Editorial Review

Regulatory Mechanism of Osteoblast Differentiation and Bone Formation

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Osteoblast differentiation is regulated by various hormones and local factors such as estrogen, parathyroid hormone, bone morphogenetic proteins (BMPs) and hedgehogs. Estrogen-deficiency increases systemic factors that stimulate osteoblast differentiation, resulting in a high turnover bone remodeling with accelerated bone formation. Parathyroid hormone has diverse effects on osteoblast differentiation depending on the exposure time *in vitro* mediated through different signal transduction systems. Among local factors, BMPs are the most potent inducers and stimulators of osteoblast differentiation: BMPs not only stimulate osteoprogenitors to differentiate into mature osteoblasts but also induce non-osteogenic cells to differentiate into osteoblast lineage cells. Sonic and Indian hedgehogs also play important roles in regulation of osteoblast differentiation by interacting with BMPs. Cbfa1, a transcription factor belonging to the runt-domain gene family, is essential for osteoblast differentiation and bone formation because *Cbfa1*-deficient mice completely lack bone formation due to maturational arrest of osteoblasts. BMPs are important local factors that up-regulate Cbfa1 expression. The phenotype of heterozygous *Cbfa1* mutation is similar to that of cleidocranial dysplasia. The intimate interaction between the local factors including hedgehogs and BMPs as well as the transcription factor Cbfa1 play crucial roles in the process of osteoblast differentiation and bone formation. Further progress in bone research will provide information important not only in the field of cell biology but also for clinical research of various bone diseases.

***Key words*: osteoblast, bone, estrogen, PTH, BMP, Hedgehog, Cbfa1**

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Introduction

Osteoblasts, chondrocytes, myoblasts, and bone marrow stromal cells including adipocytes are believed to derive from common mesenchymal progenitors (1-7) called pluripotent mesenchymal stem cells (5-7). These progenitors exhibit specific phenotypes depending on the maturational stages during their differentiation processes. In the case of osteoblasts, they express phenotypic characteristics such as high alkaline phosphatase (ALP) activity, and synthesize collagenous and non-collagenous bone matrix proteins including osteocalcin (4). Formation of mineralized bones is the most important function of osteoblasts. This phenotypic expression is regulated by various hormones including parathyroid hormone (PTH) (8, 9), 1α, 25-dihydroxyvitamin D_3 [1α, 25 $(OH)_2D_3$] (10) and estrogen (11, 12). Various local factors produced by osteoblasts also regulate their own differentiation in a paracrine and/or an autocrine fashion (4). To investigate the roles of these hormones and local factors in osteoblast differentiation, various osteoblastic cell lines have been successfully established (13-17). Experiments using these *in vitro* assay systems have contributed to understanding the regulation of osteoblast differentiation by various hormones and local factors.

In the process of cell differentiation, many transcription factors are involved in the regulatory mechanism of each cell type, and cell lineage-specific transcription factors play crucial roles in determining each cell fate. These transcription factors have been identified in several cell lineages such as myoblasts and adipocytes: MyoD family in myoblasts (18) and PPARγ-2 in adipocytes (19). Recently, three research groups independently reported that Cbfa 1,which belongs to the runt-domain gene family, is an essential transcription factor for osteoblast differentiation and bone formation (20-22).

This review describes recent advances regarding the mechanism of osteoblast differentiation and its regulation by the systemic factors and local factors including

estrogen, parathyroid hormone (PTH), bone morphogenetic proteins (BMPs), hedgehogs, and the transcription factor Cbfa1.

I. Differentiation of osteoblasts

Osteoprogenitors are present in both skeletal and extraskeletal tissues in the post-natal state. In the skeletal tissues, osteoprogenitors locate in bone marrow and the periosteum. Friedenstein and colleagues first demonstrated that bone marrow cells harvested from confluent *in vitro* cultures of marrow cells retained the ability to form bones when cultured *in vivo* within diffusion chambers (23). They also showed that single cell-derived fibroblastic colonies, which have been called colony forming units-fibroblastic (CFU-F), retained osteogenic potential. Intermittent injection of PTH increased the number of CFU-F, which explain a potential mechanism involved in an anabolic effects of PTH on bone mass (24). The osteogenic potential of the periosteum was also shown by several experiments. Using [^{3}H]-thymidine as a tracer, Tonna et al. demonstrated that the cells locating in the outer layer of the periostium differentiated mature osteoblasts and osteocytes (25). Periosteum or periosteum-derived cells generate bone nodules *in vitro* cultures (26). Transplantation of BMPs into muscle or subcutaneous sites induced ectopic bone formation (27, 28), indicating that osteoprogenitors are also present at extraskeletal sites. The characteristics of osteoprogenitors, which are present in bones and at extraskeletal sites, have not been fully determined. Identification of specific markers for osteoprogenitors is necessary to investigate the nature of osteoprogenitors.

II. Useful cell lines for analyzing the differentiation pathway of osteoblasts in vitro

Cell lines that reflect the different stages of maturation during osteogenesis are important tools for investigating the mechanism of osteoblast differentiation. Although many cell lines useful for such investigations have been established, we have used several cell lines for investigating osteoblast differentiation *in vitro* (Table 1).

Among these cell lines, C3H10T1/2 clone 8 (C3H10 T1/2) cells, a fibroblastic cell line isolated from an early mouse embryo, have characteristics corresponding to multipotent mesenchymal progenitors. This cell line is multipotent and can differentiate into various mesenchymal cells including chondrocytes, myoblasts and adipocytes by the treatment with 5-azacytidine (1). C3H10T1/2 cells also differentiate into osteoblasts in response to BMPs (16, 29-31). Cell lines possessing the characteristics of mesenchymal progenitors were also isolated from the skeletal tissue of newborn rats. ROB-C26 cells expressed only a low level of osteoblast phenotypes, but generated myotubes after reaching confluence and adipocytes following treatment with dexamethasone (3). The developmental potential of this cell line is similar to that of RCJ 3.1, which is capable of differentiating into chondrocytes in addition to osteoblasts, adipocytes and myotubes (2, 32). Successful isolation of these clonal cell lines from skeletal tissues indicates that the multipotent precursor cells which are capable of differentiating into osteoblasts, chondrocytes, adipocytes and myoblasts are present in bone tissues.

MC3T3-E1 is a clonal osteoblastic cell line isolated from calvaria of a late stage mouse embryo (15). This is a monopotent cell line, differentiating into only osteoblast lineage cells. MC3T3-E1 cells express various osteoblast phenotypes including formation of mineralized bone nodules in a long-term culture.

Bone formation is the most important function of osteoblasts. Osteoblastic cells isolated from the calvariae of newborn rats (33) or the bone marrow of adult rats (34) (primary osteoblasts) provide a suitable model to explore the bone formation process *in vitro*, because these cells generate numerous mineralized bone nodules when cultured in the presence of β-glycerophosphate and ascorbic acid. Since only a limited number of clonal cell lines retain the capacity to form mineralized bones *in vitro*, primary osteoblasts are important tools for analyzing the differentiation process of osteoblasts from osteoprogeni-

Table 1: Characteristics of cultured cells for analyzing the differentiation of osteoblasts *in vitro*

Name of cells	Origin	Characteristics
C3H10T1/2 clone 8	Early mouse embryo	A multipotential cell line, which can differentiate into osteoblasts, chondrocytes, myotubes and adipocytes on treatment with BMPs.
ROB-C26	Newborn rat calvariae	An osteoblast cell line, which can differentiate into myotubes and adipocytes.
MC3T3-E1	Calvariae of mouse embryos	A cell line, which can only differentiate into osteoblasts.
ST2	Bone marrow of adult mouse	A bone marrow stromal cell line which can differentiate into osteoblasts on treatment with BMPs.
C2C12	A subclone isolated from parental C2 myoblasts, which were isolated from the regenerating thigh muscle of an adult mouse	A myogenic cell line, which can differentiate into osteoblasts on treatment with BMPs.
Primary osteoblastic cells isolated from calvariae	Calvariae from late embryos or newborn rats	These cells are a mixed population of several cell types, but retain various osteoblastic phenotypes including bone-nodule formation.

tors to bone-forming mature osteoblasts (35, 36).

The osteogenic potential of bone marrow stromal cells has been demonstrated. We used two bone marrow-derived cell lines, ST2 (37) and MC3T3-G2-PA6 (PA6) (38), to investigate the potential to differentiate into osteoblasts. These cell lines had preadipocytic properties and retained the capacity to support hemopoiesis including osteoclastogenesis.

Transplantation of BMPs into muscular sites induced ectopic cartilage and bone formation (27, 28, 39). These findings suggest that muscular tissues contain osteo-chondrogenic progenitor cells, and BMPs alter the differentiation pathway of myogenic cells into osteochondrogenic lineage cells. Thus, myogenic cells are also useful tools for investigating the regulatory mechanism of osteoblast differentiation. We used C2C12 myoblasts for this purpose (40). C2C12 cells are believed to have originated from satellite cells, which are mononucleated cells lying along the muscle fibers in normal tissue, and are present throughout adult life as a potential source of new myoblasts. When muscles are injured or subjected to dystrophic stimuli, the satellite cells are initiated to divide and differentiate into myoblasts and fuse to form muscle fibers. These characteristics suggest that the satellite cells may be responsible for ectopic bone formation induced by BMPs in muscular tissues.

