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# SMAD-RUNX INTERACTIONS DURING CHONDROCYTE MATURATION

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**Background:** Intracellular signaling triggered by bone morphogenetic proteins (BMPs) results in activated Smad complexes that regulate transcription of BMP-responsive genes. However, the low specificity of Smad binding to regulatory sequences implies that additional tissue-specific transcription factors are also needed. Runx2 (Cbfa1) is a transcription factor required for bone formation. We have examined the role of Smads and Runx2 in BMP induction of type X collagen, which is a marker of chondrocyte hypertrophy leading to endochondral bone formation.

**Methods:** Pre-hypertrophic chondrocytes from the cephalic portion of the chick embryo sternum were placed in culture in the presence or absence of rhBMP-2. Cultures were transiently transfected with DNA containing the BMP-responsive type X collagen promoter upstream of the luciferase gene. The cultures were also transfected with plasmids, causing over-expression of Smads or Runx2, or both. After 24-48 hours, cell extracts were examined for levels of luciferase expression.

**Results:** In the presence of BMP-2, chondrocytes over-expressing BMP-activated Smad1 or Smad5 showed significant enhancement of luciferase production compared with that seen with BMP alone. This enhancement was not observed with over-expression of Smad2, a transforming growth factor beta (TGF- $\beta$ )-activated Smad. Over-expression of Runx2 in BMP-treated cultures increased transcriptional activity to levels similar to those seen with Smads 1 or 5. When chondrocytes were simultaneously transfected with both Runx2 and Smad 1 or 5, promoter activity was further increased, indicating that BMP-stimulated Smad activity can be augmented by increasing the levels of Runx2.

**Conclusions:** These results implicate the skeletal tissue transcription factor Runx2 in regulation of chondrocyte hypertrophy and suggest that maximal transcription of the type X collagen gene in pre-hypertrophic chondrocytes involves interaction of BMP-stimulated Smads with Runx2.

**Clinical Relevance:** Many skeletal abnormalities are associated with impaired regulation of chondrocyte hypertrophy in growth plates. These studies demonstrate that both BMP-activated Smads and Runx2 levels can modulate chondrocyte transition to hypertrophy.

The existence of bone morphogenetic proteins (BMPs) possessing the ability to induce ectopic bone formation was first suggested by the pioneering studies of Urist<sup>36</sup> over 30 years ago. With the cloning of BMPs, it became clear that they were a large family of growth factors from the transforming growth factor beta (TGF- $\beta$ ) superfamily. This is a group of growth factors with a unique intracellular signaling mechanism: activation of their receptors induces phosphorylation and activation of a group of intracellular transcription factors known as Smads (Fig. 1). Members of the TGF- $\beta$  superfamily bind to transmembrane cell surface receptors, which are heterodimers containing both type I and type II components. There are three general classes of receptors for members of the TGF- $\beta$  superfamily: one set for TGF- $\beta$ s, one set for activins, and one set for BMPs<sup>13</sup>. Ligand binding induces phosphorylation of a receptor dimer; this activates the type I receptor, which, in turn, phosphorylates a Smad. The receptor-activated Smad (R-Smad) then binds to a "co-

activator" Smad4 in the cytoplasm<sup>14,21</sup>. The activated Smad complex can then translocate to the nucleus where it participates in transcriptional regulation<sup>42</sup> (Fig. 1). The R-Smads include Smads 1, 2, 3, 5, and 8. Whereas TGF- $\beta$  and activin receptors induce Smad2 or Smad3 activation, or both, BMP receptors activate Smads 1, 5, or 8. Both signaling pathways are present not only in vertebrates but also in the invertebrate *Drosophila*<sup>1</sup>.

In cells of the osteoblast lineage, BMPs induce a high level expression of gene products characteristic of differentiated osteoblasts: type I collagen, alkaline phosphatase, and a variety of non-collagenous proteins found in osteoid. BMPs also act to promote hypertrophy of chondrocytes from cartilage regions destined for endochondral bone formation. They markedly stimulate expression of several hallmarks of hypertrophic chondrocytes: type X collagen, alkaline phosphatase, and matrix metalloproteinase-13<sup>24,38</sup>. However, the ability of BMPs to induce the hypertrophic phenotype is restricted to

