Hedgehog signaling enhances core-binding factor a1 and receptor activator of nuclear factor- κ B ligand (RANKL) gene expression in chondrocytes

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Abstract

Hedgehog signaling is considered to play a crucial role in chondrogenesis by regulation through a network of cytokine actions, which is not fully understood. We examined the effect of hedgehog signaling on the expression of core-binding factor a1 (Cbfa1), a critical transcription factor for the development of bone and cartilage. Primary chondrocytes prepared from the costal cartilage of newborn mice were treated with N-terminal fragment of recombinant murine sonic hedgehog (rmShh-N). Northern blot analysis indicated that Cbfa1 mRNA expression levels in the chondrocyte cultures were elevated by the treatment with rmShh-N. rmShh-N treatment enhanced 1.8 kb Cbfa1 promoter activity in chondrocytes, suggesting the presence of transcriptional control. As

Cbfa1-binding site(s) have been located in the promoter of the receptor activator of nuclear factor- κ B (RANK) ligand (RANKL) gene, we also examined RANKL expression. rmShh-N treatment upregulated RANKL and RANK mRNA expression levels in chondrocytes. Interestingly, RANKL suppressed the hedgehog enhancement of alkaline phosphatase activity in chondrocytes, suggesting the presence of a link between these signaling molecules. We conclude that hedgehog signaling activates Cbfa1 gene expression through its promoter in chondrocytes, and also activates and interacts with RANKL to maintain cartilage development.

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Introduction

Hedgehog signaling controls chondrogenesis through a network of cytokine actions. Chondrocytes are derived from mesenchymal cells and undergo a program of proliferation, hypertrophy, calcification and cell death (Erlebacher *et al.* 1995). *Indian hedgehog* (Ihh), which encodes a member of the hedgehog family of signaling factors, is expressed in chondrocytes within the early cartilaginous elements, and its expression progressively becomes restricted to prehypertrophic chondrocytes (Bitgood & McMahon 1995).

Ectopic expression of Ihh in developing chick long bones induces upregulation of parathyroid hormone-related peptide (PTHrP) in the articular perichondrium, which in turn suppresses differentiation of chondrocytes and ossification (Vortkamp *et al.* 1996). In fact, chondrocyte differentiation is enhanced in mice lacking PTHrP or its receptor (Karaplis *et al.* 1994, Lanske *et al.* 1996).

Furthermore, direct addition of hedgehog protein to limb cultures also suppresses chondrocyte differentiation (Lanske *et al.* 1996, Vortkamp *et al.* 1996). These data suggest that Ihh and PTHrP compose a signaling system to regulate cartilage development. Such regulation takes place not only in the developmental process but also during postnatal bone growth (Vortkamp *et al.* 1998, Farquharson *et al.* 2001) and fracture repair (Ferguson *et al.* 1999). Although Ihh enhances PTHrP expression, it is also reported that Ihh promotes chondrocyte proliferation in a pathway that cannot be attributed to PTHrP signaling (Karp *et al.* 2000). Thus, the target molecules for the hedgehog signaling in cartilage have not yet been fully understood.

Core-binding factor a1 (Cbfa1), a runt domain transcription factor, is prerequisite for the development of bone as well as cartilage (Komori *et al.* 1997, Otto *et al.* 1997, Inada *et al.* 1999, Kim *et al.* 1999). Cbfa1-deficient mice lack bone formation and die immediately after

birth (Komori *et al.* 1997, Otto *et al.* 1997). In these mice, terminal differentiation of chondrocytes is severely disturbed and most chondrocytes remain in the resting and proliferating zones in the femur (Inada *et al.* 1999, Kim *et al.* 1999). *Cbfa1* is expressed at high levels in the regions where mesenchymal condensation occurs around 12.5 days post-coitum. After that, *Cbfa1* expression levels in chondrocytes decline and become largely specific to osteoblasts. In chondrocytes, overexpression of *Cbfa1* induces alkaline phosphatase activity, expression of type X collagen, and calcification (Enomoto *et al.* 2000). When *Cbfa1* is overexpressed in chondrocytes in transgenic mice using type II collagen promoter, chondrocyte hypertrophy and endochondral ossification occur ectopically (Takeda *et al.* 2001, Ueta *et al.* 2001). Thus, *Cbfa1* has the ability to induce the appearance of prehypertrophic chondrocytes expressing *Ihh*.