III. Systemic factors that regulate osteoblast differentiation

1. Estrogen-deficiency increases systemic factors that accelerate bone formation

An estrogen deficiency induces a high turnover of bone remodeling, in which the accelerated bone resorption and formation simultaneously occur. In order to investigate the pathogenesis of accelerated bone formation in estrogen deficiency, we conducted several *in vitro* and *in vivo* experiments (41). We first transplanted diffusion chambers containing osteoblast-like cells isolated from newborn rat calvariae into the peritoneal cavity of sham-operated (sham), ovariectomized (OVX) rats and OVX rats with supplement of 17β-estradiol (OVX+E2). Bone formation in the diffusion chambers transplanted into OVX rats was more accelerated than that transplanted into sham rats and OVX+E2 rats, as judged by morphological and biochemical analysis. Osteoblast-like cells cultured with the sera isolated from OVX rats exhibited higher levels of the DNA content in the culture wells, alkaline phosphatase activity, mRNA expression for alkaline phosphatase and osteocalcin, calcium content in the cell layer, and formation of bone-like nodules than those exposed to the sera from sham rats and OVX+E2 rats. Antibody against IGF-I almost completely inhibited the increase in DNA contents induced by the sera isolated from OVX rats, but partially inhibited alkaline phosphatase activity. Adding IGF-I to the sera isolated from sham rats increased the DNA content to the same extent as that induced by the supplement with the sera from OVX rats, but did not increase alkaline phosphatase activity appreciably. Addition of various concentrations of 17β-estradiol, IL-1 and IL-6 to the sera isolated from sham rats did not increase the DNA content or ALP activity in the osteoblast-like cells. These results indicate that some systemic factor(s) other than IGF-I, IL-1 and IL-6 may be responsible for the stimulative effect on osteoblast differentiation in the pathogenesis of the accelerated bone formation induced by estrogen deficiency in rats.

2. The mechanism of stimulatory effects of PTH on osteoblast differentiation

In 1929, 4 years after the discovery of PTH, Bauer *et al*. (42) reported for the first time that subcutaneous injection of parathyroid extract stimulated bone formation in rats. In 1976, Reeve *et al*. (43) attempted clinical application of the synthetic fragment of human PTH [PTH(1-34)], in which they found an anabolic effect of the hormone in 4 patients with postmenopausal osteoporosis. To date, a number of *in vivo* trials have been conducted to explore the anabolic effect of PTH in bone metabolism (44-46). Among these, experiments using intact and osteopenic animals have demonstrated that intermittent administration of PTH increases bone mass, whereas continuous infusion causes a decrease (44-46). The increase in bone mass by the intermittent administration was associated with the parameters of elevated bone formation analyzed by bone histomorphometry. Continuous infusion of PTH caused osteopenia probably due to greater acceleration of bone resorption than bone formation. Thus, PTH has diverse effects on bone depending on the mode of administration. To investigate the mechanism of the anabolic effects of PTH on osteoblast differentiation, we conducted *in vitro* experiments (47). Osteoblastic cells isolated from newborn rat calvariae were cyclically treated with PTH (1-34) for the first few hours of each 48-hour incubation cycle. When osteoblastic cells were intermittently exposed to PTH only for the first 1 hour of each 48-hour incubation cycle and cultured for the remainder of the cycle without the hormone, osteoblast differentiation was inhibited by suppressing alkaline phosphatase activity, bone nodule formation, and mRNA expression of alkaline phosphatase, osteocalcin and PTH/PTHrP receptor. Experiments using inhibitors and stimulators of cAMP/PKA and Ca^{2+}/PKC demonstrated that cAMP/PKA was the major signal transduction system in the inhibitory action of PTH. In contrast, the intermittent exposure to PTH for the first 6 hours of each 48-hour cycle stimulated osteoblast differentiation. Both cAMP/PKA and Ca^{2+}/PKC systems appeared to be involved in this anabolic effect. Continuous exposure to PTH during the 48-hour incubation cycle strongly inhibited osteoblast differentiation. Although both cAMP/PKA and Ca^{2+}/PKC were involved in the effect of continuous exposure to PTH, they appeared to act independently. A neutralizing antibody against IGF-I blocked the stimula-

tory effect on alkaline phosphatase activity induced by the 6-h intermittent exposure. The inhibitory effect induced by the 1-hour intermittent exposure was not affected by anti-IGF-I antibody. These results suggest that PTH has diverse effects on osteoblast differentiation depending on the exposure time *in vitro* mediated through different signal transduction systems. These *in vitro* findings explain at least in part the *in vivo* action of PTH that varies with the mode of administration.

IV. Local factors that regulate osteoblast differentiation

1. Bone morphogenetic proteins (BMPs)

Urist reported that implantation of decalcified bone matrix into muscular tissues induced new ectopic bone formation associated with endochondral bone formation (27). He named the factor contained in the decalcified bone matrix BMP(48). A number of investigators attempted to isolate BMP from the decalcified bone matrix (49-51), but it was very difficult to obtain BMP as a single protein until the late eighties. In 1988, Wozney *et al*. (28) first cloned four cDNAs for human BMPs [BMP-1, BMP-2 A (BMP-2), BMP-2B (BMP-4) and BMP-3]. At present, at least 15 BMPs have been cloned, and these belong to TGF-β superfamily with the exception of BMP-1 (52). Recombinant proteins of several BMPs with the ability to induce ectopic bone formation *in vivo* have been successfully generated (28, 39, 53-55). Several research groups including ourselves have examined the effects of recombinant human BMPs (rhBMPs) on the differentiation of skeletal mesenchymal cells using various types of cells *in vitro*. Recombinant human BMPs are expected to be potent local factors that promote bone formation in bone defects, fracture repair and periodontal diseases.

In addition to bone formation, BMPs exert important functions in mesodermal induction (56, 57), organogenesis (58, 59) including skeletal tissue formation, and pattern formation in vertebrate development.

A. Role of BMPs in differentiation of multipotent mesenchymal cells

Untreated control C3H10T1/2 cells expressed no or extremely low levels of specific phenotypes related to osteoblasts, chondrocytes, myoblasts and adipocytes (16). BMP-2 strongly increased ALP activity, which is an early marker of osteoblasts, and parathyroid hormone (PTH)-dependent cAMP production in this cell line (16). Although control C3H10T1/2 cells produced no osteocalcin, a marker appearing at the later stage of osteoblast differentiation, BMP-2 and BMP-7 induced osteocalcin production by C3H10T1/2 cells (29, 30). These results indicate that BMP-2 and BMP-7 induce C3H10T1/2 cells to differentiate into osteogenic lineage cells. In addition, these BMPs induced C3H10T1/2 cells to differentiate into not only osteoblasts but also chondrocytes (29, 30). Ahrens *et al*. (31) also demonstrated that transfection of cDNAs encoding human BMP-2 and BMP-4 into C3H10T1/2 cells induced the cells to differentiate into both osteoblasts and chondrocytes. These results suggest that BMPs are potent inducers of osteo-chondrogenic differentiation of non-osteogenic pluripotent mesenchymal cells. BMPs also induced C3H10T1/2 cells to differentiate into adipocytes (29, 30).

B. Role of BMPs in differentiation of osteoblast lineage cells

ROB-C26 is an osteoblast precursor cell line that is also capable of differentiating into myotubes and adipocytes (3). This cell line expresses a low level of ALP activity and PTH-dependent cAMP production, and shows no osteocalcin synthesis under control culture conditions. BMP-2 stimulated ALP activity and PTH-dependent cAMP production, and induced osteocalcin synthesis in ROB-C 26 cells (17). Gitelman *et al*. (60) reported that overexpression of BMP-6 accelerated osteoblast differentiation in ROB-C26 cells, and this effect was antagonized by the addition of a neutralizing antibody against BMP-6. Nishitoh *et al*. (54) also demonstrated that growth/differentiation factor-5 (GDF-5), a member of the BMP family, stimulated ALP activity in ROB-C26, which was mediated by BMP receptor (BMPR)-IB and BMPR-II.

BMP-2 and BMP-7 promoted osteoblastic MC3T3-E 1 cells to differentiate into mature osteoblasts by increasing ALP activity, PTH-responsiveness and osteocalcin production (61, 62). IL-1β synergistically increased BMP-2-induced ALP activity in MC3T3-E1 cells, but TNF-α inhibited BMP-2-induced ALP activity in this cell line (62), suggesting that various cytokines modulate the action of BMP. Thus, it is likely that BMPs stimulate osteoprogenitors and immature osteoblastic cells to differentiate into mature osteoblasts.

