Functional involvement of the Notch pathway in osteoblastic differentiation has been previously investigated using the truncated intracellular domain, which mimics Notch signaling by interacting with the DNA-binding protein CBF-1. However, it is unclear whether Notch ligands Delta1 and Jagged1 also induce an identical cellular response in osteoblastic differentiation. We have shown that both Delta1 and Jagged1 were expressed concomitantly with Notch1 in maturing osteoblastic cells during bone regeneration and that overexpressed and immobilized recombinant Delta1 and Jagged1 alone did not alter the differentiated state of MC3T3-E1 and C2C12 cells. However, they augmented bone morphogenetic protein-2 (BMP2)-induced alkaline phosphatase activity and the expression of several differentiation markers, except for osteocalcin, and ultimately enhanced calcified nodule and in vivo ectopic bone formation of MC3T3-E1. In addition, both ligands transmitted signal through the CBF-1-dependent pathway and stimulated the expression of HES-1, a direct target of Notch pathway. To test the necessity of Notch signaling in BMP2-induced differentiation, Notch signaling was inhibited by the dominant negative extracellular domain of Notch1, specific inhibitor, or small interference RNA. These treatments decreased alkaline phosphatase activity as well as the expression of other differentiation markers and inhibited the promoter activity of Id-1, a target gene of the BMP pathway. These results indicate the functional redundancy between Delta1 and Jagged1 in osteoblastic differentiation whereby Delta1/Jagged1-activated Notch1 enhances BMP2-induced differentiation through the identical signaling pathway. Furthermore, our data also suggest that functional Notch signaling is essential not only for BMP2-induced osteoblast differentiation but also for BMP signaling itself.

Notch signaling pathway is highly conserved beyond species and plays a critical role in a variety of cellular functions, including cell proliferation, differentiation, and apoptosis (1). To date, four Notch receptors (Notch1–4) and five of their ligands (Delta1, -3, -4 and Jagged1, -2) have been identified in mammals; all are single-span transmembrane polypeptides that act by cell-to-cell contact (2). Notch receptors contain 36 epidermal growth factor-like repeats and 3 cysteine-rich Notch/LIN-12 repeats in a large extracellular domain and 6 tandem cdc10/ankyrin repeats, a glutamine-rich domain, and a PEST sequence in an intracellular domain. Notch ligands also contain epidermal growth factor repeats in the extracellular domain in addition to a unique cysteine-rich N-terminal region referred to as the Delta:Serrate:LAG2 (DSL) domain, but their intracellular domains are small and poorly conserved. The interaction of Notch with the ligands induces the nuclear translocation of the intracellular domain of Notch as a result of proteolytic cleavage at the juxtamembrane portion. In the nuclei, the intracellular domain interacts with CSL DNA-binding proteins, including CBF1/RBP-J, and transactivates target genes such as hairy enhancer of split-1 (HES-1) and HES-5. In an alternative pathway, Notch receptors have also been reported to transmit signal through CSL-independent pathways by interacting with other signaling molecules, such as mitogen-activated protein kinase, Src, and nuclear factor κB (3–5).

The Notch pathway has been demonstrated to be critical for a variety of developmental processes (1), but its functional role in bone development has been poorly understood as mice deficient in Notch1, Jagged1, or Delta1 die at an early embryonic stage before bone formation due to impaired somatogenesis and/or vascularization (6–8). In contrast, the in vitro potential of Notch pathway in osteoclastogenesis and osteoblastogenesis has been investigated in several reports. Notch activation reduces the surface expression of c-Fms, which is a receptor for macrophage colony-stimulating factor, in osteoclast precursor cells and enhances the expression of osteoprotegerin in stromal cells, which results in the down-regulation of osteoclastogenesis (9). However, controversial results have been obtained with respect to osteoblastic differentiation. Continuous expression of the intracellular domain of Notch1 (NICD), believed to act as a constitutive active form by retrovirus system or by an ordinary stable transfection study, inhibits bone morphogenetic
protein (BMP) 2-induced osteoblast differentiation in osteoblast precursor cells, including MC3T3-E1 (10, 11). In contrast, the transient expression of NICD by the adenovirus system in MC3T3-E1 cells leads to an enhanced bone mineral deposition (12). However, despite these results, it is critical to determine whether Notch ligands Delta and Jagged have an identical function in osteoblastic differentiation because these ligands have been shown to exert distinct activity in certain cells (13, 14). Moreover, it is also unclear whether Notch signaling itself is required for the osteoblastic differentiation induced by BMP2. The interaction between Notch and BMP has been demonstrated to be crucial for endothelial cell migration and myogenic differentiation (15, 16).