Cbfa1 appears to regulate several downstream genes encoding proteins related to osteoblastic phenotypes. *In vitro* studies indicated that *Cbfa1* induced the expression of alkaline phosphatase, type I collagen, osteopontin, bone sialoprotein, osteocalcin, and type III collagen (Ducy *et al.* 1997, Harada *et al.* 1999, Jiménez *et al.* 1999). The regulatory regions of the genes encoding these proteins contain *Cbfa1*-binding sites and in fact are transcriptionally regulated by *Cbfa1* during embryonic development (Ducy *et al.* 1999). In addition, *Cbfa1* also enhances expression of receptor activator of nuclear factor (NF)- κ B (RANK) ligand (RANKL) in ATDC5 cells (Akiyama *et al.* 1999), suggesting its role in coupling between osteoblasts and osteoclasts. These data indicate the importance of *Cbfa1* as a key regulatory transcription factor in skeletogenesis.

Recently, *Ihh* knock-out mice have been created and these mice lack endochondral bone formation (St-Jacques *et al.* 1999). In the perichondrium/periosteum of these mice, *Cbfa1* expression was not observed. These observations suggest that *Cbfa1* could be one of the target molecules of hedgehog signaling in chondrogenesis, but direct evidence has not yet been obtained.

As cartilage development requires degradation of skeletal matrix by chondroclasts/osteoclasts, hedgehog may regulate the expression of factors involved in osteoclastogenesis. However, the role of hedgehog in the regulation of chondrocyte differentiation has not yet been fully understood with respect to cartilage development. Therefore, we examined the downstream target molecules of the hedgehog signaling system in chondrocytes, focusing on the *Cbfa1* gene.

Materials and Methods

Cell culture

Our experiments were conducted in accordance with the guidelines of the animal care committee of Tokyo Medical and Dental University.

Chondrocytes were isolated from costal cartilage of newborn ICR mice (Lefebvre *et al.* 1994) through enzymatic digestion in 3 mg/ml collagenase (Sigma) for 50 min and then further digestion in 3 mg/ml collagenase for 5 h. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and antibiotics at 37 °C in an atmosphere of 5% CO₂. N-terminal fragment of recombinant murine sonic hedgehog peptide (rmShh-N) was purchased from R & D systems (Minneapolis, MN, USA). Recombinant human bone morphogenetic protein (rhBMP)-2 was obtained from Yamanouchi Pharmaceuticals (Tokyo, Japan). Recombinant soluble RANKL was provided by Snow Brand Milk Products Co. (Tokyo, Japan).

Northern blot analysis

Chondrocytes were plated at 1.3×10^5 cells/cm². Five days later, the medium was changed to a new one supplemented with 1% FBS and the cells were cultured in the presence or absence of 1 µg/ml rmShh-N. The dosage of hedgehog and the length of treatment were selected according to the previously published condition (Murtaugh *et al.* 1999). Four days later, total cellular RNA was prepared according to the acid guanidinium thiocyanate-phenol-chloroform method as described previously (Chomczynski *et al.* 1987). Aliquots of 10 µg total RNA per lane were electrophoresed on 1.0% agarose gels containing 0.66 M formaldehyde and transferred to nylon filters (NEN LifeScience Products, Boston, MA, USA) by electroblotting. Filters were prehybridized in hybridization buffer (50% formamide, 250 mM sodium phosphate pH 7.2, 25 mM sodium chloride, 0.5% sodium dodecyl-sulfate, 0.2 mg/ml herring sperm DNA, 10% polyethylglycol MW6000, 10 × Denhardt's solution (0.4% Ficoll type 400, 0.4% polyvinyl-pyrrolidone, 0.4% BSA fraction V)) for 24 h at 42 °C. A 1.3 kb Nco I fragment of the murine *Cbfa1* cDNA, which contains almost all the C-terminal fragment of the coding region, was used as a probe. A 1.6 kb EcoRI-BglII fragment of the mouse *Sox9* cDNA was also used and a 1.2 kb EcoRI fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (1.2 kb) was used as a control. Each DNA was labeled using the BcaBEST random primer labeling kit (Takara Shuzo Co. Ltd, Tokyo, Japan) and [α -³²P]dCTP (Amersham Biosciences, Piscataway, NJ, USA). Hybridization was performed at 42 °C for 18 h. Filters were washed twice in 2 × SSC and 0.5% SDS for 10 min at room temperature and then twice in 0.2 × SSC and 0.5% SDS for 10 min at 65 °C. Filters were exposed to X-ray films using intensifying screens at -80 °C.

