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REVIEW ARTICLE

Regulation of osteoblast differentiation mediated by BMP, Notch, and CCN3/NOV

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Summary Osteoblasts originate from common progenitors, which are capable of differentiating into other mesenchymal cell lineages such as chondrocytes, myoblasts and adipocytes. Various hormones and cytokines regulate osteoblast differentiation of mesenchymal progenitors to osteoblasts. Among these, bone morphogenetic proteins (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. BMPs are important local factors that regulate Runx2, which is an essential transcription factor for osteoblast differentiation. The Notch signaling pathway is involved in a variety of cellular function, including cell proliferation, differentiation and apoptosis. Notch signaling has a dual effect on osteoblast differentiation. In terms of stimulation, functional Notch signaling is essential not only for BMP-2-induced osteoblast differentiation but also for BMP signaling itself. CCN3/NOV, a member of the CCN family of proteins, exerts inhibitory effects on BMP-2-induced osteoblast differentiation via its involvement in the BMP and Notch signaling pathways. Thus, osteoblast differentiation is critically regulated by the intimate interaction of various signaling molecules including BMP, Notch and CCN3/NOV.

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1. Introduction

Skeletal tissue is composed of various types of mesenchymal cells such as osteoblasts, chondrocytes, myocytes, and adipocytes. These cells originate from common pluripotent progenitors called mesenchymal stem cells [1]. During the differentiation of these cell lineages, various hormones and cytokines critically regulate osteoblast differentiation. Among these, bone morphogenetic proteins (BMPs) are strong inducers of osteoblast differentiation and bone formation [2—4]. BMPs were originally identified as proteins that induced ectopic bone formation when implanted into muscular tissue [5]. Several lines of evidence have demonstrated that BMP-2 induces or promotes the expression of runt-related gene 2 [Runx2, alternatively called core-binding factor alpha 1 (Cbfa1)] [6—8] and osterix [9,10], which are essential transcription factors for osteoblast differentiation and bone formation [11—13], as well as osteoblast differentiation markers such as alkaline phosphatase (ALP), type I collagen and osteocalcin in various cells. Thus, BMPs are key molecules in the regulation of osteoblast differentiation.

The Notch signaling pathway is highly conserved among species and plays a fundamental role in a wide variety of processes during embryonic development and in the adult [14]. The *Notch* gene encodes a single transmembrane receptor member. The ligands for Notch including Delta1, Delta3, Delta4, Jagged1, and Jagged2 are also transmembrane proteins, and Notch signaling mediates cell—cell communication by regulating the choice of binary cell fate and the maintenance of stem cell populations [15]. In addition, it has been revealed that cross-talk between Notch and BMP signaling tightly regulates the cell fate in various types of cell lineages [16—19]. Although several studies using osteoblastic cell lines have demonstrated that Notch signaling is involved in the regulatory mechanism underlining osteoblast differentiation, these remain controversial [19—23].

The CCN family of proteins comprise the following six members; CCN1/cysteine-rich 61(CYR61), CCN2/connective tissue growth factor (CTGF), CCN3/nephroblastoma overexpressed gene (NOV), CCN4/Wnt-induced secreted protein 1 (WISP1), CCN5/WISP2, and CCN6/WISP3. These proteins have been demonstrated to possess a growth factor-like activity and to regulate cell growth and tissue formation. It has been reported that CCN proteins regulate the differentiation of skeletal mesenchymal cells such as muscle cells [24,25], chondrocytes [26,27] and osteoblasts [28—31]. Importantly, CTGF/CCN2 binds to BMP-4 and antagonizes its action [32]. We have also demonstrated that CCN3/NOV associates with Notch1, and that it inhibits myogenic differentiation [24]. Since these reports highlight the importance of CCN proteins in osteoblast differentiation, we investigated the role of CCN3/NOV in osteoblast differentiation [31]. Our study indicates that CCN3/NOV regulates osteoblast differentiation by interacting with BMP and Notch signalings.

Thus, osteoblast differentiation is regulated by multiple factors that interacting with BMPs. In this review, we will describe the roles of BMP, Notch and CCN3/NOV in osteoblast differentiation.