To investigate the roles of BMPs in formation of bone nodules, we investigated the distributions of BMPs and their receptors in osteoblastic cells isolated from newborn rat calvariae (36). *In situ* hybridization studies detected strong signals for *BMP-2* and *BMP-4* mRNAs in bone nodule-forming cells, but not in the cells located in inter-nodular regions. In addition, immunohistochemical analysis using an antibody reactive with both BMP-2 and BMP-4 demonstrated that positive cells first appeared in unmineralized nodules, and were then localized preferentially in mineralized nodules at a later stage in culture. Harris *et al*. (35) demonstrated by Northern blotting analysis that not only BMP-2 and BMP-4 but also BMP-6 mRNAs were expressed during bone nodule formation by osteoblasts isolated from fetal rat calvariae. The maximal levels of expression of each BMP mRNA coincided with the formation of mineralized bone nodules. These results suggested that several BMPs are involved in the mechanism of bone nodule formation in *in vitro* osteoblast culture. Hughes *et al*. (63) compared the effects of BMP-2, BMP-4 and BMP-6 on the formation of bone nodules by rat calvaria-derived osteoblastic cells; BMP-2 was less potent than BMP-4 and BMP-6 in this assay system. Re-

cently, Boden *et al*. (64) reported that glucocorticoid-induced formation of bone nodules in fetal rat calvarial osteoblasts was mediated by BMP-6: glucocorticoids preferentially increased expression of *BMP-6* mRNA, and the antisense oligonucleotide corresponding to BMP-6 strongly inhibited formation of bone nodules. We demonstrated that BMP receptors such as BMPR-IA, BMPR-IB and BMPR-II were preferentially expressed at the sites of nodule formation in calvarial culture (36). Taken together, these observations indicate that BMPs play important roles in the process of bone nodule formation through their receptors in a paracrine and/or autocrine fashion.

C. Role of BMPs in differentiation of bone marrow stromal cells

Thies *et al*. (65) first reported that BMP-2 induced the bone marrow derived cell line W-20-17 to express osteoblast phenotypes using an *in vitro* culture system. We investigated the effects of BMPs on osteoblast differentiation in ST2 (37) and PA6 (38) cells (66), which are well-characterized bone marrow-derived clonal cell lines. Neither ST2 nor PA6 cells exhibited features typical of osteoblast phenotype under control cultures. BMP-2, BMP-4 and BMP-6 induced ST2 cells to express osteoblast phenotypes such as elevated levels of ALP activity, the PTH-dependent production of cAMP and the synthesis of osteocalcin (66). More importantly, ST2 cells generated mineralized bones in diffusion chambers, when they were transplanted into peritoneal cavity of athymic mice with rhBMP-2 (Fig. 1) (66). In contrast, the stimulatory effects of these BMPs on ALP activity and PTH-dependent production of cAMP were weaker in PA6 cells than in ST2 cells. No apparent bone formation was observed when PA 6 cells were transplanted into athymic mice using diffusion chambers with rhBMP-2. BMPs failed to induce the synthesis of osteocalcin in PA6 cells (66). These results indicated that the effects of BMPs on osteoblast differentiation of bone marrow stromal cells differ in each cell line. The stimulatory effects of BMP-2 on osteoblastic differentiation were also demonstrated in human bone marrow stromal cells (67).

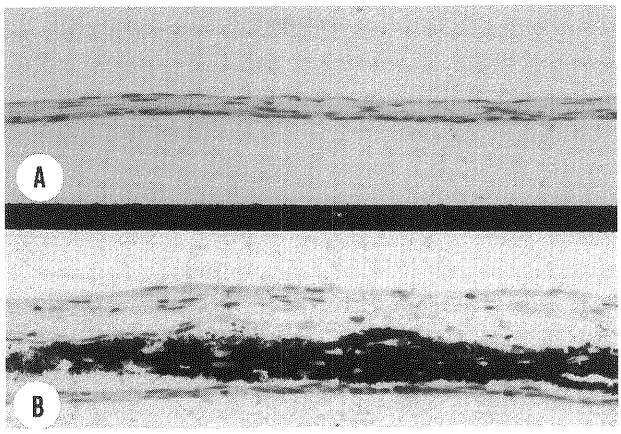

Fig. 1: Histology of ST2 cells after transplantation into the peritoneal cavities of athymic mice in diffusion chambers (66). ST2 cells transplanted without BMP-2 formed only fibrous connective tissue in the diffusion chamber (A), but the cells transplanted with BMP-2 generated mineralized bone in the diffusion chamber (B). The sections were stained by von Kossa's method and counterstained with hematoxylin.

Adipocytes are derived from common progenitors with osteoblasts. Chen *et al*. (68) investigated the roles of BMPRs in the process of BMP-induced differentiation of osteoblasts and adipocytes using 2T3 cells, which were derived from the calvariae of a transgenic mouse expressing T antigen driven by the BMP-2 promoter. BMP-2 induced this cell line to differentiate into mature osteoblasts or adipocytes. Overexpression of a kinase domain-truncated BMPR-IB in 2T3 completely inhibited osteoblast differentiation in this cell line, and the decreased level of ALP activity in the 2T3 cells with the truncated BMPR-IB was rescued by transfection with wild-type BMPR-IB. In addition, overexpression of constitutively active BMPR-IB induced formation of bone in 2T 3 cells in the absence of BMP-2. In contrast, overexpression of a kinase domain-truncated BMPR-IA blocked adipocyte differentiation, and transfection of constitutively active BMPR-IA induced adipocyte differentiation in 2T3 cells. These results suggested that BMPR-IA and BMPR-IB have different functions in the differentiation of osteoblasts and adipocytes in 2T3 cells: BMPR-IB is the major receptor involved in osteoblast differentiation and BMPR-IA is the major receptor for adipocyte differentiation. As described below, however, an important role of BMPR-IA has been demonstrated in the process of BMP-2-induced osteoblast differentiation in C2C12 myoblasts (69). Grimble *et al*. (70) also reported that BMP-2 and BMP-4 inhibited adipocyte differentiation by bone marrow stromal cells. Further studies are necessary to determine the precise roles of BMPs in the differentiation of adipocytes.

D. Role of BMPs in differentiation of myogenic cells

ROB-C 26, which is an osteoblast precursor cell line with the capacity to differentiate into myogenic cell (3), is suitable for exploring the regulatory mechanism of action of BMPs on myogenic cell differentiation. Using this cell line, we first demonstrated that BMP-2 inhibited myogenic differentiation (17). BMP-2 also inhibited myotube formation in L6 myoblasts, which originated from skeletal muscle, but failed to induce this cell line to differentiate into osteogenic or chondrogenic lineage cells (17).

To further investigate the regulatory mechanism of myogenic differentiation by BMP-2, Katagiri *et al*. (40) used C2C12 myoblasts, which originated from muscular tissue satellite cells. Interestingly, BMP-2 inhibited terminal differentiation of C2C12 cells and converted them into osteoblast lineage cells (40). The inhibitory effects of BMP-2 on myotube formation and the promotion of differentiation into osteoblast lineage cells were also identified in primary muscle cells isolated from newborn mice (40). In the process of myogenic inhibition of C2C12 cells, BMP

-2 strongly down-regulated the levels of expression of mRNAs encoding MyoD and myogenin, which are critical transcription factors regulating myogenic differentiation, by suppressing the transcriptional activity of these myogenic factors in C2C12 cells (40, 71). Wild-type C2C12 cells expressed BMPR-IA and BMPR-II mRNAs, but not BMPR-IB mRNA (69). To explore the signal transduction pathway involved in the effects of BMP-2, Namiki *et al*. (69) established a subclonal cell line of C2C12 that stably expressed a kinase domain-truncated BMPR-IA. This cell line generated numerous myotubes but failed to differentiate into ALP-positive cells after treatment with BMP-2. When wild-type BMPR-IA was transiently transfected into the BMPR-IA mutant cells, BMP-2 inhibited myogenic differentiation and induced ALP-positive cells. BMP-2 did not induce ALP-positive cells in BMPR-IA mutant cells transfected with wild-type BMPR-IB. These results suggest that BMP-2 signals in inhibiting myogenesis and inducing osteoblast differentiation are transduced via BMPR-IA, at least in C2C12 cells. Interestingly, Akiyama *et al*. (72) demonstrated that C2C12 cells stably transfected with constitutively active BMPR-IB exhibited osteoblast phenotypes, but did not express myogenic phenotypes. These results suggest that a common signal transducer (s) is involved in the signal transduction pathway via BMPR-IA and BMPR-IB during differentiation of C2C12 cells. Smads play important roles in the signal transduction pathway of ligands belonging to the TGF-β superfamily (73). C2C12 cells constitutively expressed Smad1, Smad2, Smad4 and Smad5 mRNAs (74). Yamamoto *et al*. (74) demonstrated that Smad1 and Smad5, which belong to the R-Smad family and mediate BMP signaling, are involved in the process of myogenic inhibition and induction of osteoblast differentiation in C 2C12 cells. Nishimura *et al*. (75) also reported that the activation of Smad5 and subsequent formation of the complex of Smad5 and Smad4 were key steps in the process of BMP-2-induced osteoblast differentiation in C2C12 cells.