Luciferase assay

For transfection, primary chondrocytes were plated at a density of 7×10^4 cells/cm². One day later, chondrocytes

were transfected with 1 µg reporter plasmid containing 1.8 kb *Cbfa1* promoter fragment starting -1825 bp upstream from the transcription start site linked to luciferase gene (Fujiwara *et al.* 1999). Transfections were performed using FuGENE6 (Roche, Indianapolis, IN, USA) and the cells were cultured in DMEM supplemented with 1% FBS in the presence or absence of 1 µg/ml rmShh-N for 72 h. The effect of hedgehog on *Cbfa1* gene promoter activity was evaluated by measuring luciferase activity using a Picagene kit (Toyo Ink Co., Tokyo, Japan) and a luminometer (Autolumat LB953; Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Protein concentrations in the cell lysates were determined according to the Coomassie Brilliant Blue G method as described previously (Spector 1978) and luciferase activity was normalized against protein concentration. Experiments were performed in triplicate with independent preparations of cell extracts. pGL2-control LUC-construct (Promega, Madison, WI, USA) was used as control.

The sequence of the *Cbfa1* promoter region was examined by computer-based analysis using DNASIS (Hitachi Software Engineering Co. Ltd, Kanagawa, Japan) and a Gli-binding sequence motif identical to an essential region of the minimal HNF3β floor plate enhancer (3'Gli-BS: 5'-GAACACCCA-3') (Sasaki *et al.* 1997) was found to exist between -258 and -250 bp upstream from the transcription start site. The luciferase reporter plasmids containing eight direct copies of 3'Gli-BS or m3'Gli-BS (mutated 3'Gli-BS sequence: 5'-GAAGTGGGA-3') linked to luciferase construct were transfected as described above. Expression vectors, pSRα-GLI1 and pSRα-GLI3, which express human GLI1 and GLI3 cDNAs under the control of the SRα promoter (Sasaki *et al.* 1997), were also transfected.

RT-PCR

Total RNA was extracted as described above. One microgram of total RNA was converted to cDNA using an oligo dT primer (Roche). Reverse transcription was performed by a 10 min preincubation of RNA/primer mixture at 65 °C, and then 60 min incubation with MMLV reverse transcriptase (Gibco BRL) at 37 °C.

PCR reaction was performed by repetition of the cycles consisting of 94 °C for 40 s, 60 °C for 60 s and 72 °C for 60 s. The reaction products were resolved by electrophoresis and visualized by ethidium bromide staining. The bands were quantified by densitometry and each value was normalized against that of the GAPDH band of the corresponding cDNA. As negative controls, RNA samples that were not subjected to reverse transcription were used. PCR cycles were chosen so that reactions were performed within an exponential phase of amplification. GAPDH bands were used as internal control. PCR primers and cycles used were as follows.

Patched-1 forward 5'-AACAAAAATTCAACCAAAC CTC-3', reverse 5'-TGTCTTCATTCCAGTTGATGT G-3' (Takabatake *et al.* 1997), 26 cycles; *Gli* forward 5'-CTGATTTTCAGGGAAGAGAGCAGACTGA-3', reverse 5'-CAAGCTTATGCAGCTGATCCAGCCTA-3' (Walterhouse *et al.* 1993), 29 cycles; *RANK* forward 5'-TCATCTCTGTGGTAGTAGTGGCTG-3', reverse 5'-TTAGGAGCAGTGAACCAGTCGAAG-3', 44 cycles; *RANKL* forward 5'-CAGCCATTTGCACA CCTCACCATC-3', reverse 5'-TTTCGTGCTCCCT CCTTTCATCAG-3', 41 cycles; *osteoprotegerin* forward 5'-GCACATTTGGCCTCTGCTAATTC-3', reverse 5'-ACTCTCGGCATTCACTTTGGTCCC-3', 41 cycles; *GAPDH* forward 5'-ACCACAGTCCATGCC ATCAC-3', reverse 5'-TCCACCACCCTGTTGCTG TA-3', 20 cycles.