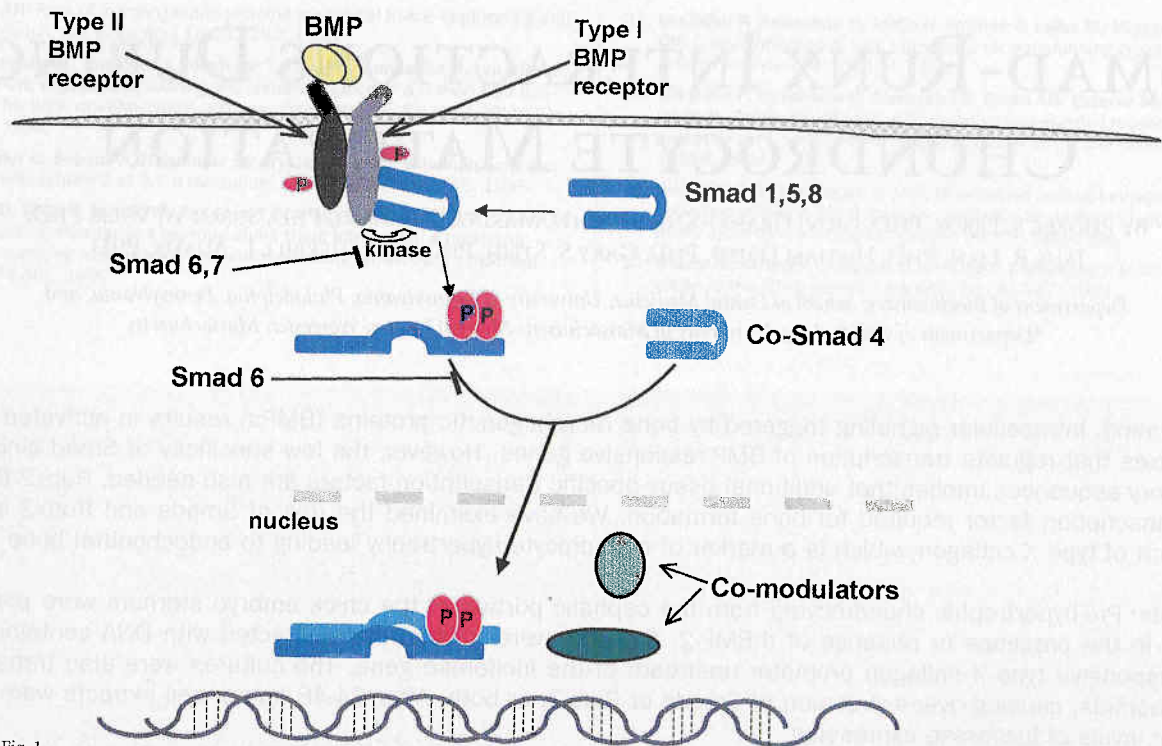


Fig. 1

Model for bone morphogenetic protein (BMP) signaling. BMPs bind to dimeric receptors containing a Type II unit (usually BMPR-II) and a Type I unit (BMPR-IA, BMPR-IB, or ALK-2). Binding causes phosphorylation of the Type II receptor, which, in turn, phosphorylates the Type I receptor. This permits binding and phosphorylation of a BMP-specific receptor Smad (Smad1, Smad5, or Smad8). The activated Smad joins with the co-Smad 4, and this complex translocates to the nucleus where it can complex with other transcription factors (co-modulators) and regulate transcription of BMP-responsive genes. Smads 6 and 7 are inhibitory Smads.

pre-hypertrophic chondrocytes: those derived from regions where endochondral bone will form<sup>24,38</sup>.

The BMP signaling cascade is active not only in bone formation but also in establishing the basic embryonic body plan and inducing the development of nearly all tissues and organs<sup>14</sup>. This raises the question of how specificity is achieved in different BMP-stimulated systems. Accumulating evidence suggests the answer: activated Smads (and probably most transcription factors) apparently do not act alone but rely on other more cell type-specific transcription factors that function as co-modulators<sup>1,43,46</sup>. As might be expected from the important role of BMPs in morphogenesis, several co-modulators identified with BMP signaling seem to be members of the homeodomain (*Hox*) group of transcription factors. These are nuclear proteins responsible for developmental patterning of embryonic structures, including limbs. The transcription factor *Hoxc-8* has been shown to bind Smad1; *Hoxc-8* represses transcription of osteoblast-related genes in the absence of BMP signaling, and Smad binding to *Hoxc-8* releases *Hoxc-8*-induced repression<sup>45</sup>. Several other homeodomain transcription factors have also been shown to bind Smad1 and regulate transcription of *Xenopus* genes involved in the formation and differentiation of mesodermal structures<sup>12,37</sup>.