Thus, BMPs play important roles in the differentiation processes of several mesenchymal cells. Further studies including signal transduction systems of BMPs are needed to clarify molecular mechanism underlying differentiation process of mesenchymal cell lineages.

2. Sonic and Indian hedgehogs

A. Sonic and Indian hedgehogs are involved in skeletogenesis

The gene *hedgehog* (*hh*) is a segment polarity gene regulating embryonic segmentation and patterning in *Drosophila* and is highly conserved in vertebrates (76). In higher vertebrates, the *Hedgehog* gene family consists of at least three members, *Sonic*, *Indian*, and *Desert hedgehog* (*Shh, Ihh, and Dhh*, respectively) (76). Shh has multiple functions during formation of various organs and tissues including formation of skeletal tissues in vertebrae and limbs (77). The phenotypes observed in *Shh* knockout mice, which include complete lack of vertebrae and partial defects of autopods, support the importance of Shh in skeletogenesis (77). These results suggested that *Shh* mutations cause some malformations in humans. Indeed, the similarity of forebrain development between *Shh* mutant mice and cases of human holoprosencephary with *SHH* mutation is reported (78-80). In addition, mutation of human *PATCHED*, which encodes a transmembrane protein that negatively regulates Shh signaling in target cells, causes the human autosomal disease termed nevoid basal cell carcinoma syndrome (81). Developmental skeletal abnormalities and a high risk of various forms of cancers, mainly basal cell carcinoma, characterize this syndrome : Mutations in the human *SHH* gene and genes that encode components of its downstream intracellular signaling pathway also cause three distinct congenital disorders : Greig syndrome, Pallister-Hall syndrome and isolated postaxial polydactyly (82). Thus, SHH signaling is involved in the pathogenesis of several diseases including those of skeletal tissues in humans.

Ihh is expressed in cartilage during skeletogenesis (83), and regulated chondrocyte differentiation through regulation of PTHrP in chicken embryos (84). The hedgehog-responsive genes *Patched* (hedgehog receptor) and *Gli* (transcriptional factor) were highly expressed in the perichondrium (84, 85), where subsequent bone formation occurred. These results suggested that the target cells for *Ihh* locate in the perichondrium, and that Ihh induces adjacent perichondrial cells to differentiate into bone-forming osteoblasts. Since Shh and Ihh have similar functions in chondrocyte differentiation (84, 86), these hedgehog proteins might be involved in osteoblast differentiation.

The hedgehog family retains structural and functional similarities between Drosophila and vertebrates. In *Drosophila*, a major role of hedgehog signaling is the activation of additional signals including *Dpp*, which is a homologue of vertebrate BMP, and wingless. Laufer et al. (87) reported that Shh is capable of regulating the expression of BMP-2 in chicken limb buds because BMP-2 mRNA was expressed adjacent to Shh-expressing cells and the ectopic transplantation of *Shh*-expressing cells induced BMP-2 expression in the cells around the transplanted cells. Bitgood *et al*. (83) showed an intimate correlation between the expression of mouseShh/Ihh genes and BMPs in various tissues. These findings prompted us to investigate whether Shh and Ihh are involved in osteoblast differentiation by a mechanism involving BMPs.

2. Regulation of osteoblast differentiation by hedgehogs

To investigate the roles of Shh in osteoblast differentiation, we first examined the effects of conditioned media collected from *Shh*- or *Ihh*-overexpressing chicken embryonic fibroblasts on differentiation of C3H10T1/2 and MC3T3-E1 cells (88). Addition of the conditioned media obtained from *Shh*-overexpressing chicken embryonic fibroblast cultures into C3H10T1/2 and MC3T3-E1 cells

significantly increased ALP activity (88). Nakamura *et al.* (89) also demonstrated that conditioned media from Ihh-overexpressing chicken embryonic fibroblasts increased ALP activity in C3H10T1/2 cells. Although no apparent increases were observed in *BMP-2, BMP-4* or *BMP-6* mRNAs in these cells after treatment with each conditioned medium, the increase in ALP activity induced by the conditioned media of *Shh*- or *Ihh*-overexpressing cells was abolished by addition of soluble BMPR-IA (Yuasa T and Yamaguchi A, unpublished data), which antagonized the action of BMP on osteoblast differentiation *in vitro* (90). The conditioned media of *Shh*-overexpressing chicken embryonic fibroblasts also increased the level of osteocalcin mRNA expression in MC3T3-E1 cells. These results suggested that Shh and Ihh induce osteoblast differentiation, and that BMPs are involved in the mechanism of action of hedgehogs. Recently, a cooperative action of Shh and BMP-7 was reported in the induction of forebrain ventral midline cells by prechordal mesoderm (91). Nakamura *et al*. (89) also demonstrated that recombinant Shh (rShh) synergistically stimulated BMP-2-induced ALP activity in C3H10T1/2 cells. Recently, Murtaugh *et al*. (92) reported that chondrogenesis of somitic tissues are regulated by intimate interaction between Shh and BMPs.

To investigate whether Shh induce ectopic bone formation, we transplanted Shh-overexpressing chicken fibroblasts cultured on type I collagen gels into intramuscular sites in athymic mice (88). Endochondral bone formation was induced at the site of transplantation. We also confirmed that chicken embryonic fibroblasts used for transplantation expressed low levels of mRNAs encoding BMP-2 and BMP-4. Taken together, these observations indicate that ectopic endochondral bone formation induced by transplantation of *Shh*-overexpressing chicken embryonic fibroblasts may be due to the synergistic effects of Shh and BMPs, which are produced by the fibroblasts. Further studies using recombinant SHH are necessary to determine the precise interaction between Shh and BMPs in osteoblast differentiation and bone formation during not only normal development as well as fracture repair. Such a study is ongoing in our laboratory.

V. Cbfa1 is an essential transcription factor that regulates osteoblast differentiation and bone formation

1. Identification of osteoblast-specific transcription factor

Osteocalcin gene is expressed only in osteoblasts with the exception of megakaryocytes (93). Three *osteocalcin* genes occur in mice (94). Two of these, osteocalcin gene 1 (OG1) and osteocalcin gene 2 (OG2), are expressed only in bone. Another gene, osteocalcin-related gene (ORG), is not expressed in bone but only in the kidney. Many investigators have analyzed the regulatory mechanism of *osteocalcin* genes to find osteoblast-specific transcription factors.

Karsenty and colleagues identified two distinct DNA sequences, osteoblast-specific cis-acting element 1 (OSE1) and osteoblast-specific element 2 (OSE2), in the promoter of the mouse *osteocalcin* gene (OG2) (95, 96). Karsenty's group and Stein's group independently identified a candidate transcription factor, which bound to OSE 2 in mouse and rat *osteocalcin* genes in osteoblast nuclear extracts (96, 97). The OSE2 sequence was identical to the consensus binding sequence of Cbfa (core binding factor), which is also called Pebp2α (polyoma enhancer binding protein). Karsenty and colleagues named this transcription factor osteoblast-specific transcriptional factor 2 (OSF2) (96). Ducy *et al*. (20) showed that OSF2 was identical to Cbfa1, also called Pebp2αA or AML3, and that Cbfa1/OSF2 was bound to OSE2 and regulated the expression of various genes expressed in osteoblasts such as osteocalcin and type I collagen. They also demonstrated that overexpression of Cbfa1/OSF2 in non-osteogenic cells such as C3H10T1/2 cells and skin fibroblasts induced them to express osteoblast-related genes (20). These results indicate that Cbfa1/OSF2 plays a crucial role in osteoblast differentiation.

2. Cbfa1-deficient mice completely lack bone formation due to maturation arrest of osteoblasts

Komori *et al*. generated *Cbfa1*-deficient mice by disruption of exon 1 of the *Cbfa1* gene, which contained the first 41 amino acids of the *runt*-domain (21). A few newborn mice of homozygout mutation of *Cbfa1* (*Cbfa1*$^{-/-}$) were delivered but soon died due to respiratory insufficiency. On embryonic day 18.5 (E18.5), however, homozygous embryos were alive with the frequency predicted by Mendelian law.

Soft X-ray examination of the wild-type embryos at E18.5 revealed that all skeletons were well calcified (Fig. 2). In *Cbfa1*$^{-/-}$ embryos at E18.5, however, parts of the tibia, radius, and vertebrae were weakly calcified, and no calcification occurred in the skull, mandibula, humerus

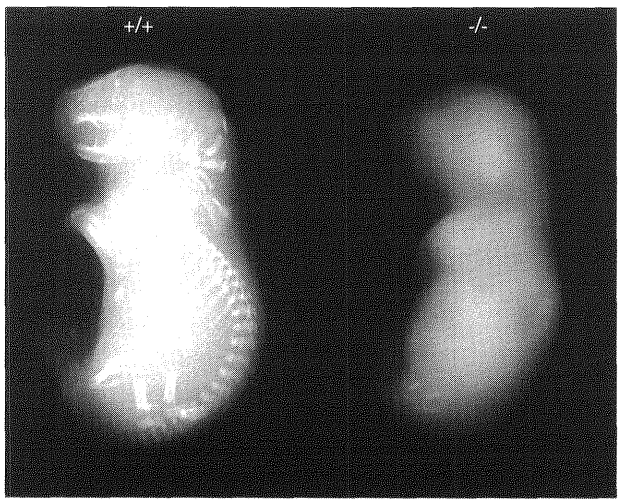

Fig. 2: Soft X-ray of E18.5 wild-type (+/+) and *Cbfa1*-deficient (-/-) embryos. Wild-type embryo exhibits well calcified skeletons, but mutant embryo has a barely calcified skeleton.

