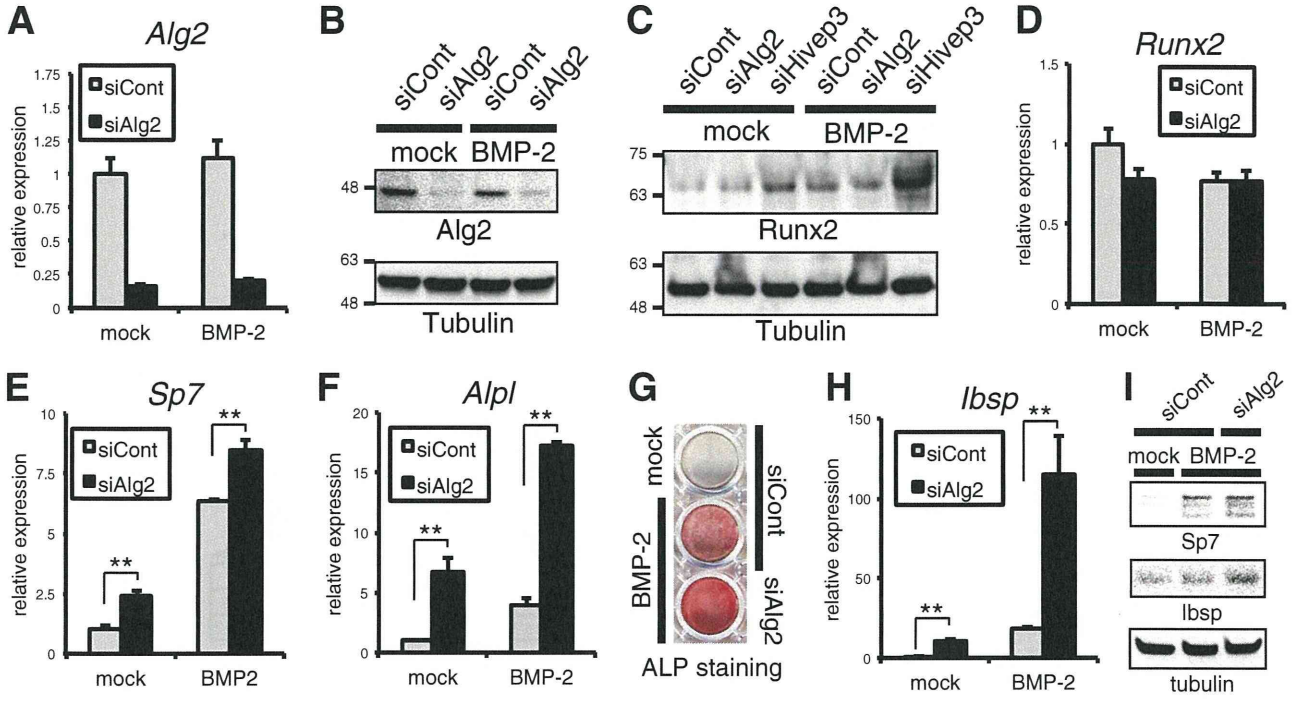


FIGURE 4. **Loss of Alg2 enhances osteoblast differentiation in ST-2 cells without affecting the protein level of Runx2.** *A* and *B*, ST-2 cells were transfected with siRNA for *Alg2* with or without BMP-2 treatment (300 ng/ml) for 3 days. Knockdown efficiency for *Alg2* was examined by qRT-PCR (*A*) or by immunoblotting (*B*). *C*, ST-2 cells were transfected with siRNA for *Alg2* or *Hivep3* with or without BMP-2 treatment (300 ng/ml) for 3 days. Cell lysates were analyzed by immunoblotting with an anti-Runx2 antibody. Tubulin served as a loading control. *D–F*, ST-2 cells were transfected with siRNA for *Alg2* with or without BMP-2 treatment (300 ng/ml) for 3 days. Expression of indicated genes was analyzed by qRT-PCR. *G*, ST-2 cells were transfected with siRNA for *Alg2* and treated with BMP-2 (300 ng/ml) for 6 days. ALP staining was performed. *H*, ST-2 cells were transfected with siRNA for *Alg2* with or without BMP-2 treatment (300 ng/ml) for 3 days. Expression of *Ibsp* was analyzed by qRT-PCR. *I*, ST-2 cells were transfected with siRNA for *Alg2* with BMP-2 treatment (300 ng/ml) for 2 days. Cell lysates were analyzed by immunoblotting with indicated antibodies. **, $p < 0.01$.