2. The role of BMPs in osteoblast differentiation

2.1. BMP signaling pathway

BMPs bind to two types of serine—threonine receptor, termed BMP type I receptor (BMPR-I) and type II receptors [33]. Both types of receptor are necessary for the transduction of BMP signals. Four BMPR-I receptors [(TRS (ALK1), ACVR1 (ALK2), BMPR-IA, and BMPR-IB)] have previously been cloned. Three type II receptors (BMPR-II, ActR-IIA, and ActR-IIB) are involved in the BMP signaling pathway. BMPR-I binds BMPs directly in the absence of type II receptors. This is in contrast to the transforming growth factor-beta (TGF-β) type I receptor that does not bind ligands in the absence of the TGF-β type II receptor [34,35]. BMPs preferentially bind at the N-terminal extracellular domain of BMPR-I, which is connected by a single transmembrane region to the C-terminal cytoplasmic kinase domain. BMPR-I has a cytoplasmic juxtamembrane region that is rich in glycine and serine residues (GS domain). Ligand-binding phosphorylates serines and threonines in the GS domain thereby activating BMPR-II; this event transmits downstream BMP signals through BMP-specific Smads and p38 MAPK to regulate BMP-responding genes.

A number of signal-transducing molecules of the TGF-β superfamily, termed Smads, have previously been identified (Fig. 1) [33,36,37]. To date, eight mammalian Smad pro-

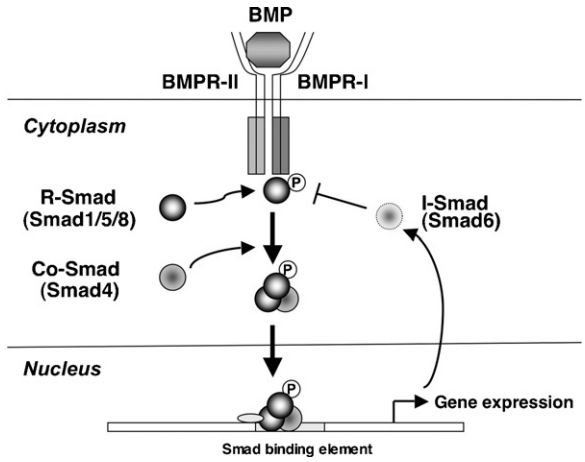

Figure 1 BMP signaling via Smad pathway. BMP binding to BMPR results in activation of BMPRs. Activated receptor complex phosphorylates R-Smad (Smad1/5/8). Activated R-Smad associates with Co-Smad (Smad4), and this complex translocates into nucleus. This complex bind to Smad binding element with transcriptional coactivator or corepressor. BMP signal stimulates expression of I-Smad (Smad6), which is capable of inhibiting the phosphorylation of R-Smad. Adapted from Ref. [37].

teins, Smad1 through Smad8, have been isolated [33,37]. These proteins are classified into three subgroups according to their structures and functions [33]. The Smads belonging to the first subgroup, referred to as pathway-restricted Smads (R-Smads) are ligand-specific and activated by the binding of ligands to type I receptors. Among these, Smad1, Smad5 and Smad8 are involved in BMP signaling [37—41], whereas Smad2 and Smad3 mediate TGF-β/activin signaling [42,43]. The second subgroup of Smads comprises the common mediator Smads (C-Smads). Smad4 (also termed DPC-4) belongs to this subgroup [42]. R-Smads are phosphorylated by the serine/threonine kinase receptors that interact with C-Smads, and then form a heterodimeric complex. This complex is translocated into the nucleus and regulates the transcription of target genes. The third subgroup of Smads comprises the inhibitory Smads (I-Smads), Smad6 and Smad7 [42—45]. These Smads inhibit ligand activity by stably binding to type I receptors. Smad6 binds to the TGF-β type I receptor, the activin type IB receptor and BMPR-IB, while Smad7 binds to the TGF-β type I receptor [44]. I-Smads compete with R-Smads for the binding to type I receptors. Smad6 also competes with Smad4 for the binding to activated Smad1 [45].