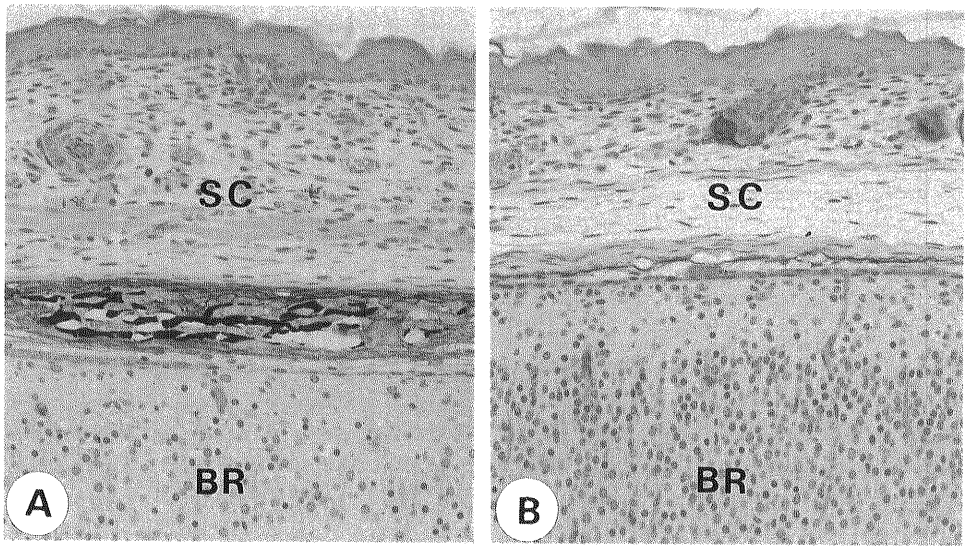

Fig. 3: Histology of calvariae of E18.5 wild-type (A) and *Cbfa1*-deficient (B) embryos. These undecalcified sections were stained by ALP and von Kossa's method. Cells stained blue represent ALP-positive cells, and matrices stained black represent calcified matrices. Note that there are no calcified matrices in *Cbfa1*-deficient mice.

and femur (Fig. 2). Histological examination revealed that the tibia and femur in E18.5 wild-type embryos consisted of cartilaginous epiphyses and a bony diaphysis. In the diaphysis, well-calcified bones were observed in the cortical and metaphysial regions. In E18.5 *Cbfa1*$^{-/-}$ embryos, the middle part of the tibia remained as calcified cartilage without formation of the bone marrow cavity. Although ALP-positive cells appeared in the perichondrial region of the calcified cartilage, no bone was formed. The midportion of the femur in E18.5 mutant mice remained as uncalcified cartilage, and no ALP-positive cells were found in the perichondrial region. Well-calcified bones were formed between brain and subcutaneous connective tissues at the calvariae of E18.5 wild-type embryos (Fig. 3A). The bone surface was covered with numerous ALP-positive osteoblasts. In E18.5 *Cbfa1*-deficient embryos, only a thin layer of the fibrous connective tissue was observed between the brain and subcutaneous connective tissue (Fig. 3B). ALP-positive cells were detected in the fibrous connective tissues, but no calcified bone was observed. These results demonstrate a lack of ossification in *Cbfa1*-deficient mice. Similar skeletal changes in *Cbfa1*-deficient mice were reported by Otto *et al*. (22).

Ducy et al. (20) demonstrated that BMP-7 induced expression of *OSF2 / Cbfa1* mRNA prior to induction of *osteocalcin* mRNA. These results suggest that OSF2/Cbfa 1 is involved in BMP-induced osteoblast differentiation, and also in the signaling pathway of BMP. On the other hand, we found that calvaria-derived cells isolated from *Cbfa1*-deficient embryos increased production of osteocalcin in response to BMP-2, although *Cbfa1*-deficient cells showed less production of osteocalcin than those from wild-type embryos (21). This suggests that transcription factors other than Cbfa1 also play some roles in BMP-2-induced osteocalcin synthesis, at least *in vitro*. Isolation of cell lines from *Cbfa1*-deficient mice may provide useful tools for investigating transcription factors involved in osteoblast differentiation, besides Cbfa1. Such studies are important to understand the regulatory mechanism of osteoblast differentiation, and they are now currently underway in our laboratories.

3. Cbfa1 is involved in osteoclastogenesis

Rodan and Martin proposed an important hypothesis concerning the possible involvement of osteoblast lineage cells in the hormonal control of bone resorption in 1981 (98). They suggested the potential direct activation of osteoclasts by the products of osteoblast lineage cells in response to bone-resorbing hormones. A series of experiments have confirmed this hypothesis (99, 100), but the precise molecular mechanism involved in the interaction between osteoblast lineage cells and osteoclasts had not been clarified until quite recently.

Recently, two molecules produced by osteoblast lineage cells, which play important roles in osteoclastogenesis, were identified. One is osteoprotegerin (OPG) (101), which is identical to osteoclastogenesis inhibitory factor (OCIF) (102). OPG is a secretary protein belonging to the tumor necrosis factor (TNF) receptor family. This protein inhibited not only formation of osteoclast-like cells (OCLs) in culture but also bone resorption both *in vitro* and *in vivo* (101, 103). OPG knockout mice exhibited severe osteopenia due to accelerated bone resorption (104, 105). The other molecule is RANKL (receptor activator of NF-kB ligand) (106), which is identical to OPG ligand (OPGL) (107), TRANCE (TNF-related activation-induced cytokine) (108) and osteoclast differentiation factor (ODF) (109). RANKL belongs to the TNF ligand family and binds to OPG. A soluble form of RANKL (soluble RANKL) together with macrophage colony stimulating factor (M-CSF) induced formation of OCLs from spleen

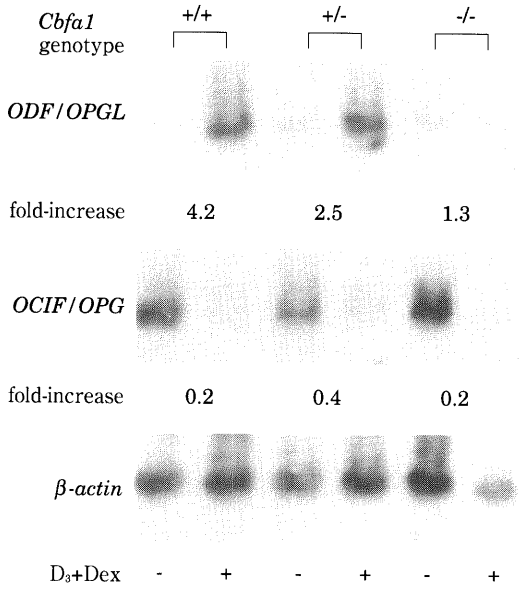

Fig. 4: Expression of *ODF/OPGL* and *OPG/OCIF* mRNAs in calvarial cells isolated from *Cbfa1*$^{+/+}$, *Cbfa1*$^{+/-}$ and *Cbfa 1*$^{-/-}$ embryos (111). Calvarial cells were cultured for 3 days in the absence or presence of 1α, 25 $(OH)_2D_3$ and dexamethasone, then northern hybridization was conducted. Fold increase was expressed by ratios of *ODF/OPGL* or *OPG/OCIF* mRNA levels, which were standardized by that of β-actin mRNA, between untreated and treated cells with 1α, 25 $(OH)_2D_3$ and dexamethasone in each cell type.

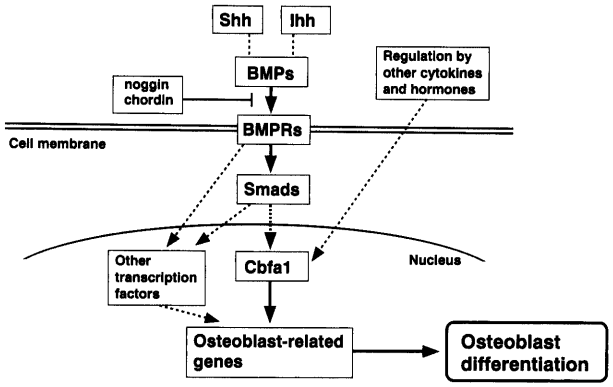

Fig. 5: A hypothetical molecular mechanism underlying osteoblast differentiation. Shh and Ihh may act cooperatively with BMPs. Noggin and chordin inhibit BMP action by competing for binding to BMPRs at an extracellular region. BMP signals may up-regulate expression of Cbfa1 through Smads. Cbfa1 effectively transcribes various osteoblast-related genes to induce osteoblast differentiation. Some transcription factor(s) other than Cbfa1 may be involved in BMP-induced osteoblast differentiation. Solid arrows indicate direct interactions and dotted arrows indicate hypothetical interactions.

cells in the absence of osteoblast lineage cells *in vitro* (107, 109). Recently, Kong *et al*. (110) reported that *opgl*-deficient mice exhibited severe osteopeterosis and they completely lacked osteoclasts as a result of an inability of osteoblasts to support osteoclastogenesis.

