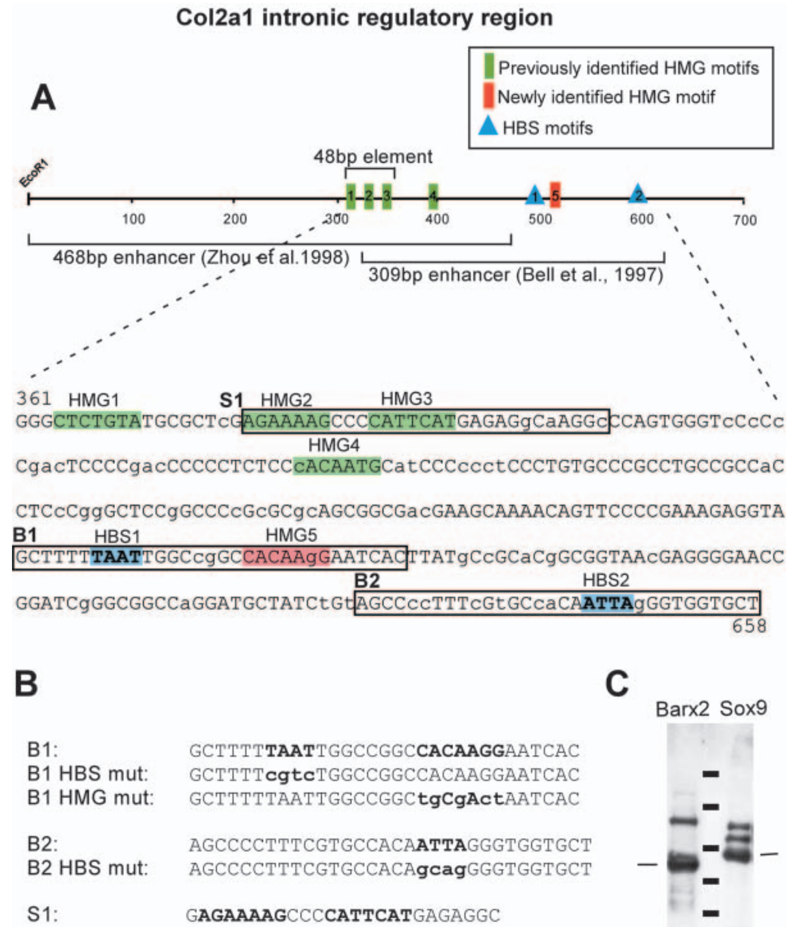

Fig. 7. Schematic of the intronic regulatory region of the *Col2a1* gene. (A) Two overlapping segments of 468 and 309 bp have previously been shown to drive chondrocyte specific expression in cell cultures and in transgenic mice (Bell et al., 1997; Zhou et al., 1995). These regions both include a 48 bp element containing three HMG-binding sites (HMG1, HMG2 and HMG3). Two additional HMG motifs (HMG4, HMG5) were identified downstream of this element. Two conserved putative homeodomain binding sites (designated HBS1 and HBS2) were identified by comparison of human and mouse genes. Boxes indicate the sequences used as probes in this study. (B) Sequences of wild-type and mutant probes. The core of binding motifs containing the sequence ATTA is indicated in bold. Mutations are indicated in lower case. (C) Western blot analysis shows that Barx2 and Sox9 are expressed in E12.5 limb nuclear extract.

consensus motif that we refer to as HMG5 (Fig. 7A). HMG5 is immediately adjacent to the HBS within the B1 probe, suggesting that it might influence binding of the probe to Barx2. Mutation of the HMG5 motif within the B1 probe reduced binding to embryonic limb nuclear extract compared with the intact B1 probe (Fig. 8C), suggesting that the HMG motif influences binding of Barx2 to this sequence and raising the possibility that Barx2 and other homeodomain proteins may bind cooperatively with Sox proteins to *Col2a1* regulatory regions.