Various BMP antagonists regulate the action of BMPs at extracellular region [46]. These antagonists bind to BMPs and prevent specific binding to the cell surface receptors. Extracellular BMP antagonists include noggin, chordin, follistatin, follistatin-related gene, twisted gastrulation, and DAN family members (Dan, Cerberus, gremlin, sclerostin/SOST). Indeed, noggin, chordin and gremlin inhibit BMP-induced ALP activity in W-20-17 bone marrow stromal cells and C3H10T1/2 cells [47—50]. During mouse embryogenesis, noggin is expressed not only in the node, notochord, and dorsal somite, but also in the condensing cartilage and immature chondrocytes [50] *Noggin* null mutant mice indicate that this molecule plays important roles in the normal patterning of the neural tube, somites and cartilage including joint formation [50].

2.2. Regulation of osteoblast differentiation by BMPs

Various mesenchymal cell lines are useful for studying the regulatory mechanism of osteoblast differentiation. C3H10T1/2 clone 8 (C3H10T1/2), a fibroblastic cell line derived from an early mouse embryo, comprises multipotential mesenchymal cells. Untreated control C3H10T1/2 cells exhibit no or extremely low levels of phenotypic characteristics related to osteoblasts, chondrocytes, myoblasts, and adipocytes [51]. We were the first to demonstrate that BMP-2 strongly enhances ALP activity, which is an early marker of osteoblast differentiation [51]. Although control C3H10T1/2 cells produced no osteocalcin, a marker appearing at the late stage of osteoblast differentiation, BMP-7-induced osteocalcin production by C3H10T1/2 cells [52]. These results indicated that BMP-2 and BMP-7 induce C3H10T1/2 cells to differentiate into osteogenic lineage cells. BMP-2, BMP-6, BMP-7, and GDF5 stimulates ROB-C26 cells, which are a committed osteoprogenitor cell line that retains the potential to differentiation into myotubes and adipocytes [53], to differentiate into osteoblasts [3]. These results indicated that BMPs are important regulators of the differentiation of osteoblasts from multipotent mesenchymal cells.

There are several osteoblast precursor cell lines, the differentiation potential of which is restricted to the osteoblast lineage. Among these, MC3T3-E1, which is a clonal osteoblastic cell line derived from the calvariae of a late-stage mouse embryo, is most frequently used to study osteoblast differentiation. This cell line expresses various osteoblast phenotypes including the formation of mineralized bone nodules in long-term culture. BMP-2 and BMP-7 increase ALP activity, PTH-responsiveness and osteocalcin production, suggesting that BMPs promote the differentiation of osteoblast precursors to more mature osteoblasts.

Osteoblastic cells isolated from the calvariae of newborn rats or the bone marrow of adult rats (primary osteoblasts) provide a suitable model in which to explore the bone formation process *in vitro*; these cells generate numerous mineralized bone nodules when cultured in the presence of β-glycerophosphate and ascorbic acid. Using these models, we demonstrated that the expression of BMP-2, BMP-4, and their receptors (BMPR-IA, BMPR-IB, and BMPR-II) is closely related to bone nodule formation, suggesting that BMPs play important roles in the process of bone nodule formation through their receptors in a paracrine and/or autocrine manner [54].

We were the first to report the inhibitory effects of BMP-2 on myogenic differentiation in the ROB-C26 cells, which are an osteoblast precursor cell line with the capacity to differentiate into myogenic cells [2]. BMP-2 also inhibits myogenic differentiation in C2C12 myoblasts, while concomitantly stimulating osteoblast differentiation in this cell line [4]. Thus, BMPs not only induce or promote osteoblast differentiation in various stages of mesenchymal cell development, but also regulate myogenesis in these cells.

Since BMP-2 knockout is embryonic lethal, Tsuji et al. [55] recently generated transgenic mice in which *BMP-2* was inactivated in a limb-specific manner before the onset of skeletal development using a *Prx1cre* enhancer. This avoided the embryonic lethality that results from the global loss of

BMP-2. These conditional knockout mice lacking BMP-2 synthesis in their limb bones exhibited few skeletal abnormalities. This might be due to the compensatory effect of other BMPs expressed in the developing limb. Interestingly, however, these mice suffer spontaneous fractures that do not heal with time; it appears that earliest steps in the fracture healing process are blocked. These results indicate that BMP-2 expression during embryogenesis is not required for the events that determine correct skeletal patterning and early osteochondrogenesis, and that BMP-2 is a critical endogenous factor necessary for fracture repair. Further studies using conditional knockout mice specifically lacking BMP signaling molecules in osteoblasts will provide more important information concerning the function of BMPs in osteoblast differentiation.

