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Runx2/Cbfa1 Stimulation by Retinoic Acid Is Potentiated by BMP2 Signaling Through Interaction With Smad1 on the Collagen X Promoter in Chondrocytes

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Abstract Chondrocyte differentiation is a fundamental process during endochondral ossification. Several factors regulate maturation via the activity of downstream signaling pathways that target specific transcription factors and regulate chondrocyte-specific genes. In this study, we investigated the mechanisms involved in the regulation of chick lower sternal chondrocyte maturation upon stimulation by retinoic acid (RA) and the bone morphogenetic protein BMP2. RA-induced Runx2 in lower sternal chondrocyte cultures and over-expression of wild-type (WT) Runx2 enhanced *colX* and alkaline phosphatase activity, while over-expression of dominant negative Runx2 was inhibitory. Furthermore, WT Runx2 enhanced the effects of both BMP2 and RA on *colX* expression, while the effects of both growth factors were completely blocked in cultures over-expressing dominant negative Runx2. Similarly, WT Runx2 enhanced the induction of *colX* by Smad1. Smad1 and Runx2 were found to act cooperatively at the chicken type X collagen promoter and elimination of either the putative Smad binding site or Runx2 binding site eliminated responsiveness to BMP2, RA, or either of the transcription factors. Altogether the results show cross talk between the BMP-associated Smads and Runx2 during chondrocyte differentiation and dependence upon both signals for induction of the type X collagen promoter. Factors or signals that alter either of these transcription factors regulate the rate of chondrocyte differentiation. J. Cell. Biochem. 90: 1287–1298, 2003.

Key words: Runx2/Cbfa1; BMP2; retinoic acid; type X collagen promoter; chondrocyte; transcription; gene expression

During endochondral bone formation a cartilage template is formed. Chondrocytes then undergo an exquisitely regulated differentiation process whereby maturing cells become hypertrophic and express type X collagen, alkaline phosphatase, and MMP13 and the surrounding matrix becomes calcified [Gerstenfeld and Shapiro, 1996; D'Angelo et al., 2000]. Terminally differentiated chondrocytes express angiogenic factors, including VEGF and undergo apoptosis [Gerber et al., 1999]. Subsequently, there is ingrowth of vascular tissues and the calcified cartilage is lined with osteoblasts and acts as a template for primary bone formation [Gerber et al., 1999]. This process of endochondral bone formation is necessary for limb development, but also occurs during most bone repair events [Vortkamp et al., 1998; Le et al., 2001; Gerstenfeld et al., 2003]. Thus, understanding the genetic and molecular pathways that regulate endochondral ossification will provide insights into developmental diseases and provide potential targets to improve fracture healing.

While the PTHrP/Ihh signaling pathway has been defined as an important negative regulator of chondrocyte maturation, less is known about the factors and signaling events that positively regulate this process [Karaplis et al., 1994; Vortkamp et al., 1996; Schipani et al., 1997]. BMPs and their Smad signaling molecules are

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important stimulators of endochondral bone formation [Asahina et al., 1996; Duprez et al., 1996; Pathi et al., 1999; Li et al., 2003]. BMP signaling induces the expression of *colX* and other maturational markers in multiple models of chondrocyte differentiation [Grimsrud et al., 1998, 1999; Ballock et al., 2000]. Similarly, retinoic acid (RA) has been demonstrated to induce chondrocyte differentiation in several models including sternal and limb chondrocytes [Pacifici et al., 1991; Koyama et al., 1999]. Responsiveness to RA has been shown to be dependent upon interactions between retinoids and endogenous factors secreted by maturing chondrocytes [Iwamoto et al., 1993]. More recent studies indicate that RA has important synergistic interactions with BMPs, and enhances BMP-mediated effects on chondrocyte differentiation [Li et al., 2003]. Since BMPs are approved for clinical use, understanding the interaction of BMP signaling molecules with other factors is critical for elucidating the precise pathways that mediate chondrocyte differentiation.

The runt related transcription factor Runx2 belongs to a family of three proteins that share a significant sequence homology [Westendorf and Hiebert, 1999]. Runx1 is essential for definitive hematopoiesis [North et al., 2002] and Runx3 plays an anti-oncogenic role in the digestive tract [Li et al., 2002], but Runx2 is essential for normal skeletal development [Komori et al., 1997; Otto et al., 1997; Choi et al., 2001]. Runx2 deficient mice die at birth due to respiratory failure resulting from the absence of mineralized bone tissue. While lack of bone formation in these animals results from a block of osteoblast maturation, subsequent work has demonstrated a role for Runx2 in chondrocyte differentiation. Although a complete cartilage template is formed, Runx2 null mice have a delay in chondrocyte differentiation [Inada et al., 1999; Kim et al., 1999]. Other studies, using in situ hybridization or animals with a *LacZ* reporter gene inserted into the Runx2 locus, show that Runx2 is expressed in hypertrophic chondrocytes, osteogenic cell lineages, and mesenchymal cell condensations during embryonic development [Ducy et al., 1997; Huang et al., 1997; Sato et al., 1997]. Recently, several transgenic mice expressing Runx2 proteins under tissue-specific promoters were described. Osteoblast-specific over-expression of Runx2 by the collagen type I promoter results in an osteopenic skeleton with multiple fractures [Liu et al., 2001]. Runx2 over-expression in cartilage using the collagen type II promoter results in dwarf phenotype due to accelerated chondrocyte maturation and premature mineralization [Ueta et al., 2001]. In contrast, over-expression of a dominant negative Runx2 results in delayed maturation and persistence of immature cartilage [Ueta et al., 2001]. Altogether, these studies suggest that Runx2 is required for cartilage tissue maturation. Thus its regulation and interaction with other signaling molecules is extremely important.