To determine whether the DNA-protein complexes formed with the intact B1 probe contain Sox9, we tested the ability of Sox9 antibodies to block the formation of these complexes. A control probe (S1) was generated containing the HMG2 and HMG3 motifs that bind to Sox9. Nuclear extracts from embryonic limb formed several complexes with the S1 probe (Fig. 8B) in accordance with previous observations (Lefebvre et al., 1998). Addition of 2 μg of Sox9 antibody reduced the intensity of these complexes by ~50%, indicating that the antibody can partially block the binding of Sox9 to the S1 probe (Fig. 8B). Significantly, addition of 2 μg of Sox9 antibody also reduced the intensity of the complex formed with the B1 probe by ~50% (Fig. 8B); a reduction similar to that caused by addition of Barx2 antibody (Fig. 8B). By contrast, the complex formed with the B2 probe was reduced by the addition of Barx2 antibody, but not by the addition Sox9 antibody (Fig. 8B). These data suggest that Sox9 and Barx2 bind cooperatively to adjacent sites in the *Col2a1* enhancer.

Barx2 and Sox9 occupy Col2a1 intronic enhancer during limb chondrogenesis

Our studies indicate that Barx2 can regulate collagen II expression in the limb mesenchymal cells. We also found that Barx2 can bind to regulatory element in the *Col2a1* enhancer in vitro. To test whether Barx2 might be actively engaged at the *Col2a1* enhancer in vivo, we performed ChIP assays. Equivalent amount of crosslinked chromatin from limb mesenchymal cells was immunoprecipitated with Barx2 antibody (Santa Cruz Biotechnology) or with an irrelevant antibody or rabbit IgG (negative control). The precipitated DNA then was subjected to PCR amplification using primers that span the region of *Col2a1* enhancer containing putative Barx2-binding site. Antibodies to Barx2 immunoprecipitated this region of *Col2a1* enhancer from limb mesenchymal cells (Fig. 9A), whereas the irrelevant antibody or normal rabbit IgG did not (Fig. 9A, lane 3). This indicates that Barx2 binds to the enhancer in vivo. In addition, we examined whether BMPs, well-known regulators of chondrogenesis, can modulate Barx2 binding to the *Col2a1* enhancer. Replicate limb micromass cultures were prepared and treated with BMP4 (200 ng/ml) or BSA and the ChIP assay was performed. The amount of *Col2a1* enhancer DNA precipitated by Barx2 antibodies was greater after BMP treatment (Fig. 9B). These results indicate that Barx2 might be an important downstream mediator of BMP signaling during chondrogenesis. To verify the specificity of Barx2 association with the *Col2a1* enhancer region, we performed a ChIP assay with D1 and C3H10 T1/2 cells transiently transfected with Myc-tagged Barx2 and cultured under differentiation conditions. The Myc antibody was used to precipitate Barx2. As shown in Fig. 9A, Barx2 binds the core regulatory region of the *Col2a1* enhancer, while binding was not observed with rabbit IgG. In addition, we tested whether Sox9 can occupy the same region of the *Col2a* enhancer. An equivalent amount of chromatin from D1 cells co-transfected with Sox9-flag and Barx2-Myc expression vectors was immunoprecipitated in parallel with Flag, Phospho-Sox9 and Barx2 antibodies. As shown in Fig. 10C, Barx2 binds the regulatory region of *Col2a1* enhancer. Both Flag and Phospho-Sox9 antibodies precipitated the *Col2a1* enhancer sequence

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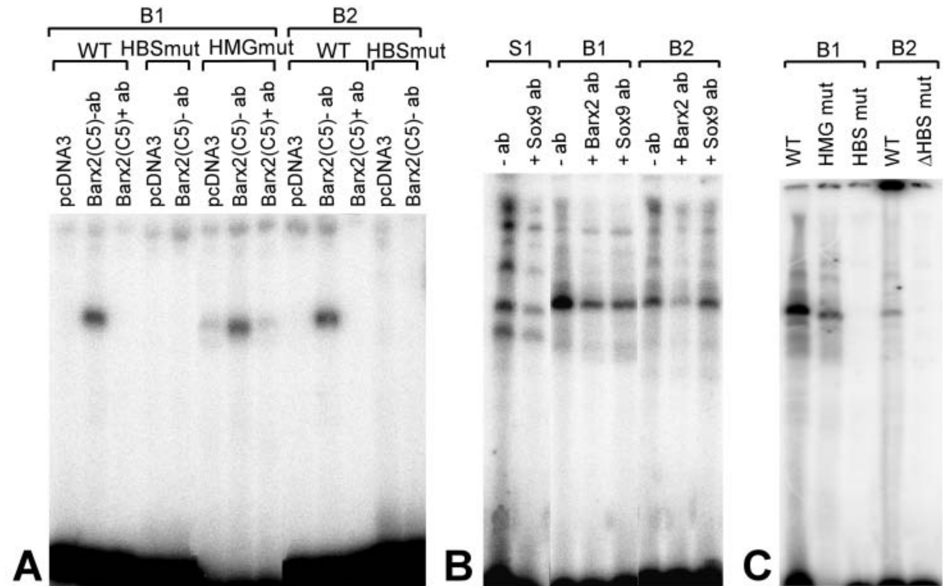