3. Role of Notch signaling in osteoblast differentiation

3.1. The Notch signaling pathway

Notch receptors contain 36 epidermal growth factor (EGF)-like repeats and 3 cysteine-rich Notch/LIN-12 repeats in a large extracelullar domain, and also 7 tandem ankyrin repeats, a proline/glutamine/serine/threonine-rich (PEST) domain, and a transactivation domain (TAD) in an intracellular region [15,56]. EGF repeats are involved in ligand interaction, the prevention of constitutive activation, and the lateral interaction for homodimerization [57]. Similar to Notch receptors, Notch ligands also contain EGF repeats in the extracellular domain, and in addition a unique cysteine-rich N-terminal region referred to as the Delta/Serrate/Lag2 (DSL) domain. The interaction of Notch with the ligands induces nuclear translocation of the intracellular domain of Notch (NICD) as a result of proteolytic cleavage at the juxtamembrane portion by γ-secretase (Fig. 2) [56]. In the nuclei, NICD interacts with CSL DNA-binding proteins, including CBF1/RBP-J, and transactivates the target genes such as *Hes1* and *Hey1*(Fig. 2). As an alternative pathway, Notch receptors have also been reported to transmit signals through CSL-independent pathways by interacting with other signaling molecules, such as MAP kinase, Src, and nuclear factor κB (NF-κB) [39].

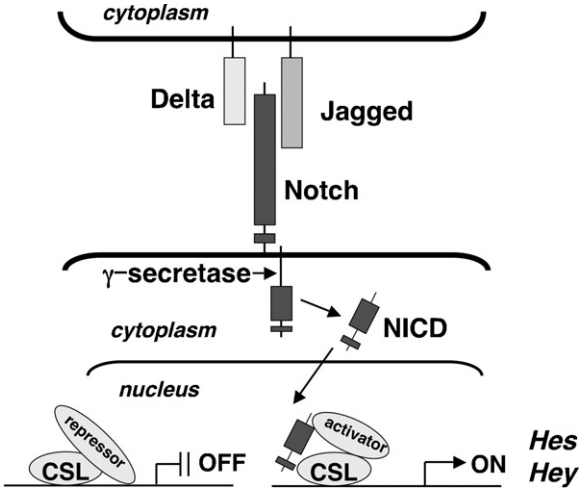

Figure 2 Notch signaling pathway. Ligands of Delta or Jagged family induce intramembranous cleavage of the Notch receptor by γ-secretase. The cleaved form of intracellular domain, NICD, translocates into nucleus, and it replaces transcriptional corepressors with activators enabling transcription of target genes, *Hes* and *Hey* by CSL.

3.2. Expression of Notch and its ligands on osteoblastic cells

To understand the role of Notch signaling in osteoblast differentiation, it is important to determine the expression profiles of Notch receptors and their ligands on osteoblastic cells under both physiological and pathological conditions. Our immunohistochemical studies using a mouse bone regeneration model revealed that Notch1 expression was weak in normal femurs, but strong expression in mesenchymal cells, including osteoblastic cells that had migrated into a bone defect at 5 days after injury [19]. At this stage, Delta1 was also expressed, and confocal microscopic analysis demonstrated the extensive colocalization between Notch1 and Delta1. Immunostaining of Jagged1 at day 10, at which time reconstruction of cortical bone is taking place, demonstrate that both Notch1 and Jagged1 also colocalize in the osteoblasts on the surface of the trabeculae. Immunohistochemistry using antibodies that recognize the active form of Notch1 revealed that the Notch1 signal is activated in the osteoblasts, and that chondrocytes appear around the sites of injury [19]. Real-time PCR also demonstrated that the expression of Notch1, Delta1, and Jagged1 was low before injury but was temporarily up-regulated at day 5 after injury (Fig. 3) [19]. These results indicate that the Notch1 signal is activated in osteoblastic cells during bone regeneration *via* the up-regulation of Delta1 and Jagged1. Moreover, the concomitant expression of Notch1, Delta1 and Jagged1 suggests the possibility of an autocrine and/or paracrine action rather than a cell-to-cell signaling mechanism, which is characterized by the contact-dependent inhibition model of nervous system development [58,59].