In the current investigation, we address the hypothesis that Runx2 is induced by RA and is essential for chondrocyte differentiation. We show that Runx2 is necessary for RA, BMP2, and Smad1 mediated differentiation. Finally, through mutational analysis of the type X collagen promoter, we demonstrate interdependence between Smad and Runx2 signaling for induction of type X collagen.

MATERIALS AND METHODS

Reagents

All-*trans*-RA was purchased from Sigma Chemical (St. Loius, MO), reconstituted in 95% ethanol at 1 mM and maintained at -20°C as recommended by the manufacturer. BMP2 was a gift from Genetics Institute (Cambridge, MA) and was obtained in solution (1 mg/ml) and maintained at 4°C. Antibodies for Smad1 and Runx2 (PEBP2αA/Cbfa1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Chondrocyte Cell Culture

Chondrocytes were isolated from 13-day-old chicken embryonic lower sterna as previously described with some modifications [Oettinger and Pacifici, 1990]. Briefly, after primary culture for 5–7 days, floating cells were plated in secondary cultures at 1.5×10^6 cells/10 cm culture plate, or at 3×10^5 cells/well/6-well plate, or at 2×10^5 cells/well/24-well plate. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% NuSerum IV (Collaborative Biomedical, Bedford, MA), 4 U/ml of hyaluronidase (Sigma Chemical), and 2 mM L-glutamate (Sigma). Chondrocytes were cultured and maintained in the presence of 50 μg/ml ascorbic acid. BMP2 (50 ng/ml), or all-*trans*-RA (100 nM) were added to the cultures in indicated experiments

after 24 h in serum-containing medium. Chondrocytes were infected with RCAS viruses at the time of secondary plating in culture medium containing 25% filtered viral supernatant and 75% sternal chondrocyte media (high glucose DMEM + 10% Nuserum IV, plus hyaluronidase. The medium was changed to 100% sternal chondrocyte medium after 2 days. Medium was changed in all experiments with identical fresh medium (±growth factors) every 2 days.

Real-Time RT-PCR Assay

Total RNA was extracted from the cultures by using RNAeasy kits (Qiagen, Santa Clarita, CA). cDNA was synthesized from 1 μg of total RNA in a 10 μl reaction mixture containing 1 U reverse transcriptase buffer (Invitrogen, Carlsbad, CA). Reaction time was 1 h at 42°C. After the reverse transcription, all the samples were diluted 1:5 and 1 μl from each dilution was used for SYBR Green PCR Master Mix assay (Applied Biosystems, Warrington, UK). For Runx2 (5′-actttgacaataactgtcct-3′; 5′-gacacctactctcatactgg-3′) and GAPDH (5′-tatgatgatatcaagagggtagt-3′; 5′-tgtatccaaactcattgtcatac-3′) primer sets, two PCR reactions were carried out with cDNA as template according to the standard SYBR Green protocol. The reactions were incubated at 50°C for 2 min to activate the uracil *N*-glycosylase (UNG) and then for 5 min at 95°C to inactivate this enzyme and activate the Amplitaq Gold polymerase. The reactions was performed for 45 cycles with denaturation at 95°C for 15 s (denaturation), followed by annealing at 45°C for 20 s, and extension and detection at 75°C for 10 s.

Northern Blot

Total RNA was isolated from the cultures using the RNAeasy kit according to the manufacturer's instructions (Qiagen). The RNA concentration was quantified by measuring absorbance at 260 nm. Northern analysis was performed on denaturing formaldehyde/agarose gels with 5 μg of RNA loaded per sample. Ethidium bromide (40 mg final concentration) was added to the loading buffer to permit visualization of the ribosomal bands. After electrophoresis, the gels were photographed with Polaroid film (Boston, MA) and RNA transferred to Gene Screen (DuPont, Wilmington, DE) nylon membranes by capillary transfer. The blotted RNA was hybridized with a DNA probe. Briefly, a synthetic collagen type X oligonucleotide was end-labeled with T4 kinase (Life Technologies, Inc., Gaithersburg, MD) as previously described [Ionescu et al., 2001]. The type X oligonucleotide probe was hybridized in QiukHyb Hybridization Solution (Stratagene, La Jolla, CA) at 73°C for 1 h according to manufacturers recommendations. Repeated washes were performed, with the final wash performed in a solution composed of 0.1× SSC and 0.1% SDS at 60°C for 30 min. Probed membranes were exposed to Kodak XAR film (Rochester, NY).

Alkaline Phosphatase Activity

Alkaline phosphatase activity was measured following a previously described protocol [Grimsrud et al., 1999]. Culture medium was aspirated from chondrocytes cultured in 24-well plates. The plates were rinsed with 150 mM NaCl, and 1 ml of reaction buffer containing 0.25 M 2-methyl-2-amino propanol, 1 mM magnesium chloride, and 2.5 mg/ml of *p*-nitrophenyl phosphate (Sigma) at pH 10.3 was added to the wells at 37°C. The reaction was stopped after 30 min by the addition of 0.5 ml of 0.3 M Na_3PO_4 (pH 12.3). Alkaline phosphatase activity was determined by measuring absorbance of light at 410 nm and comparing the experimental samples with standard solutions of *p*-nitrophenol and an appropriate blank. Alkaline phosphatase was normalized for protein concentration using BCA Protein Assay Reagent (Pierce, Rockford, IL), as measured by spectrophotometry (562 nm) and compared to standard protein concentrations. Data presented was the mean of four samples and error bars represent standard error of the mean. Statistical analysis was performed using one-way ANOVA.