Another group of transcription factors that interact with Smads are the Runx family, which are proteins contain-

ing a runt-homology domain<sup>11,41</sup>. Runx proteins are multifunctional transcription factors that modulate transcription not only by DNA binding but also through protein-protein interactions with numerous co-modulators including chromatin remodeling factors<sup>16,40,41</sup>. The gene for Runx2 (previously known as *Cbfa1*, *AML3*, *PEBP2 $\alpha$ -A*, or *OSF2*) was cloned as a transcription factor that bound to the osteocalcin promoter<sup>6,27</sup> and that was essential for bone development<sup>30</sup>. Mice lacking functional Runx2 developed neither intramembranous bone nor endochondral bone<sup>20</sup>. Subsequent investigations demonstrated that the absence of Runx2 in postnatal animals caused osteopenia, indicating that this factor was also required for osteoblast function during bone remodeling<sup>7</sup>. Consistent with these observations, culturing osteoblasts with antisense oligonucleotides for Runx2 decreased alkaline phosphatase and osteocalcin expression and diminished formation of mineralized nodules<sup>2</sup>. Although originally reported to be osteoblast-specific, Runx2 has recently been shown to be expressed in pre-hypertrophic and hypertrophic chondrocytes, which form the calcified matrix for endochondral bone formation<sup>19</sup>, and Runx2-deficient mice that lack bone also show defects in chondrocyte maturation<sup>15</sup>.

Direct evidence for Smad-Runx interactions emerged in studies examining TGF- $\beta$  induction of immunoglobulin expression; both Runx1 and Runx3 were shown to complex with activated Smad3<sup>11,31</sup>. In addition, members of the Runx family