To clarify the functional involvement of Delta/Jagged-Notch signaling pathway in osteoblastic differentiation, we first confirmed the expression of Notch, Delta, and Jagged by immunohistochemistry in an in vivo bone regeneration model. We then addressed the functional difference between Delta1 and Jagged1 by transient transfection study and by using recombiant proteins in MC3T3-E1 and C2C12 cells. We also clarified the essential role of Notch1 signaling in BMP2-induced osteoblastic differentiation by inhibiting Notch signaling with the dominant negative form of Notch1, a specific inhibitor, and siRNA.

MATERIALS AND METHODS

Bone Regeneration Model and Immunostaining—Bone defects (1.2-mm diameter) were created in the diaphysis of unilateral femurs of 8-week-old C57BL6/J mice using a dental drill with copious saline irrigation. Notch expression was analyzed after 5, 10, and 15 days of operation. For isolation of RNA, the femur was dissected into 8-mm-long pieces containing the bone defect using a bone saw. For immunohistochemistry, the sections were perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline, dehydrated with a series of ethanol, and embedded in paraffin. The deparaffinized sections were incubated with 0.3% H2O2 in methanol for 30 min, followed by 10% horse serum for 1 h. The slides were incubated with primary antibody for Notch1 (UBI) and Delta1 (F-15; Santa Cruz Biotechnology) or Jagged1 (188323; R&D Systems) for 2 h at room temperature. These primary antibodies were detected by secondary antibodies, Alexa 488 and sheep anti-rabbit Alexa 555 (Molecular Probe) and visualized by confocal laser microscopy (LSM5 Pascal; Carl Zeiss) as described previously (17). To detect the active form of Notch1, the section was incubated with anti-cleaved (Val1744) antibody (Cell Signaling), and color was developed by horseradish peroxidase-labeled anti-rabbit secondary antibody with diaminobenzidine as a substrate.

Plasmid Constructs—Expression vectors for the full length of Jagged1 and Delta1 include human cDNA (4, 18). Expression vectors of CBP-1 (wild type and dominant negative variant R218H) (19) and NICD (4) contain mouse cDNA. ALP (alkaline phosphatase)-luc and OSE-luc contain the 1838/81 promoter region of the mouse ALP gene and six copies of the Rux2 binding site on the osteocalcin 2 promoter, respectively (20). The Id-1 reporter includes four tandem copies of the Smad-binding site on the human Id-1 core promoter (21). HES-1-luc contains the 87 to 51 promoter fragment of the human HES-1 gene.

Cell Culture, Transient Transfection, and Luciferase Assay—C3H10T1/2, MC3T3-E1, and C2C12 cells were obtained from Riken Gene Bank (Tsukuba, Japan). C3H10T1/2 and MC3T3-E1 were maintained in Eagle's minimal essential medium containing 10% fetal bovine serum (Intergen), 50 units/ml penicillin-G, and 50 mg/ml streptomycin. C2C12 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and the antibiotics. Primary osteoblastic cells were isolated enzymatically from newborn mouse calvariae as described previously (22) and maintained in the same condition. Mesenchymal cells in bone marrow were obtained from mouse femurs described elsewhere. Transfection of the plasmid DNA was performed with Lipofectamine reagent (Invitrogen) according to the manufacturer's instruction. After 24 h of transfection, the cells were incubated with or without BMP2, which was kindly provided by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). Luciferase activity was determined 48 h after transfection by the dual luciferase assay kit (Promega). The processes of ligand preparation and coating on the culture dish have also been described previously (9).

ALP Staining and Enzyme-linked Immunosorbent Assay of Osteocalcin—ALP activity was determined by histochemical technique using naphthol AS-MX phosphate and fast blue BB salt as described previously (23). To determine the concentration of osteocalcin in the conditioned medium was measured by enzyme-linked immunosorbent assay (Biomedical Technologies).