Viral Infections

Viral stocks were produced as previously described in chicken embryonic fibroblasts (CEFs) following transfection with RCAS viruses using the transfection reagent Superfect (Qiagen). The CEFs were prepared from the soft tissues of day 10 chicken embryos [Morgan and Fekete, 1996]. Serum-free medium was then placed on the fibroblast cultures for 24 h and retroviral conditioned medium was harvested. The conditioned media was filtered through a 0.45-μm filter (Corning, NY) to remove cellular contaminants. The viral stocks were frozen in single use aliquots at −80°C.

Plasmid Constructs

The BMP responsive chicken type X collagen luciferase reporter construct was a generous gift from Dr. Phoebe Leboy (University of Pennsylvania). The promoter fragment used in our study is the previously designated b2/640-luciferase promoter. This promoter contains a 643 base pairs (bp) fragment derived from bases −1,792 to −2,435 of the type X collagen gene ligated to a 640 bp proximal promoter that contains the transcriptional start site and 558 bp of 5′-flanking sequence [Volk et al., 1998]. This construct was ligated into the polylinker region of the promoterless Renilla luciferase reporter plasmid, PRLnull (Promega, Madison, WI). A 20- to 30-fold induction in activity has previously been demonstrated with this reporter in response to BMP treatment [Li et al., 2003]. Wild-type (WT) and dominant negative chicken Cbfa1/Runx2 cDNAs cloned in Replication Competent Avian Leukemia virus (RCASBP[B]) were provided by Enomoto–Iwamoto (Thomas Jefferson University). WT mouse Runx2/Cbfa1 was obtained from Dr. Karsenty (M.D. Anderson, TX). Flag-tagged WT and dominant negative Smad1 were obtained from Dr. Derynck (University of San Francisco) and were cloned into RCASBP(A).

Linker-Scanning Mutation

The linker-scanning mutation, containing the Gal4 sequence, and spanning a 20-bp region of the chicken type X collagen promoter from nt −1,869 to nt −1,888, which contains Smad binding site CAGA, was created using a PCR-based method. Briefly, two primers (5′-ctgtcctccgccccaagaggag-3′; 5′-tactccgtgtctttgtgtaa-3′) each with half of the GAL4 site were designed to run PCR with the uncut b2/640-luciferase promoter. Platinum Pfx enzyme (Life Technologies, Inc.) was used in the experiments to generate blunt-end PCR products. The PCR products were purified from 0.8% agarose gel and ligated at room temperature with a rapid ligation kit (Roche Molecular Biochemicals, Indianapolis, IN). Clones were screened by PCR with a GAL4 primer (cggagtactgtcctccg) and GLprimer2 of the pRL-null Vector [Yeung et al., 2000]. The clone was confirmed by DNA sequence. Similarly, a 26-bp region of the chicken type X collagen promoter from 2,070/2,095, which has Runx binding site ACCACA, was replaced by using two primers (5′-gtcctccgattctaagggctatatatttaaatg-3′; 5′-agtactccggataatatttggttaagttagg-3′) and confirmed by DNA sequence.

Transient Transfection and Luciferase Assay

Lower sternal chondrocytes, cultured at 30–40% confluence in 6-well plates, were transfected with a total concentration of 1 μg/well of plasmid DNA 24 h after plating using the transfection reagent Superfect (Qiagen). The pGL3 firefly-luciferase reporter plasmid containing the SV40 early promoter (Promega) (10 ng/well) was co-transfected as a control for transfection efficiency. The plasmid constructs were added to the cultures in 100 μl of plain DMEM and after 10 min, medium containing 10% serum and antibiotics was added. At 3 h the medium was changed to standard culture medium. Sixteen hours later, the cultures were rinsed and serum-free DMEM containing hyaluronidase (4 U/ml), penicillin/streptomycin, 10 pM triiodothyronine (Sigma), 60 ng/ml insulin, and 1 mM cysteine (Sigma) was added. Three hours later growth factors were added to selected treatment groups. Forty-eight hours later, a chondrocyte protein extract was obtained and assayed for luciferase activity using the Promega dual luciferase assay system. Renilla luciferase values were used to normalize each sample for transfection efficiency. Data presented is the mean of triplicate samples and error bars represent standard error of the mean.

Statistical Analysis

Results from the experiments were analyzed using a one-way analysis of variance (StatView 4.5; Abacus, Berkely, CA). The P values are described in the figure legends.

RESULTS

RA Induces *Runx2* mRNA Levels in Chick Lower Strenal Chondrocytes

RAs are potent inducers of chondrocyte maturation. We have previously reported that RA enhances *colX* mRNA levels in lower sternal chondrocytes in culture for 8 and 12 days [Li et al., 2003]. This transcriptional stimulation was only observed upon treatment of these cells with 100 nM of RA. Similarly the Runx2 transcription factor was recently shown to upregulate chondrocyte differentiation in vitro and in vivo [Iwamoto et al., 2003]. We, therefore, investigated whether RA-mediated induction of chondrocyte maturation may partly result from

an up-regulation of *Runx2* expression. Continuous treatment of lower sternal chondrocytes with 10^{-7} M of all-*trans*-RA increased *Runx2* mRNA levels by 2.4- and 4.7-fold, respectively at 8 and 12 days of culture (Fig. 1A). These results suggest that RA may induce chondrocyte maturation at least partly through up-regulation of *Runx2*.