We reported that osteoclastogenesis was markedly retarded in *Cbfa1*-deficient mice (21). These results suggested that the maturational arrest of osteoblasts caused by disruption of the *Cbfa1* gene might be related to the insufficient osteoclastogenesis in *Cbfa1*-deficient mice. These observations also allowed us to speculate on the role of Cbfa1 in the regulation of RANKL and OPG because both are synthesized by osteoblast lineage cells.

We investigated the mechanism involved in retarded osteoclastogenesis in *Cbfa1*-deficient mice (111). Co-cultures of calvarial cells isolated from embryos with three different *Cbfa1* genotypes (*Cbfa1*$^{+/+}$, *Cbfa1*$^{+/-}$ and *Cbfa1*$^{-/-}$) and normal spleen cells generated TRAP-positive multinucleated osteoclast-like cells (OCLs) in response to 1α, 25 $(OH)_2D_3$ and dexamethasone, but the number and bone-resorbing activity of OCLs formed in co-culture with Cbfa1$^{-/-}$ calvarial cells were significantly decreased in comparison with those formed in co-cultures with *Cbfa1*$^{+/+}$ or *Cbfa1*$^{+/-}$ calvarial cells. The expression of *RANKL* mRNA was increased by treatment with 1α, 25 $(OH)_2D_3$ and dexamethasone in calvarial cells from *Cbfa 1*$^{+/+}$ and *Cbfa1*$^{+/-}$ mouse embryos, but not from *Cbfa1*$^{-/-}$ embryos (Fig. 4). In contrast, the expression of *OPG* mRNA was inhibited by 1α, 25 $(OH)_2D_3$ and dexamethasone similarly in all three types of calvarial cells (Fig. 4). *RANKL* and *OPG* mRNAs were highly expressed in the tibia and femur of *Cbfa1*$^{+/+}$ and *Cbfa1*$^{+/-}$ embryos (Fig. 4). In the tibia and femur of *Cbfa1*$^{-/-}$ embryos, however, *RANKL* mRNA was undetectable and the expression of *OPG* mRNA was also decreased compared with those in *Cbfa 1*$^{+/+}$ and *Cbfa1*$^{+/-}$ embryos. Thus, it is likely that Cbfa1 is involved, at least in part, in osteoclastogenesis by regulating the expression of RANKL. Recently, potential Cbfa 1 binding sites in the promoter region of murine *RANKL* were demonstrated (112). These binding sites were also reported in the promoter of *OPG*. More extensive studies on the regulation of RANKL and OPG by Cbfa1 will provide insight into the molecular mechanism involved in the classical hypothesis proposed by Rodan and Martin (107) concerning the interaction between osteoblasts and osteoclasts during bone remodeling.

4. Heterozygous mutations of Cbfa1 locus cause cleidocranial dysplasia

Cleidocranial dysplasia (CCD) is an autosomal-dominant disease showing hypoplastic clavicles, open fontanelles, supernumerary teeth, short stature and other skeletal changes (113). Mice heterozygous for mutation in the *Cbfa1* locus (*Cbfa1*$^{+/-}$) exhibited similar skeletal changes to CCD (21, 22). They exhibited hypoplastic frontal, parietal, interparietal, temporal and supraoccipital bones with open fontanelles and sutures. They also showed hypoplastic clavicles and nasal bones. Development of the primordia of tooth structures, however, was slightly delayed but structurally normal in heterozygous *Cbfa1* mice (22). In homozygously mutated mice, developmental arrest was observed at the cap stage in molar tooth germs, and neither the differentiation of mesenchymal cells in dental papilla to preodontoblasts nor the dif-

ferentiation of epithelial cells to preameloblasts was observed. The lack of supernumerary teeth in heterozygous *Cbfa1* mutant mice can be explained by the fact that mice have only one set of teeth, and deciduous teeth are not affected in humans (114).

One family with CCD showed a microdeletion in chromosome 6p21 (114, 115). CBFA1 is mapped to chromosome 6p12-p21 (116, 117). Mutations of *CBFA1* have been identified in patients with CCD (114, 118).

Perspective

Recent advances in bone cell biology revealed a part of molecular mechanism of osteoblast differentiation and bone formation. Especially, molecular cloning of BMPs and Cbfa1 was a great breakthrough in the field of osteoblast research. Figure 5 summarizes interaction of major players involved in osteoblast differentiation including BMPs and Cbfa1. However, many subjects remain obscure: regulatory mechanism of BMP action by noggin and chordin at extracellular region; signal transduction system of BMPs; interaction of BMPs and hedgehog; interaction of BMPs and Cbfa1; role of Cbfa1 isoforms (119); *etc*. In addition to these subjects, characterization of mesenchymal stem cells is important not only in the field of basic research but also in clinical research. Isolation of mesenchymal stem cells and regulation of differentiation of these cells by BMPs and Cbfa1 *in vitro* will provide a new therapeutic procedure for bone repair. Finally, advances in bone cell biology will contribute to understanding molecular pathogenesis of various bone diseases including that caused by genetic disorders.

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References

1. Taylor SM and Jones PA. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell* 1979; **17**: 771-9.
2. Grigoriadis AE , Heersche JNM and Aubin JE. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. *J Cell Biol 1988*; **106**: 2139-51.
3. Yamaguchi A and Kahn AJ. Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif. *Tissue Int* 1991; **49**: 221-5.
4. Aubin JE and Liu F. The osteoblast lineage. In: Bilezikian, J. P., L. G. Raisz, and G. A. Rodan. editors. Principles of bone biology. Academic Press, San Diego, 1996; 51-67.
5. Owen M. Marrow stromal stem cells. *J Cell Sci Suppl* 1988; **10**: 63-76.
6. Caplan AI. Mesenchymal stem cells. *J Orthop Res* 1991; **9**: 641-50.
7. Pittenger MF, Mackay AM, Beck SC, *et al*. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-7.
8. Dempster DW, Cosman F, Parisien M, *et al*. Anabolic actions of parathyroid hormone on bone. *Endocr Rev* 1993; **14**: 690-709.
9. Dempster DW, Cosman F, Parisien M, *et al*. Anabolic actions of parathyroid hormone on bone: update 1995. *Endocr Rev* 1995; **4**: 247-50.
10. Lian JB, Stein GS, Stein JL *et al*. Regulation of the bone specific osteocalcin gene by vitamins and hormones. *Vitam Horm* 1999; **55**: 443-509.
11. Turner RT, Riggs BL and Spelsberg TC. Skeletal effects of estrogen. *Endocr Rev* 1994; **15**: 275-300.
12. Boyce BF, Hughes DE, Wright KR, *et al*. Recent advances in bone biology provide insight into the pathogenesis of bone diseases. *Lab Invest* 1999; **79**: 83-94.
13. Majeska RJ, Rodan SB and Rodan GA. Maintenance of parathyroid hormone response in clonal rat osteosarcoma lines. *Exp Cell Res* 1978; **111**: 465-8.
14. Partridge NC, Alcorn D, Michelangeli VP, *et al*. Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. *Cancer Res* 1983; **43**: 4308-14.
15. Sudo H, Kodama HA, Amagai Y, *et al*. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol* 1983; **96**: 191-8.
16. Katagiri T, Yamaguchi A, Ikeda T, *et al*. The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. *Biochem Biophy Res Commun* 1990; **172**: 295-9.
17. Yamaguchi A, Katagiri T, Ikeda T, *et al*. Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. *J Cell Biol* 1991; **113**: 681-7.
18. Weintraub H. The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* 1993; **75**: 1241-4.
19. Tontonoz P, Hu E and Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPARγ 2, a lipid-activated transcription factor. *Cell* 1994; **79**: 1147- 56.
20. Ducy P, Zhang R, Geoffroy V, *et al*. Cbfa 1/Osf 2: A transcriptional activator of osteoblast differentiation. *Cell* 1997; **89**: 747-54.
21. Komori T, Yagi H, Nomura S, *et al*. Target disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 1997; **89**: 755-64.
22. Otto F, Thornell AP, Crompton T, *et al*. *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 1997; **89**: 765-71.