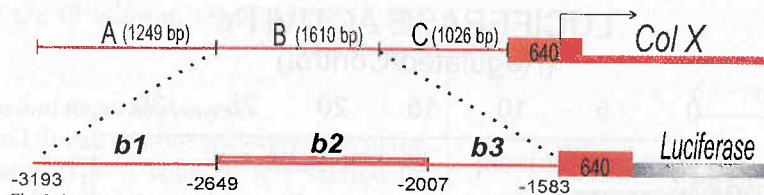


Fig. 2-A

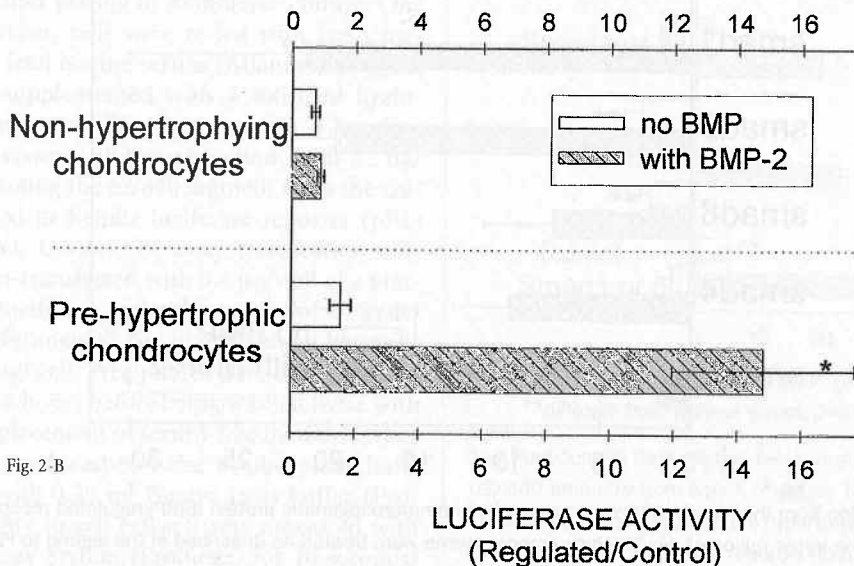


Fig. 2-B

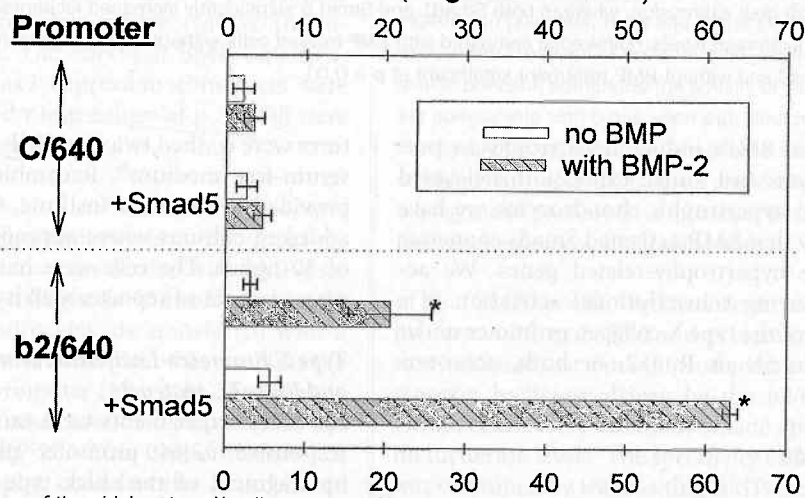


Fig. 2-C

**Fig. 2.** Analyses of promoter regions of the chicken type X collagen gene. Chondrocytes from day-15 chick embryo sternum were transfected with DNA containing pRL luciferase under the control of type X collagen promoter and plasmid containing cytomegalovirus (CMV) promoter-pGL luciferase (see Materials and Methods section). Transfection medium was replaced with serum-free medium, bone morphogenetic protein-2 (BMP-2), and 30 ng/ml, and cells were cultured for an additional 48 hours before lysis. Cell extracts were assayed for levels of type X promoter-regulated luciferase (Regulated luciferase) and luciferase controlled by the promoter (Control luciferase). To normalize for possible differences in transfection efficiency, results are expressed as the average ratio of Regulated/Control luciferase  $\pm$  SD. **Fig. 2-A:** Structure of promoter-luciferase constructs containing 5' flanking regions of the type X collagen gene. The 640-bp region contains sites of RNA polymerase binding and transcription start. **Fig. 2-B:** Comparison of activity of the b2/640 promoter from the type X collagen gene, expressed in non-hypertrophying chondrocytes derived from the lower sternal region of chick embryos and pre-hypertrophic chondrocytes from the upper sternal region. **Fig. 2-C:** Activity of the BMP-responsive b2/640 promoter versus the C/640 promoter region of the type X collagen gene. \*Difference compared with no BMP significant at  $p < 0.001$ . \*\*Difference compared with C/640 with BMP significant at  $p < 0.01$ .

co-precipitated with Smad1/Smad4 and Smad5/Smad4 complexes, suggesting that Runx proteins are also co-modulators for BMP-activated Smads<sup>11</sup>. Additional proof of Runx involve-

ment in BMP signaling has been provided by recent analysis of a Runx2 mutation found in cleidocranial dysplasia; mutant Runx2 protein failed to interact with Smads and was unable to

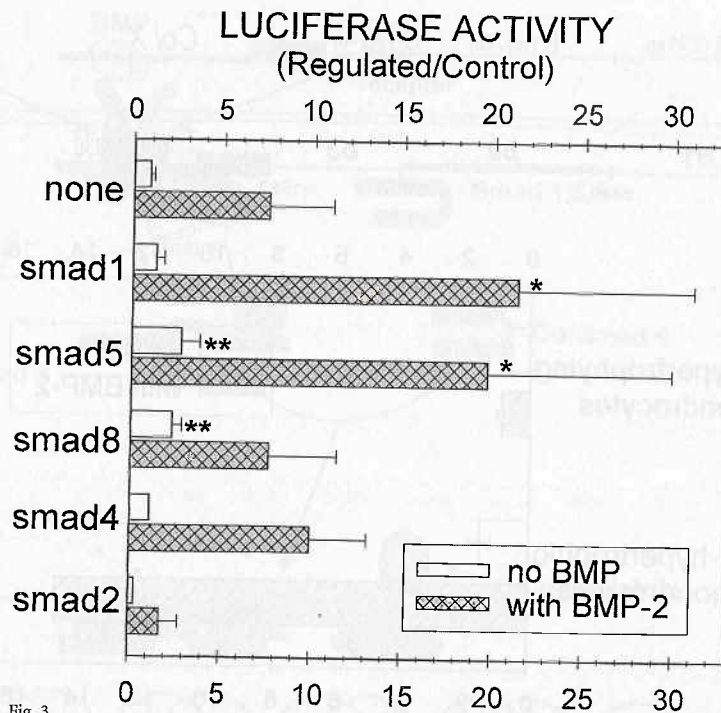


Fig. 3

Regulation of the b2/640 region from the type X collagen promoter by bone morphogenetic protein (BMP)-regulated receptor-activated Smads (R-Smads). Chondrocytes from the upper region of day-15 chick embryo sterna were treated as described in the legend to Figure 1. Transfection with Smad8 had no effect on luciferase expression, whereas both Smad1 and Smad 5 significantly increased luciferase levels and Smad 2 significantly decreased BMP-stimulated luciferase levels. \*Difference compared with BMP-treated cells without Smad significant at  $p < 0.01$ . \*\*Difference compared with cells without Smad and without BMP treatment significant at  $p < 0.01$ .

respond to BMP<sup>47</sup>. Since BMPs induce hypertrophy in pre-hypertrophic chondrocytes and Runx2 expression is elevated in pre-hypertrophic and hypertrophic chondrocytes, we have examined the possibility that BMP-activated Smads cooperate with Runx2 to induce hypertrophy-related genes. We accomplished this by assaying transcriptional activation of a BMP-responsive region of the type X collagen promoter under conditions in which Smads or Runx2, or both, are over-expressed.