Fig. 8. Barx2 binds to HBS elements within the Col2a intronic enhancer. (A) In vitro translated Barx2 proteins (HD-BBR-C) were tested for binding to the B1 and B2 probes in gel mobility-shift experiments. HD-BBRC protein formed complexes with both the B1 and B2 probes that were completely blocked by anti-Barx2 antibody. HD-BBRC did not bind to B1 and B2 probes in which ATTA sequence was mutated (B1 HBS1 mut and B2 HBS2 mut). No complexes were formed with control pcDNA3 extract. HD-BBRC formed complexes of similar intensity with the B1 and B1 HMG5 mut probes. (B) Binding of the B1,B2 and S1 probes to nuclear extracts from E12.5 embryonic limbs. The B1 probe formed stronger complexes than either the B2 probe or the B1 HMG5 mut probe. Addition of Barx2 antibodies reduced binding to both probes, indicating that Barx2 is present in the complex. Addition of Sox9 antibodies reduced binding to the probes that contain HMG motifs (S1 and B1), suggesting that Sox9 is present in these complexes. Sox9 antibody did not affect binding to the B2 probe. (C) Mutation of HBS motifs in the B1 and B2 probes (B1 HBS1 mut and B2 HBS2 mut) abolished their binding to limb nuclear extracts, whereas, mutation of the HMG motif in the B1 probe (B1 HMG5 mut) reduced binding, suggesting that the HBS and HMG sites can interact.

(Fig. 9D), showing that Sox9 also binds this region of the *Col2a1* gene during chondrogenesis.

The results of these experiments suggest that Barx2 and Sox9 occupy the *Col2a1* enhancer in limb mesenchymal progenitor cells at the same stage of chondrogenic differentiation.

GDF5 and BMP4 application induce ectopic Barx2 expression in embryonic limbs

The implied role of BMP signaling in chondrogenesis and the observation that Barx2 and GDF5 are co-expressed during limb development prompted us to explore a functional connection between Barx2 and BMP signaling. Heparin acrylic beads were soaked in BSA (0.1%), BMP4 or GDF5 (200 and 500 μg/ml), and implanted into the distal interdigital region of E12.5 mouse fore- and hindlimbs. Limbs were cultured for an additional 48 hours and were examined for Barx2 expression by in situ hybridization or immunohistochemistry. Limbs implanted with BSA-soaked beads showed no changes in the expression of Barx2 mRNA or protein (Fig. 10A,D). By contrast, application of GDF5 or BMP4 beads induced ectopic expression of Barx2 mRNA and protein within the distal region of the limb bud (Fig. 10B,C,E,F). Barx2 expression was not detected in cells immediately adjacent to the GDF5 or BMP-soaked beads, but