23. Friedenstein AJ. Osteogenic stem cells in the bone marrow. *In*: Heersche J. N. H, and J. A. Kanis. editors *Bone Miner Res* 7, Elsevier Science Publishers, 1990; 243-72.
24. Nishida S, Yamaguchi A, Tanizawa T, *et al*. Increased bone formation by intermittent parathyroid hormone administration is due to the stimulation of proliferation and differentiation of osteoprogenitor cells in bone marrow. *Bone* 1994; **15**: 717-23.
25. Tonna EA and Cronkite EP. An autoradiographic study of periosteal cell proliferation with tritiated thymidine. *Lab Invest* 1962; **11**: 455-61.
26. Tenenbaum HC and Heersche JNM. Dexamethasone stimulates osteogenesis in chick periosteum in vitro. *Endocrinology* 1985; **117**: 2211-7.
27. Urist MR. Bone: Formation by autoinduction. *Science* 1965; **150**: 893-9.
28. Wozney JM, Rosen V., Celeste AJ, *et al*. Novel regulators of bone formation: molecular clones and activities. *Science* 1988; **242**: 1528-34.
29. Wang EA, Israel DI, Kelly S *et al*. Bone morphogenetic protein-2 causes commitment and differentiation in C3H10 T1/2 and 3T3 cells. *Growth Factors* 1993; **9**: 57-71.
30. Asahina I, Sampath TK and Hauschka PV. Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. *Exp Cell Res*. 1996; **222**: 38-47.
31. Ahrens M, Ankenbauer T, Schroder D, *et al*. Expression of human bone morphogenetic protein-2 or-4 in murine mesenchymal progenitor C3H10T1/2 cells mesenchymal cell lineages. *DNA Cell Biol* 1993; **12**: 871-80.
32. Aubin JE, Heersche JNM, Merrilees MJ *et al*. Isolation of bone cell clones with differences in growth, hormone responses, and extracellular matrix production. J Cell Biol 1982; **92**: 452-61.
33. Bellows CG, Aubin JE, Heersche JNM *et al*. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *Calcif Tissue Int* 1986; **38**: 143-54.
34. Maniatopoulos C, Sodek J and Melcher AH. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* 1988; **254**: 317-30.
35. Harris SE, Sabatini M, Harris MA, *et al*. Expression of bone morphogenetic protein messenger RNA in prolonged cultures of fetal rat calvarial cells. *J Bone Miner Res* 1994; **9**: 389-94.
36. Wada Y, Kataoka H, Yokose S, *et al*. Changes in osteoblast phenotype during differentiation of enzymatically isolated rat calvarial cells. *Bone* 1998; **22**: 479-85.
37. OgawaM, Nishikawa S, Ikuta K., *et al*. B cell ontogeny in murine embryonic studies by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. *EMBO J* 1988; **7**: 1337-43.
38. Kodama H, Amagai Y, Koyama H *et al*. A new preadipose cell line derived from newborn mouse calvaria can promote the proliferation of pluripotent hematopoietic stem cells in vitro. *J Cell Physiol* 1982; **112**: 89-95.
39. Sampath TK, Maliakal JC, Hauschka PV, *et al*. Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. *J Biol Chem* 1992; **267**: 20352-62.
40. Katagiri T, Yamaguchi A, Komaki M, *et al*. Bone morphogenetic protein-2 converts the differentiation pathway of C2C 12 myoblasts into the osteoblast lineage. *J Cell Biol* 1994; **127**: 1755-66.
41. Yokose S, Ishizuya T, Ikeda T, *et al*. An estrogen deficiency caused by ovariectomy increases plasma levels of systemic factors that stimulate proliferation and differentiation of osteoblasts in rats. *Endocrinology* 1996; **137**: 469-78.
42. Bauer E, Aub J and Algright F. Studies of calcium and phosphorus metabolism. V. A study of the bone trabeculae as a readily available reserve supply of calcium. *J Exp Med* 1929; **49**: 145-62.
43. Reeve J, Williams D, Hesp P, *et al*. Anabolic effects of low doses of a fragment of human parathyroid hormone on the skeleton in postmenopausal osteoporosis. *Lancet* 1976; **1**: 1035-8.
44. Hori M, Uzawa T, Morita K, *et al*. Effect of human parathyroid hormone ((PTH(1-34)) on experimental osteopenia of rats induced by ovariectomy. *Bone Miner* 1988; **3**: 193-9.
45. Wronski TJ, Yen CF, Qi H *et al*. Parathyroid hormone is more effective than estrogen or bisphosphonates for restoration of lost bone mass in ovariectomized rats. *Endocrinology* 1993; **132**: 823-31.
46. Meng XW, Liang XG, Birchman R, *et al*. Temporal expression of the anabolic action of PTH in cancellous bone of ovariectomized rats. *J Bone Miner Res* 1996; **11**: 421-9.
47. Ishizuya T, Yokose S, Hori M, *et al*. Parathyroid hormone exerts disparate effects on osteoblast differentiation depending on exposure time in rat osteoblastic cells. *J Clin Invest* 1997: **99**: 2961-70.
48. Urist MR and Strates BS. Bone morphogenetic protein. *J Dent Res* 1971; **50**: 1392-406.
49. Sampath TK, Muthukumaran N and Reddi AH. Isolation of osteogenin , an extracellular matrix-associated , bone-inductive protein, by heparin affinity chromatography. *Proc Natl Acad Sci USA* 1987; **84**: 7109-13.
50. Wang EA, Rosen V, Cordes P, *et al*. Purification and characterization of other distinct bone-inducing factors. *Proc Natl Acad Sci USA* 1988; **85**: 9484-8.
51. Luyten FP, Cunningham NS, Ma S, *et al*. Purification and partial amino acid sequence of osteogenic, a protein initiating bone differentiation. *J Biol Chem* 1989; **264**: 13377-80.
52. Rosen V, Cox K and Hattersley G. Bone morphogenetic protein. *In*: Bilezikian J P, Raisz LG, and Rodan GA. ed. *Principles of bone biology*. Academic Press, San Diego, 1996; 661-71.
53. Hazama M, Aono A, Ueno N *et al*. Efficient expression of a heterodimer of bone morphogenetic protein subunits using a baculovirus expression system. *Biochem Biophys Res Commun* 1995; **209**: 859-66.
54. Nishitoh H, Ichijo H, Kimura M, *et al*. Identification of type

I and type II serine/threonine kinase receptors for growth/differentiation factor-5. *J Biol Chem* 1996; **271**: 21345-53.

55. Sampath TK, Rashka KE, Doctor JS, *et al*. *Drosophila* transforming growth factor β superfamily proteins induce endochondral bone formation in mammals. *Proc Natl Acad Sci USA* 1993; **90**: 6004-6008.
56. Mishina Y, Suzuki A, Ueno N *et al*. Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes Dev* 1995; **9**: 3027-37.
57. Hogan BLM. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* 1996; **10**: 1580-94.
58. Dudley AT, Lyons KM and Robertson EJ. A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev* 1995; **9**: 2795-807.
59. Luo G, Hofmann C, Bronckers ALJJ, *et al*. Bmp-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev* 1995 ; **9** : 2808-20.
60. Gitelman SE, Kirk M, Ye J-Q, *et al*. Vgr-1/BMP-6 Induces osteoblastic differentiation of pluripotential mesenchymal cells. *Cell Growth Differ* 1995; **6**: 827-36.
61. Takuwa Y, Ohse C, Wang EA, *et al*. Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen synthesis in cultured osteoblastic cells, MC3T3-E1. *Biochem Biophys Res Commun* 1991; **174**: 96-101.
62. Nakase T, Takaoka K, Masuhara K, *et al*. Interleukin-1 beta enhances and tumor necrosis factor-alpha inhibits bone morphogenetic protein-2-induced alkaline phosphatase activity in MC3T3-E1 osteoblastic cells. *Bone* 1997; **21**: 17-21.
63. Hughes FJ, Collyer J, Stanfield M *et al*. The effects of bone morphogenetic protein-2, -4, and-6 on differentiation of rat osteoblastic cells in vitro. *Endocrinology* 1995; **136**: 2671-7.
64. Boden SD, Hair G, Titus L, *et al*. Glucocorticoid-induced differentiation of fetal rat calvarial osteoblasts is mediated by bone morphogenetic protein-6. *Endocrinology* 1997; **138**: 2820-8.
65. Thies RS, Bauduy M, Ashton BA, *et al*. Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. *Endocrinology* 1992; **130**: 1318-24.
66. Yamaguchi A, Ishizuya T, Kintou N, *et al*. Effects of BMP-2, BMP-4 and BMP-6 on osteoblast differentiation of bone marrow-derived stromal cell lines, ST 2 and MC3T3-G2/PA 6. *Biochem Biophys Res Commun* 1996; **220**: 366-71.
67. Lacanda F, Avioli LV and Cheng S-L. Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. *J Cell Physiol* 1997; **67**: 386-98.
68. Chen D, Ji X, Marris MA, *et al*. Differential roles for bone morphogenetic protein (BMP) receptor type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages. *J Cell Biol* 1998; **142**: 295-305.
69. Namiki M, Akiyama S, Katagiri T, *et al*. A kinase domain-truncated type I receptor blocks bone morphogenetic protein -2-induced signal transduction in C2C12 myoblasts. *J Biol Chem* 1997; **272**: 22046-52.
70. Gimble JM, Morgan C., Kelly K, *et al*. Morphogenetic proteins inhibit adipocyte differentiation by bone marrow stromal cells. *J Cell Biochem* 1995; **58**: 393-402.
71. Katagiri T, Akiyama S, Namiki M, *et al*. Bone morphogenetic protein-2 inhibits terminal differentiation of myogenic cells by suppressing the transcriptional activity of MyoD and myogenin. *Exp Cell Res* 1997; **230**: 342-51.
72. Akiyama S, Katagiri T, Namiki M, *et al*. Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. *Exp Cell Res* 1997; **235**: 362-9.
73. Heldin CH, Miyazono K and ten Dike P. TGF-β signaling from cell membrane to nucleus through SMAD proteins. *Nature* 1997; **390**: 465-71.
74. Yamamoto N, Akiyama S, Katagiri T, *et al*. Smad 1 and Smad 5 act downstream of intracellular signalings of BMP-2 that inhibits myogenic differentiation and induces osteoblast differentiation in C2C12 myoblasts. *Biochem Biophys Res Commun* 1997; **238**: 574-80.
75. Nishimura R, Kato Y, Chen D, *et al*. Smad 5 and DPC 4 are key molecules in mediating BMP-2-induced osteoblastic differentiation of the pluripotent mesenchymal precursor cell line C2C12. *J Biol Chem* 1998; **273**: 1872-1879.
76. Hammerschmidt M, Brook A and McMahon AJ. The world according to hedgehog. *Trends Genet* 1997; **13**: 14-21.
77. Chiang C, Litingtung Y, Lee E, *et al*. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 1996; **383**: 407-13.
78. Belloni E, Muenke M, Roessler E, *et al*. Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. *Nat Genet* 1996; **14**: 353-6.
79. Roessler E, Belloni E, Gaudenz K, *et al*. Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. *Nat Genet* 1996; **14**: 357-60.
80. Seri M, Martucciello G, Paleari L, *et al*. Exclusion of the Sonic Hedgehog gene as responsible for Currarino syndrome and anorectal malformations with sacral hypodevelopment. *Hum Genet* 1999; **104**: 108- 10.
81. Hahn H, Wicking C, Zaphiropoulous PG, *et al*. Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. *Cell* 1996; **85**: 841-51.
82. Ming JE, Roessler E and Muenke M. Human developmental disorders and the sonic hedgehog pathway. *Mol Med Today* 1998; **4**: 343- 49.
83. Bitgood MJ and McMahon AP. *Hedgehog* and *Bmp* genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev Biol* 1995; **172**: 126-38.
84. Vortkamp A, Lee K, Lanske B, *et al*. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* 1996; **273**, 613-22.
85. Vortkamp A, Pathi S, Peretti GM, *et al*. Recapitulation of signals regulating embryonic bone formationg during postnatal growth and in fracture repair. *Mech Dev* 1998; **71**: 65-76.
86. Lanske B, Karaplis AC, Lee K, *et al*. Abou-Samra AB,