Cell growth

Primary chondrocytes were plated into four-well plates at a density of 2×10^4 cells/cm² and cultured for 2 days. Cells were further cultured in DMEM supplemented with 1% FBS in the presence or in the absence of 1 µg/ml rmShh-N and 1 µg/ml rhBMP-2 for 4 days. Each cell number per well was counted using a Coulter Counter (Beckman Coulter Co., Fullerton, CA, USA).

Alkaline phosphatase activity assay

Primary chondrocytes were plated into 96-well plates at the density of 130 000 cells/cm² and cultured for 5 days. Cells were treated with 1 µg/ml rmShh-N or 200 ng/ml RANKL or both and further cultured for 14 days. Cells were then subjected to alkaline phosphatase activity assay.

Cells were rinsed with saline twice and incubated with an aliquot of 50 µl cell lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, 0.1% triton X-100) on ice for 5 min, and scraped. The lysates were subjected to freezing and thawing for several times. An aliquot of 10 µl cell lysate was transferred to another plate, and incubated with 40 µl alkaline phosphatase assay mixture (6.6 mM MgCl₂, 6.6 mM p-nitrophenyl phosphate, 0.3 M 2-amino-2-methylpropanol) for 30 min at room temperature. The alkaline phosphatase activity in each well of the plates was quantified using a plate reader (BioRad Co., Tokyo, Japan), by measuring absorbance at a wavelength of 410 nm. The rest of the cell lysates were subjected to reaction with a protein assay mixture (BioRad Co.) and the protein concentrations were evaluated by measuring absorbance at a wavelength of 595 nm. The alkaline phosphatase activity levels were normalized against the protein levels.

Statistical analysis

For luciferase assay, cell growth evaluation and alkaline phosphatase assay, experimental results are expressed as

Figure 1 Gli and Patched-1 mRNA expression and their regulation by hedgehog in chondrocytes. Primary chondrocytes were plated at a density of 1.3×10^5 cells/cm². Five days later, the cells were treated with 1 µg/ml rmShh-N (SHH) or BSA (control) and cultured for 4 days. Total RNA was extracted and RT-PCR was performed using primers for *Patched-1* and *Gli*. A *GAPDH* primer set was used as internal control. Quantification was conducted based on densitometry and the values were normalized against *GAPDH*. Experiments were repeated twice, and representative data are shown. Treatment enhanced normalized values for *Gli* and *Patched-1* mRNA expression 1.52- and 3.07-fold respectively.

means $\pm$ S.D. Statistical significance of the differences was evaluated by ANOVA followed by the Fisher's PLSD method. *P* values less than 0.05 were considered to be statistically significant.

Results

Hedgehog signaling upregulates Cbfa1 mRNA levels in primary cultures of chondrocytes

We confirmed first that the primary cultures of chondrocytes express receptors for hedgehog signaling using RT-PCR. Hedgehog binds to its receptor Patched-1, which leads to the activation of transcriptional effectors, zinc finger-containing proteins of the Gli family (Marigo *et al.* 1996a,b). Patched-1 transcript was expressed constitutively, and its level was enhanced by hedgehog treatment, as reported previously (Vortkamp *et al.* 1996) (Fig. 1). Gli gene was also expressed constitutively, and hedgehog treatment upregulated its level (Fig. 1). These data revealed that the hedgehog signaling system was operating in these cells.

Examination of *Cbfa1* mRNA expression indicated that these chondrocytes expressed 6.5 kb *Cbfa1* mRNA constitutively (Fig. 2a). Treatment with hedgehog upregulated the *Cbfa1* mRNA levels. To examine the selectivity of the hedgehog effects on *Cbfa1*, we also examined the expression of Sox9, one of the transcription factors prerequisite for chondrocyte differentiation. In these chondrocytes, Sox9 mRNA was constitutively expressed, and its level was not altered by the treatment with hedgehog (Fig. 2b), indicating that the effect of hedgehog

Figure 2 *Cbfa1* and Sox9 mRNA expression and their regulation by hedgehog in chondrocytes. (a) Primary chondrocytes were plated at a density of 1.3×10^5 cells/cm². Five days later, the cells were treated with 1 µg/ml rmShh-N (SHH) or BSA (control) and cultured for 4 days and total RNA was extracted. The aliquot of 10 µg RNA per lane was electrophoresed and transferred to nylon membrane. Northern hybridization was performed using ³²P-labeled *Cbfa1* probes. 18S ribosomal RNA served as an RNA-loading control. Experiments were repeated four times, and representative data are shown. (b) Northern hybridization was performed with ³²P-labeled Sox9 probe. 28S ribosomal RNA served as an RNA-loading control.

on *Cbfa1* is selective and not an overall upregulation of any genes in the cells.