In Vivo Bone Formation—MC3T3-E1 cells (3 x 10^4 in number) were inoculated into poly-D,L-lactic-co-glycolic acid/gelatin sponge (PGS) (kindly provided by Yamanouchi Pharmaceutical Co. Ltd.), a scaffold carrier that was preincubated with 1 μg of either BMP2, Delta1, and/or Jagged1. The PGS/cell complex was then transplanted into the subfascial region of nude mice as described previously (20) and subjected to soft x-ray imaging and histological analysis 2 weeks after transplantation.

siRNA and Immunoblotting—siRNA specific for mouse Notch1 was introduced into MC3T3-E1 and C2C12 cells at the final concentration of 1 μmol with Oligofectamine reagent (Invitrogen) according to the manufacturer’s instruction. The siRNAs designed for Notch1 were as follows: RNAi1, AGCCGAAAGAGGAGAAGAGAU; RNA2, CCAAAGCAACGCGAAACAA; RNA3, UGAAAGCCGAAACUGGAAAGAA. All of these duplex siRNAs carry the overhanging AG and UA extranucleotides for sense and antisense sites, respectively (I gene, Tsukuba, Japan). The scramble sequence ACCCGUAAACCGGGA-AUUU was used as the control. Four hours later, the cells were incubated with or without recombinant human BMP2 and subjected to ligand incubation. ALP staining, ALP activity, or immunoblotting, the transfected cells were harvested in a buffer of 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA, and a protease inhibitor mixture (Roche Applied Science), and the supernatant was resolved by 6% SDS-PAGE and electrotransferred to a nitrocellulose membrane. Notch1 expression was detected by anti-Notch1 cytoplasm antibody (UB1) and ECL plus (Amersham Biosciences).

Real-time PCR—cDNA was synthesized from 1 μg of total RNA using AMV reverse transcriptase and oligo(dT) primer, and the mRNA expression was quantified by the Light Cycler system (Roche Diagnostics). Primer sequences, MgCl2 concentration, and the annealing temperature are available on request. The relative amount of mRNA was determined at 50% levels of PCR product and normalized by glyceraldehyde-3-phosphate dehydrogenase expression.

RESULTS

Notch Expression during Bone Regeneration—Notch receptors and their ligands have been demonstrated to express in certain osteoblasts, but their in vivo expression profile remains unclear. To clarify this, immunohistochemical analysis of Delta1, Jagged1, and Notch1 was performed using an adult mouse femur carrying a bone defect in the diaphysis. Immunoreactivity to Notch1 was subtle in untreated femurs. However, strong expression was detected in the mesenchymal cells that had migrated into the bone defect 5 days after injury (Fig. 1, A and B). At this stage, Delta1 was also expressed, and confocal microscopic analysis demonstrated extensive colocalization between Notch1 and Delta1. Immunostaining with Jagged1 on day 10, when the cortical bone was in a reconstruction phase, showed that both Notch1 and Jagged1 colocalized in the osteoblasts on the surface of the trabeculae. Immunohistochemistry using antibodies that recognize the active form of Notch1 (23) showed that the Notch1 signal is activated in the osteoblasts and chondrocytes appeared around the bone defect (Fig. 1D). Real-time PCR demonstrated that the expression of Notch1, Delta1, and Jagged1 was temporarily up-regulated on day 5 after the drill hole injury was introduced (Fig. 1E). These results indicate that the Notch1 signal is activated in osteoblast-committed cells during bone regeneration via up-regulation of Delta1 and Jagged1.

ALP Activity and Osteocalcin Production by Transient Expression of Notch1, Delta1, and Jagged in MC3T3-E1 and C2C12 Cells—Prior to investigating the functional role of Notch signaling in osteoblast differentiation, the expression of Notch1, -4, Delta1, -3, -4, and Jagged1, -2 was examined in three osteoprogenitor cell lines, namely C3H10T1/2, MC3T3-E1, C2C12, and in the primary culture of calvaria- and bone marrow-derived osteoblastic cells. Reverse transcription PCR demonstrated that although the expression of several ligands,
such as Notch4 and Delta4, was absent or very low in several cells, other molecules, including Notch1, Delta1, and Jagged1, were expressed well in the examined cells (Fig. 2A). Because the functional property of ligand-induced Notch activation in osteoblast differentiation is not yet characterized, we determined to compare the functional differences of Delta1 and Jagged1. For this, we first carried out a transient transfection study using the expression vector of NICD and full length of Delta1 and Jagged1 in the cultured MC3T3-E1 and C2C12 cells. ALP staining showed that overexpression of these molecules alone did not influence ALP activity but enhanced BMP2-induced ALP activation in both cell lines (Fig. 2B). Luciferase reporter assay carrying the ALP promoter showed that Delta1 and Jagged1 transactivated the promoter activity by Notch activation. MC3T3-E1 cells were transiently transfected with the luciferase reporter construct carrying the mouse ALP gene promoter along with empty vector or the indicated expression plasmids. All the data, including the following figures, are expressed as mean ± S.D. from four independent transfection experiments. D, inhibition of BMP2-induced osteocalcin production by Notch activation in C2C12 cells. The concentration of osteocalcin in condition medium was measured by enzyme-linked immunosorbent assay. E, inhibition of osteocalcin (OSC) promoter activity by Notch activation in C2C12 cells.