We next investigated the effects of Runx2 on chick lower sternal chondrocyte differentiation by over-expressing WT Runx2 and its dominant negative form using the RCAS retroviral system. Runx2 increased alkaline phosphatase activity by sevenfold in 8-day cultures, while viral over-expression of Runx2-DN resulted in a 68% decrease in alkaline phosphatase activity (Fig. 1B). Since alkaline phosphatase is a marker of chondrocyte differentiation, the results indicate that RA may enhance maturation through mechanisms that include induction of *Runx2*.

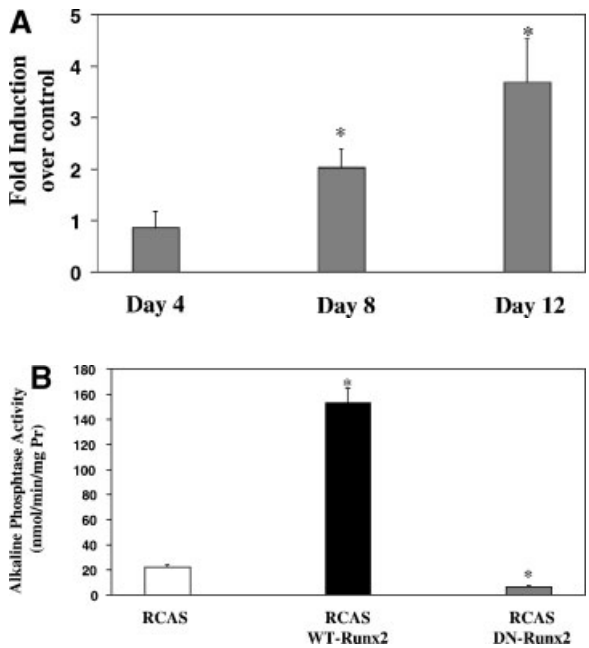

Fig. 1. Retinoic acid (RA) induces *Runx2* in lower sternal chondrocytes. **A**: Lower sternal chondrocyte cultures were treated continuously with RA (100 nM) for 8 days. Cultures were the harvested and RNA prepared and *Runx2* expression assessed by real-time RT-PCR as described in Materials and Methods. The data represents the mean of three replicate experiments and the symbol (*) represents significance compared to the control ($P < 0.05$). **B**: Lower sternal chondrocytes were infected with either an empty RCAS virus, or viruses expressing wild-type (WT) or dominant (DN) Runx2 as described in Materials and Methods. Cultures were harvested and alkaline phosphatase measured as described in Materials and Methods. The symbol (*) represents significance compared to the control (Fig. 2A; $P < 0.05$).

Runx2 Is Necessary for *colX* Induction by BMP2 and RA

We examined the possibility that Runx2 contributes to BMP2- and RA-induced chondrocyte differentiation. Chick caudal sternal chondrocytes were infected with the replication competent chicken retrovirus, RCAS, expressing either WT or dominant negative Runx2 in a series of gain and loss of function experiments. In the presence of control (empty) RCAS virus, there was absent *colX* expression in 8-day cultures, while addition of BMP2 (50 ng/ml) or RA (100 nM) was associated with the induction of *colX* expression. As previously shown, the induction was greater with BMP than with RA [Li et al., 2003] (Fig. 2A).

Over-expression of WT Runx2-induced *colX* expression under basal conditions and further enhanced the expression of *colX* in BMP2 and RA-treated cultures. In contrast, over-expression of dominant negative Runx2 completely inhibited the expression of *colX* in both BMP2 and RA-treated chondrocyte cultures. Thus, gain of Runx2 function results in spontaneous maturation and enhances the effects of BMP2 and RA, while loss of function inhibits these effects (Fig. 2A).

Prior work has demonstrated that the BMP-related Smad transcription factors can also induce chondrocyte differentiation [Li et al., 2003]. In order to determine if there is a cooperative interaction between Smads and Runx2, we investigated the effect of Runx2 and Smad1 over-expression alone and in combination on *colX* expression. Both Runx2 and Smad over-expression equally induce colX expression in 8-day cultures. However, a synergistic effect is observed when both transcription factors are co-expressed (Fig. 2B) indicating that Runx2 and Smad1 cooperatively induce cell differentiation of chick lower sternal chondrocytes. Altogether the findings suggest that Runx2 enhances Smad1 effects on chondrocyte differentiation and that basal Runx2 effects are necessary for BMP2 mediated chondrocyte differentiation.

Runx2 Enhances BMP-Effects at the Collagen Type X Promoter

A BMP responsive region of the chicken colX promoter has previously been identified and we used this b2-640 colX promoter fragment to further investigate the cooperative effect