## Materials and Methods

### Cell Culture

Cells were isolated from the upper (cephalic) and lower (caudal) one-third portions of sterna from 15-day chick embryos (B&E Eggs, Stevens, PA, U.S.A.) by digestion for 3.5 hours at 37°C, 5% CO<sub>2</sub> in calcium and magnesium-free Hanks' balanced salt solution (CMF-HBSS) containing 0.6 mg/ml collagenase and 0.04% trypsin<sup>3</sup>. The cells were initially resuspended in a complete medium composed of high glucose Dulbecco's modified Eagles medium (DMEM) with 10% NuSerum IV (Collaborative Biomedical Products/Becton-Dickinson, Bedford, MA, U.S.A.) and 100 units/ml penicillin/streptomycin (Pen/Strep) and were cultured for 5 days as described previously<sup>23,24</sup>. The floating chondrocytes were then replated in 12-well culture dishes at  $5 \times 10^4$  cells/cm<sup>2</sup> in 1 ml complete medium supplemented with 4 units/ml hyaluronidase (HA) to promote attachment<sup>25</sup>. After transfection, cul-

tures were washed twice in CMF-HBSS before a switch to 1 ml serum-free medium<sup>24</sup>. Recombinant human BMP-2 (kindly provided by Genetics Institute, Cambridge, MA, U.S.A.) was added to cultures where appropriate at a final concentration of 30 ng/ml. The cells were harvested 24-48 hours later for measurement of reporter activity.

### Type X Promoter-Luciferase, Smad, and Runx2 Constructs

All of the experiments were carried out with use of a BMP-responsive "b2/640 promoter" plasmid<sup>28</sup> that contained a 642-bp fragment of the chick type X collagen promoter representing -2649/-2007 (relative to the transcriptional start site) upstream of a 640-bp fragment containing 558 bp of the proximal promoter region plus 82 bp of transcribed DNA (Fig. 2-A). The ability of this type X promoter-luciferase construct (b2/640) to respond to BMP has been previously described<sup>28</sup>. When transfected into pre-hypertrophic chondrocytes, this promoter permits relatively high level luciferase expression in pre-hypertrophic chondrocytes from the upper region of embryonic chick sternum; however, it shows little activity when transfected into chondrocytes from the lower sternal region that do not undergo hypertrophy during development. All Smad expression plasmids contained the Smad gene controlled by the cytomegalovirus (CMV) promoter and were kindly provided by Dr. Peter ten Dijke (Netherlands Cancer Institute, Amsterdam, The Netherlands)<sup>17</sup>. The Runx2 ex-

pression plasmid was the *til* isoform, regulated by a CMV promoter<sup>16,33</sup>.

#### Transient Transfections and Reporter Assays

The ability of Smads and Runx2 to stimulate expression of the hypertrophy-related gene type X collagen was assayed by transiently transfecting chondrocytes with type X promoter-luciferase DNA 1 day after plating in monolayer culture. One hour prior to transfection, cells were re-fed with fresh medium containing 10% fetal bovine serum (Atlanta Biological, Atlanta, GA, U.S.A.) supplemented with 4 units/ml hyaluronidase and 100 units penicillin/streptomycin. Chondrocytes were transfected using CaPO<sub>4</sub> precipitation, with 2.2 µg/well of a plasmid containing the *b2/640* segment from the collagen X promoter fused to Renilla luciferase reporter (pRL; Promega, Madison, WI, U.S.A.). To assay transfection efficiency, the cells were co-transfected with 0.4 µg/well of a plasmid containing firefly luciferase under the control of an SV40 early promoter (pGL; Promega)<sup>38</sup>. Smad and Runx2 plasmids were added at 0.1-2.0 µg/well. Precipitates were allowed to remain on the cells for 5-6 hours before being washed twice with CMF-HBSS and the replacement of serum-free medium. After 24-48 hours, the cells were washed twice in phosphate buffered saline and lysed with 0.25 ml Passive Lysis buffer (Promega). Luciferase activity in cell extracts was measured with the Dual-Luciferase Assay System (Promega) for 10 seconds/reaction using an Opticomp luminometer (MGM Instruments, Hamden, CT, U.S.A.). The effects of BMP, Smad expression constructs, and Runx2 expression constructs were compared with use of a paired *t* test; values of  $p \leq 0.05$  were considered statistically significant.