Loss of Alg2 Promotes Osteoblast Differentiation in ST-2 Cells without Affecting the Protein Level of Runx2—To investigate the possible role of Alg2 in osteoblast differentiation, siRNA for *Alg2* was transfected into ST-2 cells to obtain an ~80% decrease in mRNA expression (Fig. 4*A*) and in protein level (Fig. 4*B*). Although silencing of *Hivep3* increased the level of Runx2 protein, siAlg2 had no effect (Fig. 4*C*). As expected, loss of Alg2 also did not change the RNA level of Runx2 (Fig. 4*D*). However, *Alg2* knockdown mildly enhanced *Sp7* expression (Fig. 4, *E* and *I*), although it dramatically increased the expression (Fig. 4*F*) and activity (Fig. 4*G*) of ALP. A similar effect was seen on the level of *Ibsp* mRNA (Fig. 4*H*) and protein (Fig. 4*I*), suggesting a suppressive role of Alg2 in osteoblast maturation.

Forced Expression of Alg2 Inhibits Osteoblast Differentiation and Bone Formation—We investigated the effect of overexpression of Alg2 in osteoblasts by infection of adenovirus or lentivirus carrying an Alg2 expression cassette. In ST-2 cells, forced expression of Alg2 showed no effect on Runx2 protein level (Fig. 5*A*), whereas it strongly suppressed the expression of *Sp7*, *Alpl*, and *Ibsp* (Fig. 5*B*). The lentivirus-mediated expression of the Alg2 transgene product was confirmed at the protein and mRNA level (Fig. 5, *C* and *D*). Combined induction of *Hivep3* siRNA with the Alg2 lentivirus completely negated the enhanced expression of *Ibsp* by siHivep3, suggesting that Alg2 is a downstream mediator of Hivep3 for blocking osteoblast differentiation (Fig. 5*E*). To assess the role of Alg2 in osteoblastic bone formation, we employed the *ex vivo* culture system of calvarial bone harvested from E17.5 mouse embryo. The infection efficiency of lentivirus in bone culture was evaluated by immunofluorescence, and the V5-tagged transgene product was detected by anti-V5 antibody (Fig. 5*F*). The rate of osteoblastic intramembranous bone formation can be examined by measuring the width of the fontanelle (20). Application of BMP-2 promoted the bone formation, and it significantly decreased the fontanelle width, whereas combined induction of Alg2-expressing lentivirus cancelled the narrowing (Fig. 5*G*), indicating that Alg2 inhibited BMP-induced osteoblastic bone formation.

Alg2 Knockdown Does Not Affect ER Stress nor BMP Signaling in ST-2 Cells—A defect in ALG may affect the quality control of protein folding in the ER, which might subsequently evoke ER stress (26, 27). In addition, because physiologically mild ER stress is required for proper osteoblast differentiation and maturation (28, 29), we investigated the effect of *Alg2* siRNA on ER stress-related genes by qRT-PCR (Fig. 6*A*). Atf4, a downstream target of PKR-like endoplasmic reticulum kinase of ER stress transducer, is crucial for the expression of *Bglap2* and synthesis of type I collagen during osteoblast maturation (28, 30). *Alg2* silencing showed no remarkable effect on the *Atf4* mRNA level (Fig. 6*A*). An ER stress transducer called cAMP-responsive element-binding protein 3-like 1 (*Creb3l1*), alternatively known as Oasis, is also crucial in osteoblast differentiation (29). However, the level of *Creb3l1* was unchanged by Alg2 silencing (Fig. 6*A*). DNA damage-inducible transcript 3 (*Ddit3*), a target gene of