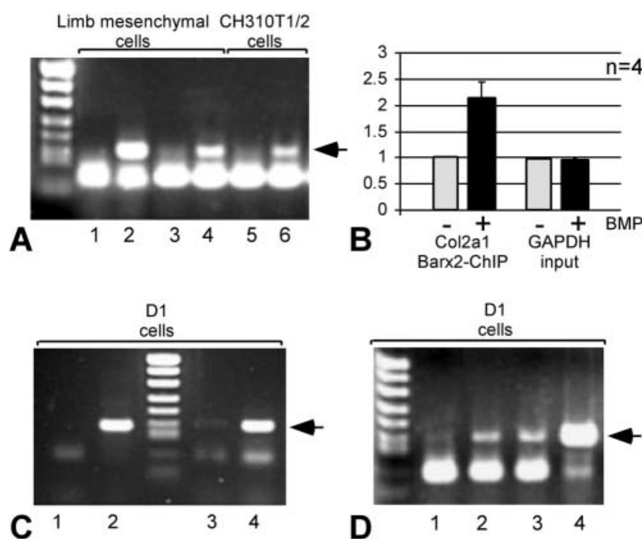

Fig. 9. Barx2 and Sox9 bind to endogenous *Col2a1* enhancer during chondrogenic differentiation. (A) Crosslinked chromatin from limb mesenchymal cells and C3H10T1/2 was precipitated with Barx2 or nonspecific antibody and analyzed by PCR using primers spanning the *Col2a1* enhancer. Lanes 1-6, amplification of *Col2a1* enhancer: 1, no Barx2 antibody added; 2, amplification of *Col2a1* enhancer from input DNA; 3, immunoprecipitation with non-specific antibody; 4, immunoprecipitation with Barx2 antibody; 5, immunoprecipitation with normal rabbit IgG; 6, immunoprecipitation with Barx2 antibody. (B) Barx2 has stronger association with *Col2a1* enhancer after the BMP treatment. Equivalent amount of crosslinked chromatin from treated with BMP and untreated limb mesenchymal cells were immunoprecipitated with Barx2 antibody and analyzed by PCR using primers spanning *Col2a1* regulatory region. *Gapdh* control confirming that equivalent amounts of chromatin were used in each ChIP assay. (C,D) During chondrogenic differentiation the *Col2a1* enhancer is occupied by both Barx2 (C) and Sox9 (D). D1 cells were simultaneously transfected with Myc-tagged Barx2 and Flag-tagged Sox9 expression vectors and cultured under differentiation conditions. Approximately equivalent amounts of crosslinked chromatin were immunoprecipitated in parallel with antibodies specific for Myc, flag, Phospho-Sox9 or normal rabbit IgG, and *Col2a1* enhancer region was amplified by PCR. (C) Lane 1, no antibody; 2, input DNA; 3, rabbit IgG; 4, Myc antibody. (D) Lane 1, negative control; 2, immunoprecipitation with Flag antibody; 3, with Phospho-Sox9 antibody; 4, positive control, input DNA. Arrows indicate *Col2a1* product.

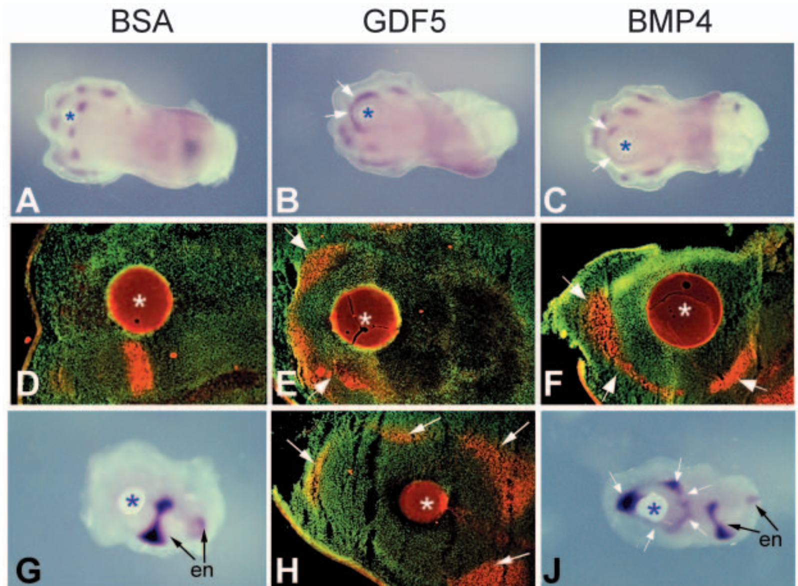

Fig. 10. Implantation of GDF5 (B,E,H) and BMP4 (C,F,J) beads (*) into the distal regions of E11.0-12.5 limbs results in ectopic expression of Barx2. mRNA and protein expression were visualized using in situ hybridization (A-C,G,J) and immunohistochemistry (D-F,H), respectively. Nuclei were stained with Oli-green. Arrows indicate ectopic Barx2 expression. Black arrows indicate endogenous Barx2 expression; white arrows indicate ectopic Barx2 expression; en, endogenous expression.

was observed only at a defined distance (~200 μm) from the bead, implying that a particular concentration of GDF5 or BMP4 may be required to induce the expression of Barx2. There was also a difference in the response of fore- and hindlimbs to GDF5 and BMP4 (Table 1). A higher proportion of limbs exhibiting induced expression of Barx2 were obtained when GDF5 or BMP4 beads were implanted in the hindlimb versus the forelimb (89% versus 40%) (Table 1). These results are consistent with the idea that the forelimb is more developmentally advanced than the hindlimb at this stage and thus less responsive to developmental signals. Taken together, these data suggest that GDF5 and BMP4 regulate Barx2 expression in limb bud mesenchyme in a stage-dependent manner.