**FIG. 1. Expression of Notch1, Delta1, and Jagged1 during bone regeneration.** A, hematoxylin and eosin stain of a bone defect lesion 5 days after the drill hole injury was introduced. A drill hole defect 1.2 mm in diameter was created in the middle shaft of the mouse femur. Note that a number of mesenchymal cells were invaded into the bone defect (original magnification ×25). B, immunohistochemistry of Notch1 and Delta1 at 5 days after the drill hole injury was introduced. Expression of Notch1 and Delta1 was detected by Alexa647- and Alexa488-labeled secondary antibodies, respectively, and colocalization was visualized by confocal microscopy. B and C, dotted lines represent the margin of the original cortical bone. C, colocalization of Notch1 and Jagged1 in osteoblastic cells reconstructing cortical bone 10 days after bone defect. Notch1 and Jagged1 were extensively colocalized in the numerous osteoblasts (original magnification ×50). D, localization of the active form of Notch1 5 days after the bone defect. The active form of Notch1 was detected by specific antibody to the cleaved form of Notch1 and visualized by diaminobenzidine as a substrate. E, up-regulation of Notch1, Delta1, and Jagged1 expression during bone regeneration. Relative expression of each mRNA was quantified by real-time PCR.
FIG. 3. Requirement of ligand immobilization and in vivo ectopic bone formation. A, enhanced ALP activity by immobilized, but not in medium, Delta1 and Jagged1. In the presence of BMP2 (500 ng/ml), MC3T3-E1 and C2C12 cells were cultured either on dishes precoated with recombinant Delta1 or Jagged1 (coat) or in a condition of each protein suspended in a culture medium (1 µg/ml) (medium). B, suppression of BMP2-induced osteocalcin production by immobilized Delta1 and Jagged1 in C2C12 cells. C, real-time PCR analysis of osteoblastic differentiation markers. The data are expressed as mean ± S.D. after normalization by glyceraldehyde-3-phosphate dehydrogenase expression. OSC, osteocalcin; OSP, osteopontin; Col1α, type I collagen. D, calcified nodule formation induced by Delta1 and Jagged1. MC3T3-E1 cells were inoculated on Delta1- or Jagged1-coated plates and incubated with 0.1 µg/ml ascorbic acid and 5 mM β-glycerophosphate for 3 weeks. At the end of the incubation, the cells were subjected to real-time PCR of ALP and osteocalcin (upper graph) or to von Kossa staining (lower panel). E and F, enhanced ectopic bone formation by Delta1 and Jagged1. MC3T3-E1 cells were attached into a solubl scaffold carrier containing recombinant Delta1 or Jagged1 in the presence or absence of BMP2 and transplanted into subfascial regions of nude mice. E, soft x-ray image of the scaffold/cells complex 2 weeks after transplantation. F, hematoxylin-eosin and von Kossa staining of one of the representative results in panel D.