Prior to investigating the function of Notch signaling in osteoblast differentiation using various osteoblastic cells, we

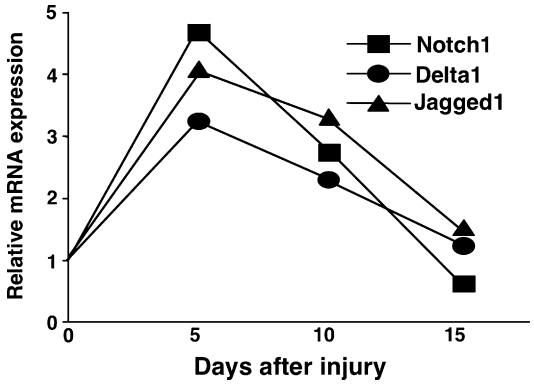

Figure 3 Expression profile of mRNA for *Notch1*, *Delta 1*, and *Jagged 1* during bone regeneration. The expression level of these mRNA is low before injury, but these mRNA is highly up-regulated on day 5 after injury. Relative expression of each mRNA was quantified by real-time RT-PCR. Adapted from Ref. [19].

also confirmed the expression of the mRNA of Notch1—4, Delta1, Delta3, Delta4, Jagged1, and Jagged2 by RT-PCR. C3H10T1/2, MC3T3-E1, C2C12, and primary osteoblastic cells isolated from newborn mice calvariae and from adult mouse bone marrow expressed substantial levels of Notch1 and Jagged1 mRNA; however, the expression of Notch3 and Delta4 was extremely low [19]. These results indicate that Notch signaling is involved in the regulation of various osteoblastic cells.

3.3. Notch signaling exerts diverse action on osteoblast differentiation

There have been several reports that describe the role of Notch signaling in osteoblasts differentiation. In 1999, Nofziger et al. [20] reported using C2C12 cells that differentiate into myoblasts and osteoblasts that Notch signaling inhibited not only myogenesis but also osteogenesis induced by BMP-2. These results are in agreement with the general concept that Notch signaling blocks differentiation toward a primary differentiation fate or that it forces the cell to remain in an undifferentiated state [14,59]. Shindo et al. [60]. demonstrated that using KusaA^{NICD} and KusaO^{NICD}, which stably overexpress *NICD* that Notch signaling suppresses the osteoblastic differentiation of mesenchymal progenitor cells. Sciaudone et al. [22] also demonstrated that constitutive overexpression of *NICD* by retroviral transduction inhibited osteoblast differentiation in ST2 and MC3T3-E1 cells. Recently, these authors proposed that the inhibitory effects of Notch signaling on osteoblast differentiation are induced by the suppression of Wnt/beta-catenin signaling [23].

In contrast to the inhibitory effects of Notch signaling on osteoblast differentiation, Tezuka et al. [21] demonstrated using C3H10T1/2 and MC3T3-E1 cells that Notch signaling stimulated osteoblast differentiation. We also demonstrated the stimulatory effects of Notch signaling on the differentiation [19]. We transiently overexpressed *Delta1* and *Jagged1* by adenoviral transduction in MC3T3-E1 cells. This induced no apparent changes in osteoblast differentiation, but did augment BMP-2-induced osteoblast differentiation, including bone nodule formation associated with the up-regulation of *Hes1*, a direct target gene of the Notch pathway. In addition, the injection of MC3T3-E1 cells overexpressing *Delta1* and *Jagged1* into muscle enhanced BMP-2-induced ectopic bone formation *in vivo*. When Notch signaling was inhibited by the dominant negative extracellular domain of Notch1, specific γ-secretase inhibitor (L685,458) or siRNA impaired BMP-2-induced osteoblast differentiation and the promoter activity of *Id-1*, a target gene of the BMP pathway. These results indicated the functional redundancy between Delta1 and Jagged1 in osteoblastic differentiation, whereby Delta1/Jagged1-activated Notch1 enhances BMP-2-induced osteoblast differentiation. Furthermore, our data also suggest that Notch signaling is essential not only for BMP-2-induced osteoblast differentiation but also for BMP signaling itself.