Hedgehog signaling enhances transcriptional activity of the Cbfa1 gene promoter in primary chondrocytes

To elucidate the mode of hedgehog action in the regulation of *Cbfa1* gene expression, *Cbfa1* promoter fragment

Figure 3 Regulation of 1.8 kb *Cbfa1* promoter activity by hedgehog. Primary chondrocytes were plated at a density of 7×10^4 cells/cm². One day later, cells were transfected with the 1.8 kb *Cbfa1* promoter fragment linked to luciferase gene (1.8 kb *Cbfa1*). Cells were cultured in the presence (SHH) or absence (C) of 1 µg/ml rmShh-N for 3 days. Luciferase activity was measured using the Picagene kit and a luminometer. PGL2 control vector was used as a control. Representative data are shown. Data are means $\pm$ S.D. from six cultures. * $P < 0.05$.

Figure 4 Activation of Gli-binding sites by gli in chondrocytes. Chondrocytes were co-transfected with Gli (Gli-1 or Gli-3) expression vectors and a reporter construct containing HNF3β-Gli-binding site (GAACACCCA) linked to luciferase (HNF3β-Gli-BS-Luc). A mutant construct, in which mutation was introduced into HNF3β-Gli binding sequence (GAAGtgggA), was also used (mHNF3β-Gli-BS-Luc). The cells were cultured for 3 days and luciferase activity was measured. Representative data are shown. Data are means $\pm$ S.D. from six cultures. * $P < 0.05$.

linked to luciferase gene was transfected into chondrocytes and the cells were cultured in the presence or in the absence of hedgehog. Luciferase assay indicated that the 1.8 kb *Cbfa1* promoter activity was enhanced by the hedgehog signaling in chondrocytes and this was a specific effect as control pGL2 activity was not altered by hedgehog (Fig. 3).

Figure 5 The effect of hedgehog and BMP on chondrocyte proliferation. Primary chondrocytes were plated at a density of 2×10^4 cells/cm². Two days later, cells were treated with 1 µg/ml rmShh-N (SHH) or 1 µg/ml rhBMP-2 (BMP) and cultured for 4 days. Cell number was determined using a Coulter counter. Representative data are shown. Data are means $\pm$ S.D. from eight cultures. * $P < 0.05$.

Gli-binding site is functional in chondrocytes

Sequence analysis revealed the presence of a 9 base-consensus Gli-binding sequence between -258 and -250 bp upstream from the transcription start site of the *Cbfa1* promoter. As Gli gene was expressed in the primary cultures of chondrocytes (Fig. 1), it seems to mediate hedgehog signaling in these cells. To examine whether the Gli-binding sequence is functional in these chondrocytes, we co-transfected Gli-1 expression vector constructs and a reporter containing Gli-binding sites into the chondrocytes. The luciferase activity was enhanced by the co-transfection with Gli-1 expression vector (Fig. 4). This was a sequence-specific effect, since the luciferase activity was not altered when mutation was introduced into the Gli-1-binding site (Fig. 4). In addition, this effect was specific to Gli-1 since Gli-3 expression vector did not enhance luciferase activity (Fig. 4).

Hedgehog signaling augments BMP effect on cell growth

To examine whether hedgehog has any effect on proliferation of these chondrocytes, the cells were grown in the presence or in the absence of hedgehog. Hedgehog had no significant effect on cell proliferation, but augmented the effect of BMP, which enhanced chondrocyte proliferation by itself (Fig. 5).