contact. To confirm that recombinant Delta1 and Jagged1 also display identical functions as demonstrated by transient expression study and to test whether ligand immobilization is necessary, MC3T3-E1 and C2C12 cells were incubated with recombinant Delta1 or Jagged1 under two culture conditions wherein the recombinant proteins were suspended in culture medium or immobilized on the culture plate. The result showed that for BMP2, an augmentation in BMP2-induced ALP activity was observed in the presence of immobilized Delta1 and Jagged1 but not in the presence of Delta1 and Jagged1 in the medium (Fig. 3A). In addition, inhibition of osteocalcin expression was also observed in the immobilized condition (Fig. 3B). The expression profile of osteoblastic differentiation markers was investigated by real-time PCR in C2C12 cells. In addition to ALP, mRNA levels of type I collagen (Col1α) and Runx2 were increased by Jagged1 coating, whereas osteopontin levels did not change and osteocalcin levels decreased slightly (Fig. 3C). Similar results were obtained with Delta1 treatment (data not shown). However, 3 weeks of culture in the presence of β-glycerophosphate and ascorbic acid resulted in enhanced mineralization in the presence of Delta1 and Jagged1 with concomitant up-regulation of ALP and osteocalcin mRNA (Fig. 3D). We also examined the in vivo potential of Delta1 and Jagged1 for bone formation; a scaffold carrier carrying MC3T3-E1 cells along with Delta1, Jagged1, and/or BMP2 was transplanted into the subfascial region of nude mice for 2 weeks. The results showed that Delta1 or Jagged1 alone did not trigger calcification but enhanced ectopic bone formation induced by BMP2 (Fig. 3E). The histology results showed that larger numbers of mineralized bone trabeculae could be observed in cases carrying both BMP2 and Delta1 or Jagged1 compared with BMP2 alone (Fig. 3F). These results indicate that both Delta1 and Jagged1 need to be immobilized and act as positive regulators for osteoblastic differentiation, although osteocalcin production is down-regulated in an early stage of differentiation.

CBF-1-dependent Activation and HES-1 Regulation—Notch1 transmits signals through CSL DNA-binding protein-dependent and -independent pathways. Wild type or dominant negative (R218H) CBF-1 was transfected with or without Delta1 or Jagged1 to observe the requirement of CBF-1 for Delta/Jagged-induced Notch activation. The results showed that CBF-1 alone is able to enhance BMP2-induced ALP activation and that R218H CBF-1 blocked Delta1/Jagged1-induced ALP activity (Fig. 4, A and B). Similar inhibitory effects caused by R218H were observed in the expression of Runx2 and Col1α (data not shown). In addition, both Delta1 and Jagged1 induced the expression of HES-1, a target gene of Notch, through the CBF-1-dependent pathway (Fig. 4, C and D). Considered collectively, these results suggest that CBF-1 is involved in the osteoblast differentiation induced by Delta1 and Jagged1.

Suppression of Notch1 Signaling by Dominant Negative Extracellular Domain and Specific Inhibitor—Notch signaling has been demonstrated to play a crucial role for cell fate deter-
the suppression of ALP, Runx2, and Col1, the expression of which was dramatically decreased (Fig. 6A) except for osteocalcin, transcriptional levels (Fig. 6D). MC3T3-E1 cells were transiently transfected with or without the expression vector of dominant negative form of CBF-1 (R218H) and subjected to ALP staining after 5 days of culture in the presence of BMP2 (500 ng/ml). C, induction of HES-1 expression by Delta1 and Jagged1. MC3T3-E1 cells were incubated with or without the indicated precoated Notch ligands for 3 days and subjected to real-time PCR of HES-1. D, involvement of CBF-1 in the induction of HES-1 promoter activity. MC3T3-E1 cells were transiently transfected with the indicated plasmids and subjected to luciferase assay.

**Fig. 4. Involvement of CBF-1 in Delta1/Jagged1-induced ALP activation and HES-1 expression.** A, enhanced ALP activity by CBF-1. MC3T3-E1 cells were transiently transfected with the indicated plasmids and subjected to ALP staining after 5 days of culture in the presence of BMP2 (500 ng/ml). B, involvement of CBF-1 in Delta1- and Jagged1-induced ALP activity. MC3T3-E1 cells were transiently transfected with or without the expression vector of dominant negative form of CBF-1 (R218H) and subjected to ALP staining after 5 days of culture in the presence of BMP2 (500 ng/ml). C, induction of HES-1 expression by Delta1 and Jagged1. MC3T3-E1 cells were incubated with or without the indicated precoated Notch ligands for 3 days and subjected to real-time PCR of HES-1. D, involvement of CBF-1 in the induction of HES-1 promoter activity. MC3T3-E1 cells were transiently transfected with the indicated plasmids and subjected to luciferase assay.