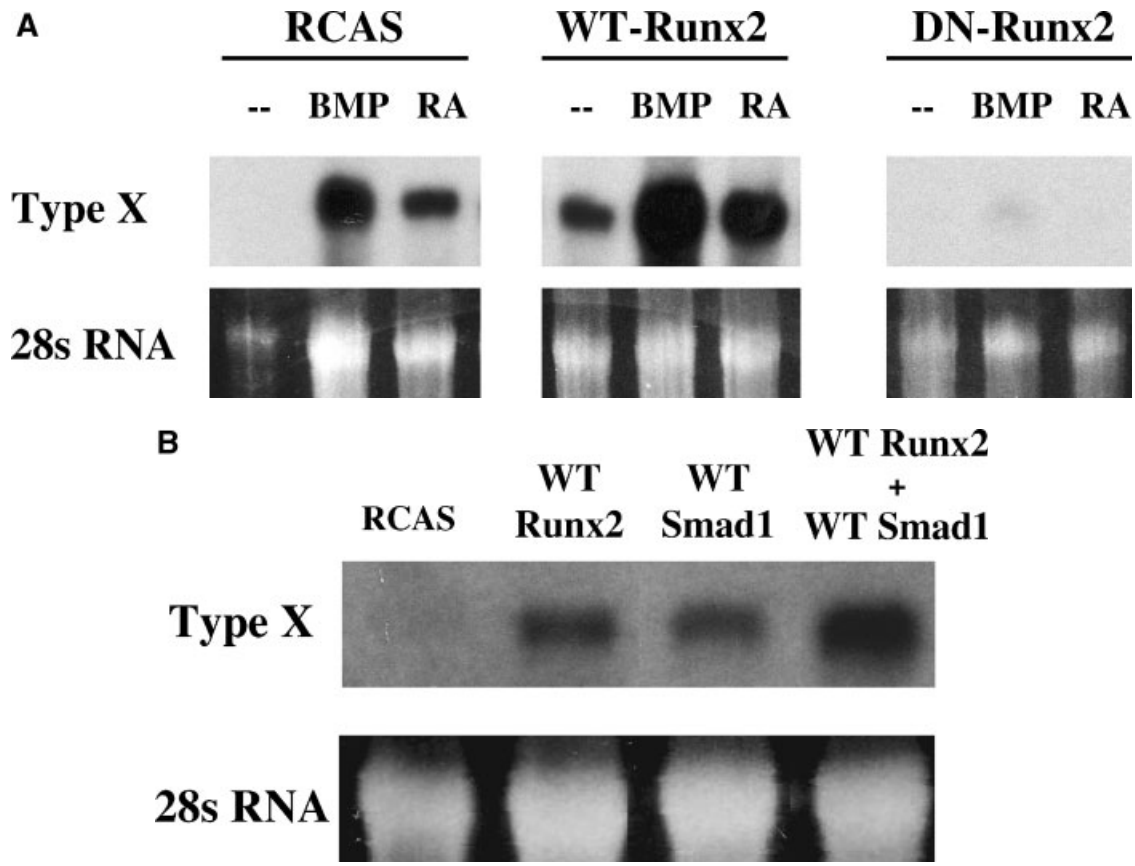

Fig. 2. Runx2 stimulates chondrocyte differentiation. The cultures were then treated with either control medium or medium containing RA (100 nM) or BMP2 (50 ng/ml) continuously for 8 days as indicated in the figures. **A**: Cultures were harvested and *colX* expression assessed by Northern blot. **B**: Cultures were infected with either RCAS viruses expression WT Runx2 or WT Smad1 alone or in combination and *colX* expression assessed by Northern blot. The 28S rRNA was used as a loading control.

between BMP signaling and Runx2 [Volk et al., 1998]. The promoter has a putative Smad response element between nt −2,572 and nt −2,569 and a Runx2 consensus sequence in a region between nt −2,359 and nt −2,354 (Fig. 3A). Runx2 over-expression markedly enhanced both basal and BMP-induced activation of the promoter. In RCAS-infected controls BMP2 induced the type X collagen promoter by 2.5-fold. In contrast, Runx2 over-expression induced promoter activity fivefold, and the addition of BMP2 to Runx2 over-expressing cultures resulted in a 23-fold induction compared to basal levels (Fig. 3B). These results clearly show that Runx2 acts in a cooperative manner with BMP2 to induce type X collagen transcription.

Smad1 Enhances RA-Effects at the Collagen Type X Promoter

Since RA induces Runx2 (Fig. 1), we also examined whether over-expression of Smad1 would enhance the effects of RA on the type X collagen promoter. Similar to BMP2, a cooperative effect occurred between Smad1 and RA on the type X collagen b2/640 promoter. RA and Smad1 enhanced promoter activity in 8-day sternal chondrocyte cultures. While RA alone induced a threefold increase of promoter activity, Smad1 enhanced expression of the luciferase reporter gene (*LUC*) by 4.8-fold (Fig. 3C). Transcript induction was markedly enhanced in cultures simultaneously over-expressing Smad1 in presence of RA (21-fold) suggesting a cooperative synergy between the Smad responsive region on the type X collagen promoter and the Runx2 putative response element downstream of the Smad response domain (Fig. 3A).

Runx2 and Smad1 Cooperatively Transactivate Specific Regions of the b2/640 Type X Collagen Promoter

To directly examine cooperation between Smad1 and Runx2 at the colX promoter, we

selectively eliminated the 20 bp of the GC-rich Smad binding sequence at nt −2,553 to nt −2,572 by substitution with the bacterial Gal4 sequence. A similar substitution was made for the putative Runx2 site between nt −2,359 and nt −2,354 (Fig. 4A). In cultures transfected with the WT promoter, over-expression of Smad1 and Runx2 alone induce luciferase activity and when Smad1 and Runx2 are co-transfected, the induction is synergistic (Fig. 4B,C). Mutation of the Smad binding region abolished responsiveness of the type X collagen promoter to both Smad1 and Runx2, suggesting that the Smad binding site is necessary for Runx2 activity (Fig. 4B). Similarly, mutation of the putative Runx2 binding site eliminated induction following Smad1 and Runx2 transfection alone and in combination (Fig. 4C), and also eliminated induction in RA and BMP2 treated cultures (data not shown). Our results clearly demonstrate a