Hedgehog enhances RANKL mRNA expression in chondrocytes

Cartilage development requires concerted actions of chondrocytes and chondroclasts for formation and degradation

Figure 6 RANK and RANKL expression and their regulation by hedgehog in chondrocytes. Chondrocytes were plated at a density of 1.3×10^5 cells/cm². Five days later, the cells were treated with 1 µg/ml rmShh-N (SHH) or BSA (control) and cultured for 4 days. Total RNA was extracted and RT-PCR was performed using primers for RANK and RANKL. A GAPDH primer set was used to detect GAPDH bands as internal control. Quantification was conducted based on densitometry and the values were normalized against GAPDH. Experiments were repeated twice, and representative data are shown. Hedgehog treatment enhanced normalized values for RANK and RANKL mRNA expression 1.82- and 1.79-fold respectively.

respectively. To further examine the mechanism by which hedgehog signaling regulates cartilage development, we also examined expression of factors involved in the regulation of matrix degradation. We found that RANKL, which is a critical activator for osteoclast differentiation, was constitutively expressed in chondrocytes (Fig. 6), and that hedgehog moderately enhanced its expression. Interestingly, RANK (a receptor for RANKL) mRNA expression was not observed in the chondrocytes, while hedgehog treatment induced RANK mRNA expression in these cells. Osteoprotegerin mRNA level was not altered by the hedgehog treatment (data not shown). Thus, hedgehog effects on RANKL and RANK mRNA were selective to these genes.

RANKL suppresses the hedgehog effect on alkaline phosphatase activity

To examine whether RANKL has any effect on chondrocyte function, alkaline phosphatase activity assay was performed. Hedgehog treatment enhanced alkaline phosphatase activity in these chondrocytes (Fig. 7). RANKL suppressed this hedgehog effect (Fig. 7). BMP also enhanced alkaline phosphatase activity in these chondrocytes; however, RANKL did not reduce such BMP-induced alkaline phosphatase activity, suggesting specificity of RANKL action on the hedgehog effect. BMP and hedgehog additively enhanced alkaline phosphatase activity, and in the presence of both hedgehog and BMP, RANKL also suppressed alkaline phosphatase activity, but suppression was partial and alkaline

Figure 7 The effect of RANKL, hedgehog, and BMP on alkaline phosphatase activity in chondrocytes. Chondrocytes were plated into 96-well plate at a density of 1.3×10^5 cells/cm². Five days later, cells were treated with 200 ng/ml soluble RANKL and/or 1 µg/ml rmShh-N (SHH) and/or 1 µg/ml rhBMP-2 (BMP), and cultured for 14 days. Cell lysates were prepared and 10 µl of them were subjected to alkaline phosphatase assay using an alkaline phosphatase assay mixture. The reaction activity of each well in the plates was quantified with a plate reader, by measuring absorbance at a wavelength of 470 nm. The alkaline phosphatase activity levels normalized against protein levels were analyzed. Representative data are shown. Data are means $\pm$ S.D. from five cultures. * $P < 0.05$.

phosphatase level was similar to that after the BMP treatment alone.

Discussion

We demonstrate here that hedgehog upregulates *Cbfa1* gene expression. This hedgehog action was through the activation of the transcriptional activity of the 1.8 kb fragment of *Cbfa1* gene that leads to subsequent *Cbfa1* mRNA expression in chondrocytes. Chondrocyte differentiation occurs through a multistep pathway, and *Ihh* expressed predominantly in prehypertrophic chondrocytes enhances the expression of PTHrP in periarticular perichondrium to regulate the rate of hypertrophic chondrocyte differentiation. However, *Ihh*-deficient mice do not show bone collar formation, and such absence of bone collar formation correlates with an absence of *Cbfa1* expression in the perichondrium/periosteum, and possibly not directly related to PTHrP regulation. *Cbfa1*-deficient mice show impairment in osteoblast differentiation as well as terminal differentiation of the growth plate chondrocytes (Inada *et al.* 1999), indicating that *Cbfa1* also plays an important role in chondrogenesis. Our data indicated that *Cbfa1* is one of the targets of hedgehog signaling in regulation of chondrocyte differentiation.

The hedgehog regulation of *Cbfa1* gene expression depends on the enhancement of the 1.8 kb *Cbfa1* promoter activity. This fragment reveals promoter activity in various cells (Fujiwara *et al.* 1999). We found the presence of a Gli-binding consensus sequence in this region. In addition, a reporter gene containing a Gli consensus sequence is functional in chondrocytes. As the hedgehog treatment was conducted for 4 days, whether the changes in *Cbfa1* expression could be either direct results of hedgehog signaling via Gli or other secondary consequences of phenotypic changes induced by hedgehog treatment is still to be elucidated.