**Inhibition of Endogenous Notch1 Expression by siRNA—**To further confirm the requisite role of Notch pathway, three distinct small interference RNA (siRNA), which were designed based on the coding region of mouse Notch1, were transfected into the cultured cells. The transfection efficiency of Cy3-labeled control siRNA was ~40% (data not shown). Immunoblots of total cell lysates of Notch1-siRNA-introduced MC3T3-E1 cells showed specific inhibition of endogenous Notch1 (Fig. 6A). Similar to the case of DN-Notch1, the siRNA-mediated suppression of Notch1 resulted in a strong inhibition of BMP2-induced ALP activity as well as osteocalcin expression (Fig. 6B and C) by affecting the transcriptional levels (Fig. 6D). However, the expression levels of osteoblastic differentiation markers were not necessarily concomitant with the promoter activity except for osteocalcin, the expression of which was dramatically decreased (Fig. 6E). The suppression of ALP, Runx2, and Col1α was ~40% of the scramble RNA. Considered collectively, however, these results indicate that endogenous Notch1 signaling is essential for BMP2-induced osteoblast differentiation.

**Interaction between Notch and BMP Signaling—**The interference of signaling pathway between Notch and BMP/transforming growth factor-β (TGFβ) has been previously demonstrated (16, 24). To study a mechanism of Notch1-induced augmentation of BMP activity, we first examined whether Notch activation could enhance BMP signaling. For this purpose, the expression and transcriptional control of Id-1, a target gene of BMP signaling, were examined in cells treated with immobilized Delta1 and Jagged1 in the presence or absence of BMP2. However, the results showed that mRNA levels and Id-1 promoter activity induced by BMP2 could not be influenced by Notch activation (Fig. 7A and B). In addition, it was determined by real-time PCR that Notch1 activation did not alter the expression levels of BMP2 antagonists such as Noggin, Chordin, Follistatin, and Tsg (data not shown). These data suggest that the enhancement of BMP2 activity by Notch1 is not due to the activation of BMP signaling. Next, we investigated a mechanism by which the enhancement of the Notch signal leads to a dramatic decrease in BMP activity. Incubation with L685,458 reduced the expression levels of Id-1 (Fig. 7C). In addition, the transient transfection of siRNA resulted in the reduction of Id-1 promoter activity (Fig. 7, C and D). Considered collectively, these data indicate that the suppression of BMP2-induced osteoblastic differentiation by Notch inhibition is at least partially due to suppressed BMP signaling.

**DISCUSSION**

Notch receptors are known to play an important role in epithelial-mesenchymal interactions and are required for the organization of many different tissues, including vascular system, hematopoietic system, skin, and pancreas (2). Although the expression of Notch-related molecules in developing bone remains unclear, a recent study has demonstrated that Notch1 is expressed in the mesenchymal condensation area and subsequently in the hypertrophic chondrocytes during chondrogenesis (26). From this study, it appears that Notch1, Delta1, and Jagged1 are expressed in cultured osteoblast precursor cells as
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well as in differentiating osteoblasts during bone regeneration and that Notch1 is actually activated in these cells. These results suggest that in addition to the maintenance of hematopoietic stem cell niche (27), Notch signaling plays an important role in the commitment of mesenchymal cells to the osteoblastic cell lineage. Moreover, the concomitant expression of Notch1, Delta1, and Jagged1 may give rise to a possibility of an autocrine and/or paracrine action instead of a cell-to-cell signaling mechanism that is characterized by a contact-dependent inhibition model in the development of the nervous system (28).