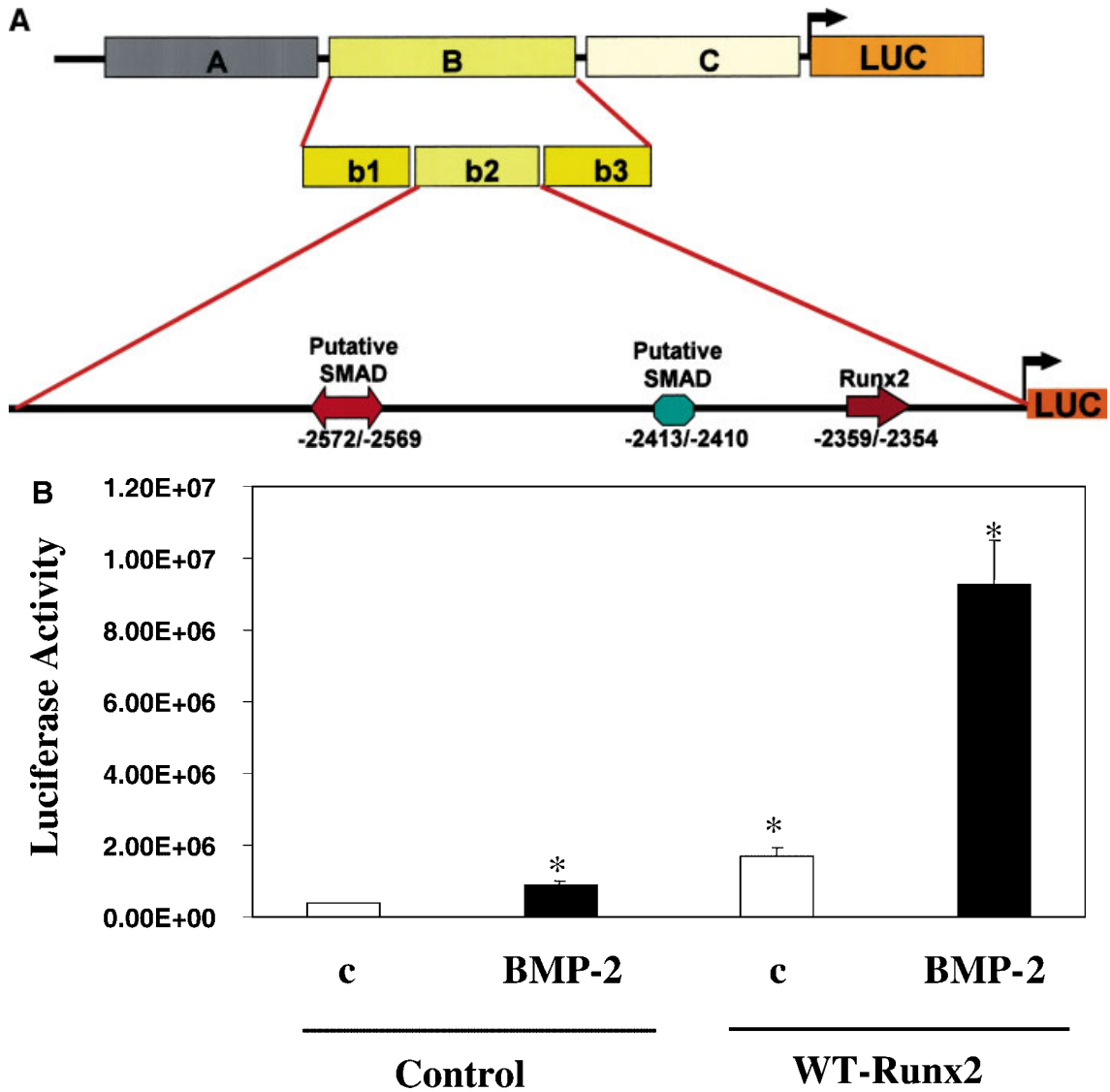

Fig. 3. Runx2 and Smad1 enhance the induction of the type X collagen promoter by BMP2 and RA, respectively. Chondrocyte cultures were transiently co-transfected with either control plasmid or with plasmids expressing WT Runx2 or Smad1 and the type X collagen promoter after 24 h in culture as described in Materials and Methods. The cultures were subsequently continuously treated with either BMP2 (50 ng/ml) or RA (100 nM) for 48 h. **A**: Schematic representation of the collagen type X promoter driving the luciferase reporter gene (*LUC*). This promoter is organized into three regions A, B, and C. The BMP responsive region contains the b2/640 fragment of which carries putative Smad and Runx2 binding sites as indicated. **B**: Transfected cultures were treated with or without BMP2. **C**: Transfected cultures were treated with or without RA. The symbol (*) represents significance compared to the control (B and C; $P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