One interpretation of this dual action of Notch signaling on osteoblast differentiation is that the effect of Notch activation may differ depending on the period of activation; transient and short-term activation of the Notch pathway may enhance osteoblastic differentiation, whereas long-term, continuous activation may lead to the inhibition of osteoblastic commitment. Further studies will be necessary in order to elucidate the culture period-dependent functional difference of Notch signaling in osteoblastic differentiation.

3.4. Cross-talk between Notch and BMP signaling during osteoblast differentiation

In order to explore the mechanism underlying the cooperative interaction between Notch and BMP signaling in osteoblast differentiation, we examined the cross-talk of BMP and Notch signaling. Recent studies have revealed that Notch and BMP/TGF-β cooperatively activate the opposite pathway, such as Hes1 and Hes-5, or Smad target gene *PAI-1* [17,61,62]. In the Notch pathway, NICD interacts with Smad1/5, and recruits p300 and P/CAF to the transcription machinery; this results in the cooperative activation of Notch target genes such as *Herp2* and *Hes* [17,62]. In our study, however, Notch1 did not enhance BMP signaling leading to *Id-1* expression. Furthermore, BMP-2 failed to induce *Hes-1* gene expression (unpublished data), indicating the presence of other interaction mechanisms between BMP and Notch. It is most likely that other Notch target genes, such as *Lfng*, *Hey1*, and *Tcf7* which could be up-regulated in C2C12 cells in response to BMP-2 [63], may be involved in the cooperative interaction between BMP-2 and Notch in osteoblast differentiation, although *Hey1*, another target gene of both Notch and BMP, may act as a negative regulator [64]. More importantly, we revealed that Notch activation is required for BMP signaling [19]. The inhibition of Notch signaling leads to a dramatic decrease of *Id-1* promoter activity, indicating that Notch signaling is required for BMP signaling itself.

4. CCN3/NOV inhibits BMP-2-induced osteoblast differentiation by interacting with the BMP and Notch signaling pathways

4.1. The CCN family of proteins

The CCN family of proteins comprises the following six members; CYR61, CCN2/CTGF, CCN3/NOV, CCN4/WISP1, CCN5/WISP2, and CCN6/WISP3. Several lines of evidence demonstrate that these proteins regulate the differentiation of skeletal mesenchymal cells such as muscle cells [24—26], chondrocytes [26,27], and osteoblasts [28—31].

The CCN family of proteins basically contain four conserved modular domains with sequence similarities to IGF-binding protein (IGFBP), von Willebrand type C (vWC), thrombospondin type 1 (TSP1), and C-terminal cysteine knot (CT) domains [25]. WISP2/CCN5 retains similar structure lacking the CT domain. These modular structural domains are shared among the members of the CCN family, with the exception of proteins of CCN5/WISP2 that lacks the CT module [26].

4.2. Biological activity of CCN3/NOV

The N-terminal truncated form of CCN3/NOV can transform chicken embryonic fibroblasts by its oncogenic activity, and

aberrant expression of CCN3/NOV is associated with the development of several tumors of different origins including Wilms' tumor, renal cell carcinoma, neuroblastoma, glioblastoma, adrenocortical carcinoma, and musculoskeletal tumors [17—20]. During normal development, *CCN3/NOV* is expressed in a wide variety of tissues including the notochord, central nervous system, kidney, adrenal cortex, muscle and cartilage. Several studies have demonstrated that CCN3/NOV physically associates with fibulin 1C [65], rpb7 [66], connexin 43 [67], and Notch [24]. Although the biological activities of CCN3/NOV are to a certain extent attributed to the interactions with these molecules, other factors may interact with CCN3/NOV and are likely to regulate its downstream reactions.

The expression of *CCN3/NOV* is detected in the dermomyotome [68]. Interestingly, the expression in the dermomyotome coincides with that of the transmembrane receptor, Notch and its ligand, Delta. The expression of *Notch* frequently overlaps with *CCN3/NOV* expression. For example, both genes are expressed in chondrocytes and are assumed to participate in cartilage formation [69]. *CCN3/NOV* is also involved in vascular formation [70], and its role in response to injury has been postulated, based on the prominent upregulation of CCN3/NOV observed in smooth muscle cells after balloon catheter injury of rat carotid arteries [71,72]. In this experimental model, a remarkable up-regulation of Notch and its ligands has also been demonstrated [72]. These observations suggest a hypothesis that CCN3/NOV may exert some of its effects through the Notch signaling pathway.