Hedgehog signaling seems to interact with BMP signaling in regulation of several systems (Murtaugh *et al.* 1999, Pathi *et al.* 1999, Grimsrud *et al.* 2001), and *Cbfa1* expression can be induced by BMPs in C3H10T1/2 fibroblasts (Ducy *et al.* 1997). We also observed that BMP enhances proliferation and alkaline phosphatase activity in chondrocytes. However, BMP neither altered the expression level of *Cbfa1* mRNA nor enhanced promoter activity (data not shown), revealing that in the primary cultures of chondrocytes, hedgehog signaling may act through a pathway independent of BMP signaling as regards the regulation of *Cbfa1*.

We also showed that in the primary chondrocytes, hedgehog signaling upregulated RANK and RANKL mRNA levels. RANKL, expressed on the surface of osteoblasts, is a potent stimulator of osteoclastogenesis and osteoclast activity (Lacey *et al.* 1998, Yasuda *et al.* 1998). It is also expressed in hypertrophic chondrocytes of the growth plate (Lacey *et al.* 1998), and its receptor RANK also detected at a somewhat lower level in hypertrophic chondrocytes (Hsu *et al.* 1999). It was reported that hedgehog upregulates RANKL expression in ATDC5 cells (Akiyama *et al.* 1999), while no previous papers referred to RANK. RANK mRNA, which is not expressed in a basal state, is induced by hedgehog treatment. Hedgehog upregulated RANKL expression, while RANKL suppressed alkaline phosphatase activity induced by hedgehog signaling. These data revealed that hedgehog signaling plays a role not only in positive regulation through *Cbfa1* but also in negative regulation. Since the promoter structure of RANKL gene contains a *Cbfa1* binding domain (Kitazawa *et al.* 1999), hedgehog effects on RANK and RANKL could be in part mediated through *Cbfa1* in primary chondrocytes. Thus, hedgehog and RANKL actions are mutually related in regulating cartilage development. It is possible that RANKL expression by chondrocytes is involved in osteoclast or chondroclast formation and/or activation which is necessary for cartilage resorption at the growth plate. The RANK expression by growth plate chondrocytes is intriguing, although its role is not obvious at this point.

A dual action of Ihh has been suggested to be an important issue (Karaplis *et al.* 1997). The attachment of cholesterol moieties to the C-terminal region of the

cleaved N-terminal region of Ihh (Porter *et al.* 1996, Tabin *et al.* 1997) results in both its tethering to the plasma membrane and sequestration at the site of the Patched receptor. The resultant accumulation of hedgehog protein may lead, through short-range and PTHrP-independent effects, to the promotion of the terminally differentiated phenotype. These pro-differentiating actions of Ihh are in contrast to those elicited by the long-range diffusion of Ihh and activation of the PTHrP signaling pathway. These direct effects of Ihh are possibly related to the observations described in this paper. Such direct action of hedgehog has been also described in different cell systems such as ATDC5 (Akiyama *et al.* 1999) and micromass cultures of limb bud cells (Stott *et al.* 1997).

As chondrocytes have been reported to express PTHrP, we cannot exclude the possibility that the effect of hedgehog on cell number, alkaline phosphatase activity, and expression of RANK and RANKL could be indirect, possibly via PTHrP. It is intriguing if there is involvement of the two different pathways in our system.

Since the promoter structure of RANKL gene contains a *Cbfa1*-binding domain (Kitazawa *et al.* 1999), *Cbfa1* might mediate hedgehog effects on RANKL in primary chondrocytes. A recent study has shown that *Cbfa1* does not regulate RANKL gene activity in stromal/osteoblastic cells, although it binds to the *Cbfa1*-binding domain of the RANKL promoter (O'Brien *et al.* 2002), while the other observation reveals that overexpression of *Cbfa1* in the cells of the osteoblastic lineage increases RANKL expression (Geoffroy *et al.* 2002). It is possible that *Cbfa1* may regulate RANKL gene activity in a different manner from that of the other known targets of *Cbfa1*, such as osteocalcin. Although the effect of *Cbfa1* on RANKL expression in chondrocytes remains to be elucidated, hedgehog effects on RANK and RANKL could be in part mediated through *Cbfa1*.

We conclude that hedgehog signaling activates *Cbfa1* gene expression through its promoter region in chondrocytes and also activates RANKL gene expression to regulate the balance of the cartilage development. These targets of hedgehog signaling contribute to concerted regulation of both cartilage and bone formation during endochondral bone formation.

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