#### Results

When pre-hypertrophic chondrocytes from the upper region of chick embryo sterna are treated with BMP-2 or BMP-4, mRNA for type X collagen is induced within 24 hours<sup>24</sup>. Similarly, when chondrocytes are transfected with a type X collagen promoter-reporter construct that contains the *b2/640* region of the type X promoter (Fig. 2-A), these chondrocytes show a 6-10-fold stimulation of reporter gene expression at addition of BMP-2<sup>38</sup>. This responsiveness is characteristic of pre-hypertrophic chondrocytes found in regions of endochondral bone formation and is not seen with non-hypertrophying chondrocytes from the lower region of chick embryonic sterna (Fig. 2-B). The BMP responsiveness of *b2/640*-containing promoters is also not seen if the *b2* region is replaced with the "C" region, a 1.026-kb sequence immediately upstream of the 640 sequence in the chick type X collagen gene (Fig. 2-C)<sup>38</sup>.

A Smad5 expression plasmid was co-transfected along with promoter-luciferase plasmids containing either the BMP-responsive *b2* region or the BMP-nonresponsive C region from the type X collagen gene (Fig. 2-A). Smad5 markedly stimulated *b2/640* luciferase expression in the presence of added BMP-2 but had no effect on the C/640 promoter (Fig. 2-C). The role of individual Smads in transmitting this response was examined by transfecting chondrocytes with each

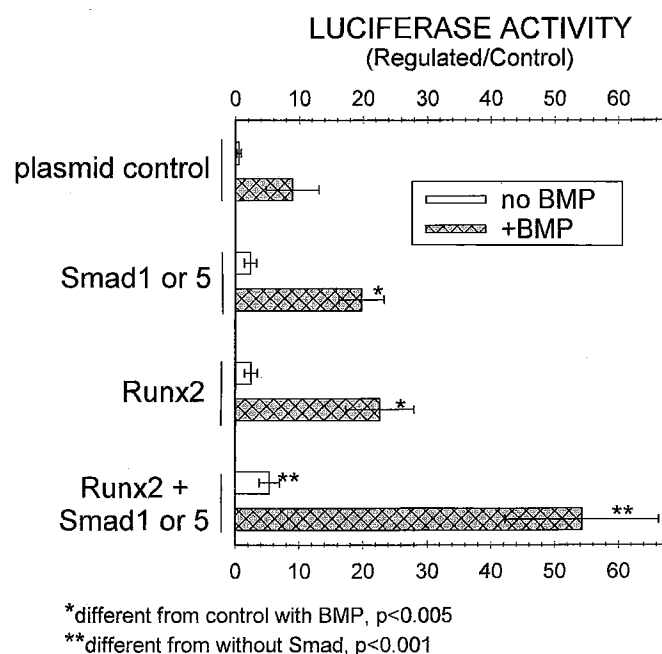


Fig. 4 The Runt-domain transcription factor Runx2 increases activity of the *b2/640* promoter from type X collagen. Two different forms of Runx2 cDNA were transfected into pre-hypertrophic upper sternal chondrocytes, along with luciferase reporter plasmids with or without Smad expression plasmids. Runx and Smad plasmids were added at 0.5 µg/well. In the presence of bone morphogenetic protein-2 (BMP-2), Runx2 over-expression stimulated the activity of the *b2/640* promoter to levels comparable with those seen with Smads 1 or 5, and the stimulation by combining Runx plus Smads was additive. Even in the absence of added BMP (open bars) Runx2 plus Smads 1 or 5 showed a small but significant stimulation of luciferase levels, suggesting that the chondrocytes are producing some BMP.

of the BMP-specific R-Smad constructs along with *b2/640*-luciferase. When BMP-2 was present in the cultures, the addition of Smad1 or Smad5 produced a 2-3-fold stimulation of reporter luciferase driven by the *b2/640* promoter (Fig. 3). However, over-expression of Smad8 appeared to have no effect on luciferase levels. The specificity of R-Smad over-expression was examined by transfecting BMP-treated chondrocytes with Smad4, which is a Co-Smad, or with Smad2, which is an R-Smad activated by TGF- $\beta$  signaling. Over-expression of Smad4 yielded luciferase levels that were not significantly different from those produced by BMP stimulation of chondrocytes without additional Smad, whereas over-expression of the TGF- $\beta$ -specific Smad2 appeared to inhibit the BMP-stimulated type X promoter activity (Fig. 3).