To further explore this hypothesis, BMP4, GDF5 or BSA beads were implanted into the early E11.0 mouse limbs. Limbs were cultured for an additional 48 hours and Barx2 expression was studied by in situ hybridization. In cultured control limbs with or without implanted BSA beads, endogenous Barx2 expression was rapidly lost from the distal part of the limb, while proximal expression of Barx2 remained (Fig. 10G, en).

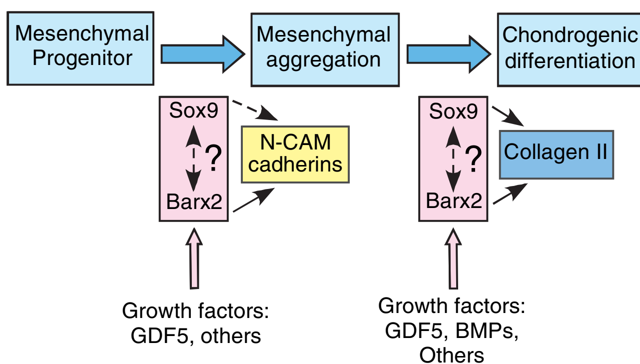

Fig. 11. A model of limb bud skeletal development showing molecules that may influence the various stages of cellular morphogenesis and differentiation. We propose that GDF5, Barx2 and Sox9 act at two stages of skeletal development: cellular aggregation and chondrogenic differentiation. By contrast, BMPs are most probably involved only in the second step of skeletal development – chondrogenic differentiation. Barx2 expression in the limb bud mesenchymal cells first promotes cellular aggregation by inducing the expression of certain cell-adhesion molecules. Later, Barx2 promotes chondrogenic differentiation via the regulation of the expression of collagen II and other genes. The nature of the relationship between Barx2 and Sox9 remains to be determined.

In contrast to controls limbs (Fig. 10G) and to the limbs at E12.5 (Fig. 10B,C), application of GDF5 or BMP4-soaked beads induced ectopic expression of Barx2 all around the bead (Fig. 10H,J).

Discussion

Chondrogenesis begins with formation of mesenchymal aggregations followed by differentiation of the condensed cells into chondrocytes that produce cartilage specific matrix proteins including collagen II. BMPs and Sox proteins are essential regulators of chondrogenesis (Tsumaki et al., 2002; Wagner et al., 1994; Zehentner et al., 1999), and the results presented here suggest that Barx2 is also necessary for chondrogenesis and interacts functionally with both BMPs and Sox9.

Barx2 regulates mesenchymal condensation and chondrogenic differentiation during limb development

Our experiments indicate that Barx2 is required for adhesion and aggregation of mesenchymal cells (Fig. 11). Several adhesion molecules, including NCAM and cadherins, particularly N-cadherin, have been implicated in the formation of mesenchymal aggregations, initiation of chondrogenesis and limb patterning (Tavella et al., 1994; Widelitz et al., 1993; Yajima et al., 2002). Our previous experiments showed that NCAM, which mediates Ca^{+2}-independent cell adhesion, is

Table 1. Ectopic Barx2 expression in E12.5 mouse limbs in response to GDF5 and BMP4

Protein	Forelimbs, ectopic Barx2 expression	(%)	Hindlimbs, ectopic Barx2 expression	(%)
BSA (0.1%)	0/12	0	0/8	0
GDF5 (200 μg/ml)	4/10	40	8/9	89
GDF5 (500 μg/ml)	6/11	54	7/8	88
BMP4 (200 μg/ml)	3/7	43	5/7	71
BMP4 (500 μg/ml)	2/5	40	3/4	75

regulated by Barx2 (Edelman et al., 2000a). However, although inhibition of Barx2 blocks condensation of mesenchymal cultures, condensation and chondrogenesis are not disrupted in NCAM knockout mice (Fang and Hall, 1999), suggesting that Barx2 must regulate additional CAMs or cadherins. The finding that Barx2 is involved in regulation of Ca^{+2}-dependant cell adhesion suggests that Barx2 can also modulate cadherin expression. This possibility is supported by a study showing that Barx2 regulates cadherin 6 in ovarian carcinoma cells (Sellar et al., 2001). However, further experiments are required to determine which cadherins are regulated by Barx2 in developing limb.