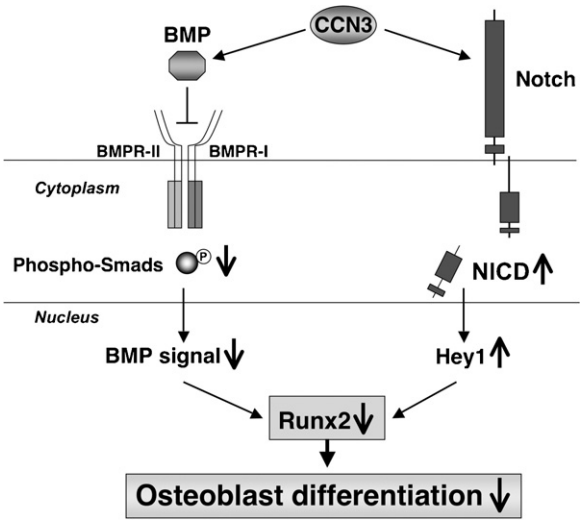

Figure 4 A possible mechanism underlying the inhibitory effects of CCN3/NOV on osteoblast differentiation. CCN3/NOV binds to BMP-2, though extracellular binding of these two molecules should be further confirmed, and inhibits phosphorylation of Smad1/5/8. This effect attenuates BMP signaling, and down-regulate Runx2 transcription. CCN3/NOV also Notch at extracellular region, and increased NICD. NICD increases expression of *Hey1*, and then Hey1 inhibit transcription of Runx2. Thus, the inhibitory effects of CCN3/NOV on osteoblast differentiation are mediate by interacting with both BMP and Notch signaling pathways.

4.3. CCN3 inhibits osteoblast differentiation by interacting with BMP signaling

Several reports [28—30] have indicated that the members of the CCN family participate in regulating osteoblast differentiation; however, the distinct role of CCN3 in osteoblast differentiation has not been well documented. CTGF/CCN2 binds to BMP-4 and antagonizes its action [32]. It also inhibits BMP-9-induced osteoblast differentiation [29]. These reports prompted us to explore that CCN3 itself also interacts with BMP and affects BMP signaling. In order to confirm this hypothesis, we first overexpressed *CCN3* in MC3T3-E1 osteoblastic cells by using adenoviruses with or without transduction with *AdBMP-2* [32]. This experiment demonstrated that the overexpression of *CCN3* inhibited the expression of osteoblast-related markers induced by BMP-2 such as *Runx2*, *osterix*, *ALP* and *osteocalcin*. These results indicate that CCN3 exerts an inhibitory effect on BMP-2-induced osteoblast differentiation. To explore the mechanism involved in this effect, we examined the interaction between CCN3 and BMP-2, and revealed the direct physical association between these two proteins. As of yet, however, we have not confirmed that NOV inhibits BMP-2 action by preventing it from binding to BMP receptors [32]. Such a study is currently being undertaken in our laboratories. In addition, the overexpression of CCN3 inhibited Smad1/5/8 phosphorylation and the expression of *Id1*, *Id2* and *Id3*, all of which are induced by BMP-2 [32]. These findings indicate that CCN3 inhibits BMP-2-induced osteoblast differentiation by its involvement in BMP signaling (Fig. 4).

4.4. CCN3 interacts with the Notch signaling pathway

We demonstrated that the C-terminal cysteine knot of CCN3 is associated with the EGF-like repeats of Notch1, and that it inhibits myogenic differentiation in C2/4 myoblasts [24]. This suggests that the involvement of CCN3 in the Notch signaling pathway also plays a role in the inhibitory effect of CCN3 on BMP-2-induced osteoblast differentiation. To confirm this, we performed western blot analysis to examine the expression of the cleaved Notch1 protein, which is an active form of Notch [32]. This study revealed that the overexpression of *CCN3* elevated the expression level of cleaved Notch1, and that simultaneous transduction with *AdCCN3* and *AdBMP-2* retained the expression level of cleaved Notch1 in MC3T3-E1 cells. Further, CCN3 increased the mRNA expression levels of *Hes1* and *Hey1*, which are involved in downstream Notch signal transduction, and CCN3 also stimulated the promoter activities of these genes [32]. These results indicate that CCN3 is involved in Notch signaling during osteoblast differentiation (Fig. 4).