Juppner H, Segre GV and Kronenberg HM. PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 1996; **273**: 663-6.

87. Laufer E, Nelson CE, Johnson RL, *et al*. Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* 1994; **79**: 993-1003.
88. Kinto N, Iwamoto M, Enomoto-Iwamoto M, *et al*. Fibroblasts expressing Sonic hedgehog induce osteoblast differentiation and ectopic bone formation. *FEBS Letters* 1997; **404**: 319-23.
89. Nakamura T, Aikawa T, Iwamono-Enomoto M, *et al*. Induction of osteogenic differentiation by hedgehog proteins. *Biochem Biophys Res Commun* 1997; **237**: 465-9.
90. Natsume T, Tomita S, Iemura S, *et al*. Interaction between soluble type I receptor for bone morphogenetic protein-4. *J Biol Chem* 1997; **272**: 11535-40.
91. Dale JK, Vesque C, Lints TJ, *et al*. Cooperation of BMP 7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. *Cell* 1997; **90**: 257-69.
92. Murtaugh LC, Chyung JH and Lassar AB. Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. *Genes Dev* 1999; **13**: 225-37.
93. Thiede MA, Smock SL, Petersen DN, *et al*. Presence of messenger ribonucleic acid encoding osteocalcin, a marker of bone turnover, in bone marrow megakaryocytes and peripheral blood platelets. *Endocrinology* 1994; **135**: 929-37.
94. Desbois C, Hogue D and Karsenty G. The mouse osteocalcin gene cluster contains three genes with two separate spatial and temporal patterns of expression. *J Biol Chem* 1994; **269**: 1183-90.
95. Ducy P and Karsenty G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol Cell Biol* 1995; **15**: 1858-69.
96. Geoffroy V, Ducy P and Karsenty G. A PEBP 2α/AML-1-related factor increases osteocalcin promoter activity through its binding to an osteoblast-specific cis-acting element. *J Biol Chem* 1995; **270**: 30973-9.
97. Merriman HL, van Wijnen AJ, Hiebert SW, *et al*. The tissue–specific nuclear matrix protein, NMP-2, is a member of the AML/CBF/PEBP 2/Runt domain transcriptional factor family : Interaction with the osteocalcin gene promoter. *Biochemistry* 1995; **34**: 13125-32.
98. Rodan GA and Martin TJ. Role of osteoblasts in hormonal control of bone resorption - a hypothesis. *Calcif Tissue Int* 1982; **33**: 349-351.
99. Suda T, Takahashi N and Martin TJ. Modulation of osteoclast differentiation. *Endocr Rev* 1992; **13**: 66-80.
100. Chambers TJ, Owens JM, Hattersley G, *et al*. Generation of osteoclast-inductive and osteoclastogenic cell lines from the H-2 KbtsA 58 transgenic mouse. *Proc Natl Acad Sci USA* 1993; **90**: 5578-5582.
101. Simonet WS, Lacey DL, Dunstan CR, *et al*. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997; **89**: 309-319.
102. Tsuda E, Goto M, Mochizuki S, *et al*. Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem Biophys Res Commun* 1997; **234**: 137-142.
103. Yasuda H, Shima N, Nakagawa N, *et al*. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG) : a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology* 1998; **139**: 1329-37.
104. Bucay N, Sarosi I, Dunstan CR, *et al*. Osteoprotegerin- deficient mice develop early onset osteoporosis and arterial calcification. *Gene Dev* 1998; **12**: 1260-1268.
105. Mizuno A, Amizuka N, Irie K, *et al*. Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. *Biochem Biophys Res Commun* 1998; **247**: 610-5.
106. Anderson DA, Maraskovsky E, Billinsley WL, *et al*. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 1997; **390**: 175-9.
107. Lacey DL, Timms E, Tan HL, *et al*. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998; **93**: 165-76.
108. Wong BR, Rho J, Arron J, *et al*. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem* 1997; **272**: 25190-4.
109. Yasuda H, Shima N, Nakagawa N, *et al*. Osteoclast differentiation factor is a ligand for osteoprotegerin / osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 1998; **95**: 3597 -602.
110. Kong YY, Yoshida H, Sarosi I, *et al*. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999; **397**: 315-323.
111. Gao YH, Shinki T, Yuasa T, *et al*. Potential role of Cbfa 1, an essential transcriptional factor for osteoblast differentiation, in osteoclastogenesis: Regulation of mRNA expression of Osteoclast Differentiation Factor (ODF). *Biochem Biophys Res Commun* 1998; **252**: 697-702.
112. Kitazawa, R, Kitazawa S and Maeda S. Promoter structure of mouse RANKL/TRANCE/OPGL/ODF gene. *Biochem Biophys Acta* 1999; **1445**: 134- 141.
113. Jarvis JL and Keats TE. Cleidocranial dysostosis, a review of 40 new cases. *Am J Radiol* 1974; **121**: 5-16.
114. Mundlos S, Otto F, Mundlos C, *et al*. Mutations involving the transcriptional factor CBFA 1 cause cleidocranial dysplasia. *Cell* 1997; **89**: 773-9.
115. Mundlos S, Mulliken JB, Abramson DL, *et al*. Genetic mapping of cleidocranial dysplasia and evidence of a microdeletion in one family. *Hum Mol Genet* 1995; **4**: 71-5.
116. Levanon D, Negreanu V, Bernstein Y, *et al*. AML 1, AML 2 and AML 3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. *Genomics* 1994; **23**: 425-32.
117. Zhang Y, Bae S, Takahashi E *et al*. The cDNA cloning of the transcripts of human *PEBP2αA/CBFA1* mapped to 6 p12.3-p21.1, the locus for cleidocranial dysplasia. *Oncogene* 1997; **15**: 367-71.
118. Lee B, Thirunavukkarasu K, Zhou L, *et al*. Missense mutations abolishing DNA binding of the osteoblast-specific tran-

scriptional factor OSF2/CBFA1 in cleidocranial dysplasia. *Nature Genet* 1997; **16**: 307-10.

119. Harada H, Tagashira S, Fujiwara M, *et al*. Cbfa 1 isoforms exert functional differences in osteoblast differentiation. *J Biol Chem* 1999; **274**: 6972-8.

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