Collectively, our observations suggest that Barx2 influences condensation of limb bud mesenchyme via regulation of particular adhesion and matrix molecules. Interestingly, in the absence of Sox9, chondrogenesis is also blocked at the stage of cell condensation, indicating that both Barx2 and Sox9 are necessary for this process (Akiyama et al., 2002; de Crombrugghe et al., 2000). Whether Barx2 and Sox9 act coordinately or sequentially during condensation is as yet to be determined.

Barx2 and Sox9 regulate Col2a1 gene expression during chondrogenesis

Overexpression of Barx2 in micromass cultures increased Alcian Blue staining and nodule formation, suggesting that Barx2 controls chondrogenic differentiation. It is a formal possibility that Barx2 promotes chondrogenesis by inducing prechondrogenic condensation alone. However, our results suggest that Barx2 induces expression of the major cartilage matrix protein collagen II. We found that Barx2 binds to two different conserved HBS motifs in the cartilage-specific *Col2a1* enhancer region, and that overexpression of Barx2 in the limb mesenchymal progenitor cells can activate *Col2a1* gene expression. Previous studies showed that Sox9 activates this same enhancer, and identified at least two Sox9-specific HMG motifs (Bell et al., 1997; Zhou et al., 1998). We identified a fifth conserved element that matches the HMG-box consensus motif (HMG5), adjacent to one of the Barx2-binding sites (HBS1). We also found that Barx2 and Sox9 can occupy the same regulatory element of Col2a1 enhancer during chondrogenic differentiation. This and our other experiments showing that binding of limb nuclear proteins to the HBS1 element was reduced by mutation of this adjacent HMG motif and partially disrupted by addition of Sox9 antibodies, strongly suggests a functional interaction between Barx2 and Sox9.

GDF5 and BMP signaling regulates Barx2 expression during limb development

Previous and current studies indicate that both Barx2 and the closely related factor Barx1 are regulated by BMPs. We showed that BMP4 and GDF5 can induce ectopic expression of Barx2 in developing limbs and promote binding of Barx2 to Col2a1 enhancer during chondrogenesis. By contrast, BMP4 inhibits expression of Barx1 and restricts its expression to the proximal, presumptive molar mesenchyme of mouse embryo (Barlow et al., 1999; Tucker et al., 1998). This difference in responses to BMPs might be due to activation of various downstream signaling pathways in each cellular context or to interaction with other developmental signals (Yoon and Lyons, 2004). BMP signals are mainly mediated through ligand binding to receptors followed by activation of Smad proteins (Chen et al., 2004; Nishimura et al., 2003; Nohe et al., 2004). Recent studies have shown a distinct, structurally related class of SMADs which inhibits, rather than induces, TGFβ family signals (Christian and Nakayama, 1999; Nakayama et al., 1998).

BMPs are crucial regulators of chondrogenesis that increase condensation of limb mesenchyme and directly induce chondrogenic genes, including Sox9 (Zehentner et al., 1999). Moreover, mice carrying different combinations of mutations in the genes encoding BMPs, GDF5 and their receptors have more severe defects in limb development than mice carrying single mutations in these genes (Tsumaki et al., 2002; Vortkamp, 1997; Yi et al., 2000). This suggests that multiple BMPs and GDFs may have redundant or synergistic functions in the regulation of chondrogenesis and skeletal development.

In our experiments, we found that both BMP4 and GDF5 could induce Barx2 expression in cultured mouse limbs, indicating that various BMPs might regulate Barx2 expression at different stages of limb development. GDF5 has been reported to regulate both cellular condensation and chondrogenic differentiation in cultured limbs and in micromass cultures (Akiyama et al., 2000; Buxton et al., 2001; Francis-West et al., 1999; Hatakeyama et al., 2004; Spiro et al., 2001). BMPs (BMP2 and BMP4), however, appear to be crucial for later stages of skeletogenesis involving chondrogenic differentiation and skeletal patterning (Kameda et al., 2000; Tsumaki et al., 2002). Hence, GDF5 can induce chondrogenesis in mesenchymal cells that have not yet condensed, while BMPs induce chondrogenic differentiation only after condensation (Fig. 11). This conclusion is reinforced by observations that the effectiveness of BMPs in inducing chondrogenic differentiation in mesenchymal micromass cultures is greater in high-density cultures or after induction of cell-cell interactions (Denker et al., 1999). Based on these data and our own observations, we propose that GDF5 and BMPs act in a sequential manner to regulate Barx2 during mesenchymal condensation and chondrogenesis (Fig. 11).

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