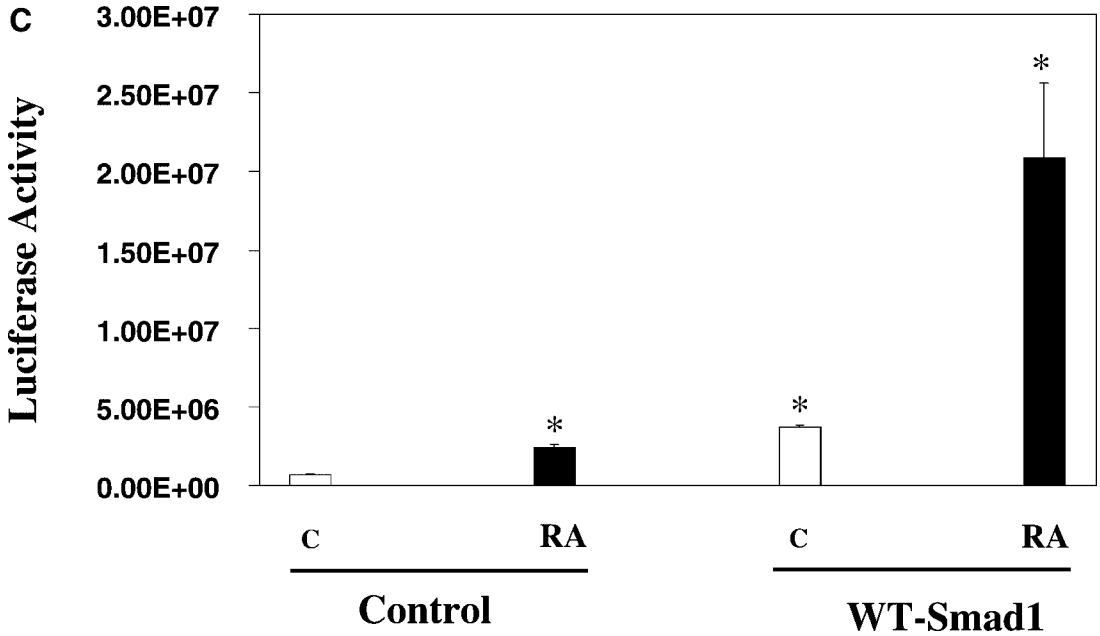

Fig. 3. (*Continued*)

cooperative function between the BMP signaling and Runx2 transcriptional regulation of chondrocyte differentiation.

DISCUSSION

Others and we have shown that stimulation of chondrocyte maturation by BMP is enhanced by RA [Iwamoto et al., 2003; Li et al., 2003]. In the present study, we show that RA induces Runx2, and furthermore that Runx2 is a necessary co-factor for chondrocyte differentiation in response to BMP2 signaling. While RA and BMP2 both induce chondrocyte maturation, this effect was absent in cells with loss of Runx2 function. In contrast, gain of Runx2 function enhanced the maturational effects of RA, BMP2, and Smad1. Finally, through a series of mutations of the type X collagen promoter, we demonstrated that both Runx2 and Smad binding regions are necessary and interdependent for activation of transcription. Thus, our results show that factors that regulate Smad or Runx2 expression or signaling control chondrocyte maturation and implicate RA as an important mediator of this process.

The lower sternum contains a cell population that exhibits delayed differentiation compared to the upper sternal chondrocytes and thus is a useful model to examine factors that accelerate this process [Pateder et al., 2001]. Previous studies have shown that BMP2 up-regulates transcription of the bone related (MASNS) isoform of Runx2 in both osteoblasts and chondrocytes [Stewart et al., 1997; Gori et al., 1999; Drissi et al., 2000; Takazawa et al., 2000; Li et al., 2003]. However, little is known about the mechanisms by which RA induces chondrocyte differentiation. We assessed the expression of chicken Runx2 by real-time RT-PCR using specific primers for the (MASNS) isoform and found that RA potently induces transcriptional levels of Runx2 after 8 days of culture and to a higher extent at 12 days. In primary isolates of chondrocytic cells from the rat rib RA also induces Runx2, but this is associated with a

Fig. 4. The Smad and Runx binding sites are essential for transcriptional activity of the type X collagen promoter. **A**: The b2/640 type X collagen promoter construct was mutated with replacement of the Smad and Runx2 binding sites with Gal4 sequences as described in Materials and Methods. **B**: Cultures were co-transfected with either WT or a mutated promoter lacking the Smad binding region and WT Smad1 and Runx2 alone or in combination as described in Materials and Methods. **C**: Cultures were co-transfected with either WT or a mutated promoter lacking the Runx2 binding region and WT Smad1 and Runx2 alone or in combination as described in Materials and Methods. The symbol (*) represents significance compared to the control (B and C; $P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