Although all cultures lacking exogenous BMP had low luciferase levels compared with those with added BMP (Fig. 3, clear bars), the over-expression of Smads 1 and 5 yielded a small but significant elevation of the *b2/640*-luciferase compared with cultures transfected with a control plasmid. This is consistent with our observations<sup>39</sup> that pre-hypertrophic and hypertrophic chondrocytes produce low levels of BMP-2 that should be capable of inducing activation of BMP-specific R-

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-2600  TATAATCATCCCTATTTACACAAAGACACAGAGATTATTCACCTCTCTCC
-2550  CCAAGAGGAGACAAGGCAGGAAGTGAGACTGTCTGAACAAATAGGGGTGTA
-2500  CATTATTGGTGCCTTTGTAGAATAAGTGCCTCTGCCTTGGGGGTATTTAT
-2450  GAATTAGGCTAGAAATTAGCATGAAACGGCGTGTGCACAGAACTGTTGCC
-2400  TGCTGTTCTTAACCTAACCAATATTATCAAATTAGCAACAACCACACT

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Fig. 5

Sequence of a 250-bp region within the b2 segment of the chick type X collagen promoter. Numbers indicate base pairs upstream of the transcription start site. This sequence contains two CAGA and three reverse CAGA motifs (underlined), which are potential binding sites for R-Smads and Smad4. It also contains the consensus sequence ***ACCACA*** (boldface italics) for Runx binding.

Smads. Combining Smad1 and Smad5, with or without Smad4, had no better results than those from Smad1 or Smad5 alone (data not shown), suggesting that the over-expression of either BMP-specific Smad alone is sufficient for maximal effect.

The same approach was employed for examining the possible role of Runx2 in expression of hypertrophy-specific genes. Pre-hypertrophic chondrocytes over-expressing the Runx2 gene in the presence of the b2/640 type X promoter-luciferase construct were analyzed for luciferase levels. The effect of Runx2 was similar to that seen with either Smad1 or Smad5: 2-3-fold stimulation in the presence of exogenous BMP-2 (Fig. 4). When both Runx2 and Smad1 or 5 were co-transfected into pre-hypertrophic chondrocytes, the combined over-expression resulted in an additional stimulation of the type X promoter construct; luciferase levels were 6-10-fold higher than those of cultures exposed to BMP-2 and control (empty) expression plasmids.

### Discussion

Our observations using pre-hypertrophic chick chondrocytes demonstrate that over-expression of Smad1 or Smad5, but not Smad8, promotes BMP-stimulated activity of the type X collagen promoter (Fig. 3). Several previous studies have used the approach of over-expressing individual Smad genes to analyze the role of Smads 1, 5, and 8 in BMP signaling<sup>5,18,29,44</sup>. As in our type X collagen promoter studies, myogenic C2C12 cells induced to undergo osteogenesis with BMP plus exogenous Smads showed no differences between Smad1 and Smad5<sup>44</sup>. However, in the developing Zebrafish these two Smads appear to differ in their response to BMPs<sup>5</sup>.

There have been few functional studies with Smad8, which shows high homology to Smads 1 and 5. It has been cloned from mammalian systems; however, evidence for a positive effect of Smad8 on BMP-stimulated genes is thus far limited to *Xenopus* promoter systems<sup>8,18</sup>. Since there is evidence that Smad8 is absent in BMP-responsive cell lines<sup>34</sup>, it is not surprising that it is ineffective in modulating BMP re-

sponsiveness of the type X collagen promoter.

Our studies with Smad4 indicate that its over-expression has no effect on the type X promoter (Fig. 3), suggesting that amounts of this co-modulator are not limiting in pre-hypertrophic chondrocytes. This is consistent with evidence that Smad4 is broadly expressed in all zones of the developing growth plate<sup>32</sup>. In contrast, Smad2, which is an R-Smad in the TGF- $\beta$ /activin-signaling pathway, markedly inhibited BMP stimulation of the type X collagen promoter (Fig. 3). This implies that elevated levels of Smad2 may effectively compete with the BMP-activated R-Smads for a downstream signaling component. Evidence for a competition between Smad2 and BMP-regulated Smads is provided by the observation that Smurf1, an enzyme promoting ubiquitination and degradation of Smads 1 and 5, increases cellular responsiveness to Smad2<sup>43</sup>.

Immunohistochemical studies<sup>32</sup> have shown that both Smad1 and Smad5 are expressed in chondrocytes undergoing maturation. Our studies indicate that the combination of Smad1 and Smad5 is no more effective than either alone, implying that they are functioning interchangeably. If Smad1 and Smad5 were each present in amounts that limited high-level BMP signaling, as suggested by the fact that over-expression induced 2-3-fold stimulation of type X promoter activity, independent regulation might provide more precise control over BMP signaling rates.