Zamurovic et al. [64] reported that BMP-2 stimulated the expression of *Hey1* in osteoblastic cells, including MC3T3-E1 cells, and that Hey1 inhibited osteoblast differentiation by abrogating the transcriptional activity of *Runx2*. These results suggest that Hey1 is a negative regulator of BMP-induced osteoblast differentiation. We revealed that the overexpression of *CCN3* promoted the mRNA expression and promoter activity of *Hey1*, suggesting that Hey1 is involved in the inhibitory effect of CCN3 on BMP-2-induced

osteoblast differentiation [32]. This finding is supported by our experiments using *Hey1*-deficient osteoblastic cells (dHey1 cells); CCN3 induced no apparent inhibitory effects on BMP-2-induced osteoblast differentiation in these cells [32]. It will be an important issue to consider whether the changes observed in dHey1 cells is due to *Hey1* deficiency and/or chromosomal abnormality of appropriate gene expression during establishment of the cell lines. In order to assess this possibility, we rescued the expression of *Hey1* using adenovirus in dHey1 cells. This experiment confirmed that the recovery of *Hey1* expression in dHey1 cells suppressed the osteoblast-related markers induced by *AdBMP-2*. These results further supported Zamurovic's proposal that Hey1 is a negative regulator of BMP-induced osteoblast differentiation [64]. Interestingly, Lee et al. [73] reported that Hes1, which is another downstream transducer of Notch signaling, stimulated Runx2 activity by cooperating with retinoblastoma protein (pRb). In our study [32], although CCN3 stimulated the expression of both *Hes1* and *Hey1* in MC3T3-E1 cells, its stimulatory effect on *Hey1* was greater than that on *Hes1*. It will be interesting to explore the distinct roles of these two downstream transducers during osteoblast differentiation.

Recently, Rydziel et al. [74] reported that CCN3 inhibited osteoblast differentiation by inhibiting the BMP signaling pathway. They suggested, however, that Notch signaling is not involved in the inhibitory effect of CCN3 on osteoblast differentiation. Further studies are required in order to resolve these conflicting results on the role of Notch signaling in the inhibitory effects of CCN3 on osteoblast differentiation. Rydziel et al. [74] also reported that CCN3 transgenic mice that specifically overexpressed CCN3 in osteoblasts exhibited osteopenia due to reduced bone formation activity. Our CCN3 transgenic mice also exhibited similar phenotypes (unpublished results). These results indicate that CCN3/NOV exerts inhibitory action on osteoblast differentiation *in vitro* as well as *in vivo*. Since our studies revealed that the expression of CCN3/NOV was dramatically up-regulated concomitant with the molecules involved in the Notch and BMP signaling pathways at an early phase of bone regeneration (unpublished results), it will be of great interest to investigate the bone regeneration process in CCN3/NOV transgenic and knockout mice. Such investigations are now under way in our laboratory.

A recent report demonstrated that CCN3/NOV is a central regulator of human primitive hematopoietic cells [75]. Since the osteoblast lineage cells are essential to maintain hematopoietic stem cells in bone marrow [76], CCN3/NOV expressed in osteoblast lineage cells might be involved in regulation of hematopoietic stem cells.

5. Summary

After cloning of human *BMP* genes [77], the role of BMPs in osteoblast differentiation has been extensively investigated by using the recombinant proteins. Several lines of evidence indicate that BMPs stimulate or enhance Runx2 expression and activity in osteoprogenitors and osteoblastic cell. This BMP-Runx2 axis plays crucial roles in osteoblast differentiation and bone formation. In addition to these findings, recent studies revealed that BMP signals cross talk with other signaling pathways such as Notch and Wnt. Further studies concerning cross talk of BMP-Runx2 axis and other signaling pathways are required to clarify the regulatory mechanism of osteoblast differentiation and bone formation. These studies will provide important information on the molecular mechanism of bone regeneration that will be useful for developing effective therapeutic approach to bone repair.

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