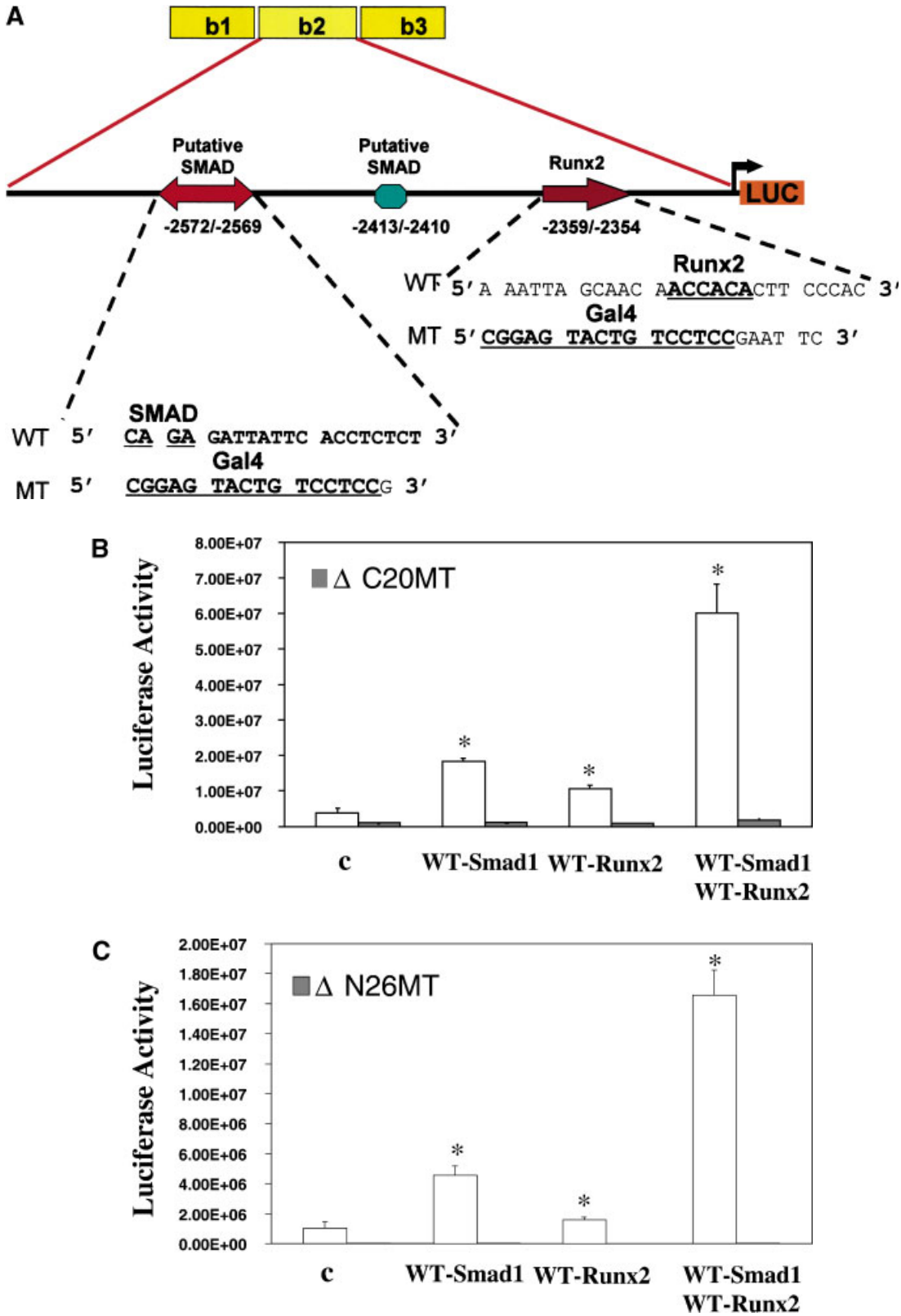
A
b1
b2
b3
Putative SMAD
Putative SMAD
Runx2
LUC
-2572/-2569
-2413/-2410
-2359/-2354
Runx2
WT 5′ A AATTA GCAAC AACCACACTT CCCAC 3′
Gal4
MT 5′ CGGAG TACTG TCCTCCGAAT TC 3′
SMAD
WT 5′ CA GA GATTATTC ACCTCTCT 3′
Gal4
MT 5′ CGGAG TACTG TCCTCCG 3′
B
Luciferase Activity
8.00E+07
7.00E+07
6.00E+07
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
Δ C20MT
c
WT-Smad1
WT-Runx2
WT-Smad1 WT-Runx2
C
Luciferase Activity
2.00E+07
1.80E+07
1.60E+07
1.40E+07
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
Δ N26MT
c
WT-Smad1
WT-Runx2
WT-Smad1 WT-Runx2

Fig. 4.

decrease in *col2* and an induction in bone cell differentiation [Jimenez et al., 2001]. In contrast our data shows that RA induces chondrocyte differentiation and suggests that Runx2 may mediate this event.

Runx2 over-expression induced chick sternal chondrocyte maturation as measured by increased alkaline phosphatase activity and enhanced *colX* expression. In contrast, dominant negative Runx2 over-expression abrogated the expression of chondrocyte differentiation markers. Iwamoto et al. [2003] also found induction of Runx2 by RA and reported enhanced maturation in chondrocyte cultures over-expressing chick Runx2 and delayed maturation in cultures over-expressing dominant negative Runx2. Previously we showed in this culture model that RA induces *colX* only after 8 days of continuous treatment [Li et al., 2003]. Here we demonstrate that the induction of Runx2 is also only apparent following 8 days of treatment, and thus show a temporal association between the induction of *Runx2* by RA and the onset of chondrocyte maturation. More importantly, we show that the ability of both BMP2 and RA to stimulate chondrocyte differentiation is absent in the cultures with loss of Runx2 function. Thus Runx2 is a required cofactor for chondrocyte differentiation in this model. These findings were confirmed in experiments in which the Runx binding site was eliminated from the type X collagen promoter.

Prior work in our laboratory has shown that RA increases the protein levels of Smad1 and Smad5, thus enhancing chondrocyte responsiveness to BMPs [Li et al., 2003]. Other studies have reported responsiveness of the b2 fragment of the colX promoter to both Smad1/5 and Runx2 in upper sternal chondrocytes. However, significant induction of the promoter required stimulation with BMP proteins [Leboy et al., 2001]. Here, using less mature chondrocytes, we show that Smad1 alone is sufficient to activate the type X collagen promoter. Furthermore, there is strong cooperation between Smad1 and Runx2 in inducing both the promoter and the endogenous transcript in chondrocytes from the chick lower sternum. Collectively, our data shows that Smad1 alone is sufficient to induce the type X collagen promoter as long as there is basal Runx2 signaling, and suggest that less mature chondroprogenitors are more sensitive to signals that stimulate differentiation.

These data show that both Smad and Runx binding sites are required for activation of the collagen type X promoter by BMP2 and RA. Smad proteins have been previously shown to interact with Runx family members in various cell systems [Hanai et al., 1999; Lee et al., 2000]. Two molecular mechanisms may be responsible for the cooperation between Smad1 and Runx2 on the collagen type X promoter. The first involves a direct interaction of the factors while associated their respective DNA consensus binding sites, while the second mechanism would involve other associated intermediary factors, the identity and role of which requires further investigation. Taken together, our findings define Runx2 and BMP-mediated Smad signaling as critical molecular events that converge to induce chondrocyte differentiation. Defining the factors that target these signaling events and the interaction of these signals with other important modulators of chondrocyte differentiation will provide additional insights into endochondral bone formation.

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