Activated R-Smad/Smad4 heteromeric complexes can bind to DNA, and several consensus sequences for the binding have been reported. Mutations in these binding elements decrease promoter activation by BMP or TGF- $\beta$ , which suggests that they are sites involved in regulating target genes. However, these Smad-binding elements tend to be sequences that occur relatively frequently in DNA and would provide only limited specificity. This fact, plus the low binding affinities of Smads for their binding sites, implies that Smad-mediated modulation of specific genes requires additional transcription factors capable of binding DNA at other specific sites. A diverse array of co-modulators has been reported in the past several years<sup>35</sup>. In activin and TGF- $\beta$ -stimulated signaling,

these include some transcription factors that are widely expressed in many cell types, e.g., the fos/jun complexes. Other TGF- $\beta$  signaling co-modulators are DNA binding proteins with more tissue-restricted expression, such as the vitamin D receptor and the FAST family of nuclear proteins<sup>28,35</sup>.

Runx proteins are multifunctional transcription factors that also show tissue-specific expression. They not only serve as DNA binding proteins but also modulate transcription through protein-protein interactions with numerous co-modulators<sup>40,41</sup>. Runx-containing transcription factors are dimers of  $\alpha$  and  $\beta$  subunits in which the major determinant of tissue specificity is apparently the  $\alpha$  or Runx subunit<sup>40,41</sup>. Runx1 (AML1) is expressed in cells of the hematopoietic lineage, where it activates myeloid and lymphoid-specific genes. Runx3 (AML2) is highly expressed in B lymphocytes and regulates immunoglobulin class switching. In contrast, expression of Runx2 is highest in bone-forming cells, and lack of Runx2 expression prevents both intramembranous and endochondral bone formation.

In chondrocytes, Runx2 has been reported to regulate chondrocyte maturation, and mice deficient in Runx2 show defective chondrocyte maturation<sup>15,19</sup>. These mice showed no type X collagen mRNA in growth-plate regions of the humerus and femur, whereas other long bones lacked expression of other genes normally transcribed during later stages of hypertrophy<sup>15</sup>. Enomoto et al.<sup>9</sup> recently reported that Runx2 is expressed in hypertrophic and pre-hypertrophic chondrocytes and that over-expression of Runx2 in chick immature chondrocytes could induce hypertrophy-related genes such as alkaline phosphatase, MMP-13, and type X collagen. Our results expand on this finding, demonstrating that Runx2 over-expression increases activity of a BMP-responsive region of the type X promoter and that the combination of elevated Runx2 and activated Smad1 or Smad5 produces additive effects on the promoter activity (Fig. 4).

All promoter analyses were performed by assaying luciferase levels 24-48 hours after transfection with luciferase, Smad, or Runx constructs, or all three. Since this is sufficient time for induction of new mRNAs and proteins, it is formally possible that Smad and Runx modulation of the type X collagen promoter are secondary effects resulting from these factors inducing one or more other transcription factors, which, in turn, act on the type X promoter. However, if Smad and Runx act directly on the type X promoter, one would expect to find both Runx-binding and Smad-binding elements contained within the *b2* segment of the type X promoter. The consensus sequence for Runx binding is ACCACA<sup>27</sup>. Several consensus sequences for binding activated Smads have been reported; they include CAGA or AGAC and GCCGnCGC<sup>22,28,31</sup>. A 250-bp section of the type X collagen *b2* promoter region is presented in Figure 5. It contains a Runx consensus sequence at -2360 to -2355 (in boldface) as well as two CAGA sequences and several reverse CAGA sequences (underlined) further

upstream. This region of the type X collagen promoter therefore possesses sites with the potential for both Smad binding and Runx binding and is consistent with the hypothesis that these factors directly regulate the *b2* region within the type X promoter.

Both BMP signaling and TGF- $\beta$  signaling have been reported to modulate Runx2 levels. BMP up-regulates Runx2<sup>10,25</sup> and might therefore promote chondrocyte hypertrophy by not only activating transcription of hypertrophy-related genes but also increasing levels of a co-modulator required for maximal transcription of these genes. In contrast, over-expression of TGF- $\beta$ -activated Smad2 has been reported to suppress Runx2 gene expression<sup>26</sup>. Because Runx2 acts as a co-modulator, decreased endogenous levels should limit the ability of BMP-activated Smads to stimulate transcription of hypertrophy-related genes. It is therefore plausible that the marked inhibition of BMP-stimulated luciferase expression seen with Smad2 over-expression (Fig. 3) is caused by limiting levels of Runx2.

Although our results implicate Runx2 as a co-modulator for Smad1 or Smad5 in regulating type X collagen expression in chondrocytes, it may not be the only transcription factor capable of serving as a co-modulator for BMP-regulated Smads. The report that Hoxc-8 can bind to Smad1 and stimulate osteopontin expression in osteogenic 2T3 cells<sup>45</sup> suggests that this homeodomain transcription factor can serve as a co-modulator in Smad1-mediated activation of osteoblast-specific gene expression. The co-modulators of BMP-induced Smad function may therefore vary, depending on the cell type. ■

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