Although there is no functional difference between Delta and Jagged in a majority of cases, these ligands show distinct expression patterns in a part during development (29, 30). This suggests that specific ligand-receptor pairs may perform distinct functions. Indeed, a recent study has demonstrated the role of Delta and Jagged in the differential commitment of T lymphocytes (13). Fringe, an extracellular regulatory protein, glycosylates Notch, which in turn leads to specific inhibition of Jagged binding (5). In this study, we demonstrated that, like NICD, Delta1 and Jagged1 show identical biological activity in the case of osteoblastic differentiation. Although Delta1 or Jagged1 alone is unable to modulate differentiated status, these ligands enhance BMP2-induced expression of several differentiation markers, such as ALP, Runx2, and Col1α, in the same manner. In addition, Delta1 and Jagged1 are capable of enhancing bone mineral deposition and BMP2-induced ectopic bone formation. Furthermore, it appeared that both Delta1 and Jagged1 transmit signals through CBF-1 and transactivate the HES-1 gene. Similar results have been demonstrated by Tezuka et al. (12), who utilized the adenovirus system to express NICD. Osteocalcin expression is likely to be suppressed in an initial phase of differentiation when Notch1 is activated (12, 31). In general, Notch signaling is thought to either block differentiation toward a primary differentiation fate or to force the cell to remain in an undifferentiated state (1). Considering the concomitant expression of Delta1 and Jagged1 in vivo bone regeneration, these results suggest that there is a functional redundancy between Delta1 and Jagged1 and that these ligands direct osteoprogenitor cells to the differentiated status through identical signaling pathways. However, contradictory results have been reported from a retrovirus or an ordinary stable transfection study in which NICD introduction impairs osteoblastic differentiation of stromal cell ST-2 and MC3T3-E1 and Kusza cells (10, 11). One interpretation of this difference is that the effect of Notch activation may vary depending on the period of Notch activation; transient and short-term activation of the Notch pathway may enhance osteoblastic differentiation as shown in our study, whereas long-term continuous activation may lead to the inhibition of osteoblastic commitment. Further studies would be necessary to elucidate the culture period-dependent functional difference of Notch signaling in osteoblastic differentiation.

In this study, we demonstrated that the inhibition of Notch signaling by DN-Notch or L685,458 leads to a dramatic decrease in ALP and osteocalcin production. Furthermore, the inhibition of endogenous expression of Notch1 by siRNA also
resulted in a dramatic decrease in osteoblastic differentiation associated with the decrease in differentiation markers, such as Col1a and Runx2 expression. In particular, osteocalcin expression was severely suppressed at the transcriptional level. Although this observation is not consistent with the results of the ligand stimulation study, it suggests that a functional Notch signal may be essential for osteocalcin expression through a distinct regulatory mechanism compared with other marker genes. Considered collectively, these results indicate that constitutive Notch1 signaling is critical for BMP2-induced osteoblastic differentiation. In this regard, it has been demonstrated that the inhibition of Notch signal through L685,458 prevents BMP4-induced suppression of myogenesis of C2C12 cells (15), indicating that functional Notch signaling may be necessary, to some extent, for BMP-induced differentiation.

To explore a mechanism of the cooperative interaction between Notch and BMP signaling in osteoblast differentiation, we examined the interaction between BMP and Notch signaling. Recent studies have revealed that Notch and BMP/TGFβ cooperatively activate opposite pathways such as HES1 and HES-5 or Smad target gene PAI-1 (16, 24, 25). In Notch pathway, NICD interacts with Smad1/5 and recruits the transcription machinery, including p300 and P/CAF, resulting in the cooperative activation of Notch target genes such as Herp2 and cooperative interaction between BMP2 and Notch in osteoblast differentiation, although Hey1, another target gene of both BMP and Notch. It is possible that other Notch target genes such as Lfng, Hey1, and Tcf7 that could be up-regulated in C2C12 cells with response to BMP2 (32) may be involved in the cooperative interaction between BMP2 and Notch in osteoblast differentiation, although Hey1, another target gene of both Notch and BMP, acts as negative regulator (33). More importantly, our data revealed that the inhibition of Notch signaling or expression leads to a dramatic decrease in Id1 promoter activity, indicating that Notch signaling is required for BMP signaling itself and that BMP signaling locates upstream of Notch as described previously in the regeneration process in Xenopus tail (34). However, it is most likely that Notch signaling may be critical for TGFβ pathway as well, because TGFβ-induced reporter activity using 3TP-luc and ARE-luc was also inhibited by DN-Notch and L685,458 in our examined cells. Further experiments are necessary to explore the underlying mechanism that links Notch inhibition to the suppressed BMP/TGFβ signaling.

In conclusion, our results demonstrate that both Delta1 and Jagged1 transmit signals through the CBF1-dependent pathway and induce osteoblastic differentiation by cooperating with BMP. In addition, functional Notch1 activation is essential not only for BMP-induced osteoblast differentiation but also for BMP signaling itself. Our results suggest a therapeutic potential for the signaling pathway from Delta1 and Jagged1 to CBF1-dependent gene expression in bone regeneration as well as osteoporosis.

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