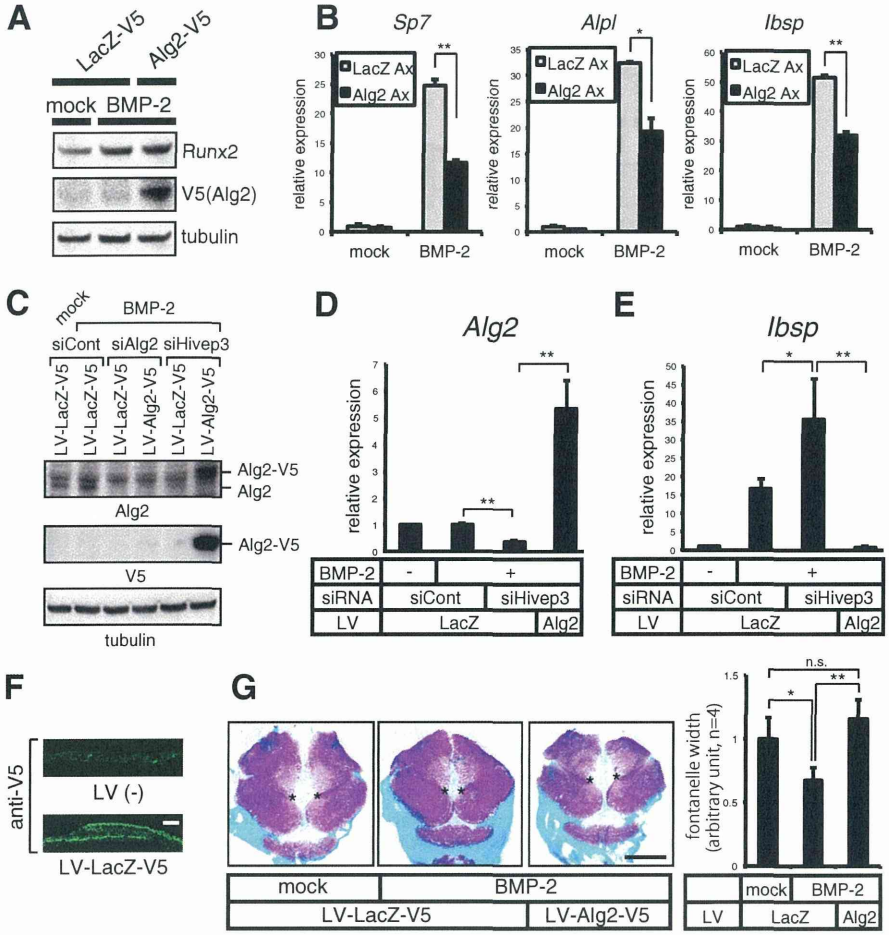


FIGURE 5. **Gain of Alg2 expression suppresses osteoblast differentiation and bone formation.** *A* and *B*, ST-2 cells were infected with V5-tagged LacZ or Alg2 adenovirus (*Ax*) and subsequently treated with BMP-2 (300 ng/ml). Cell lysates were analyzed by immunoblotting with anti-Runx2 and anti-V5 antibodies at day 6 (*A*). The expression of the indicated genes was evaluated by qRT-PCR (*B*). *C–E*, ST-2 cells were infected with LacZ or Alg2 lentivirus (*LV*). The infectants were transfected with the indicated siRNAs and stimulated with BMP-2 (300 ng/ml) for 4 days before analysis by immunoblotting with anti-Alg2 or anti-V5 antibodies (*C*). Expression of *Alg2* (*D*) or *Ibsp* (*E*) was evaluated by qRT-PCR. *F* and *G*, calvarial bones of E17.5 mouse embryos were infected with the indicated lentivirus for 16 h. Immunostaining using FITC-linked anti-V5 antibody on bone coronal sections was performed at day 2 of culture (*F*). *Scale bar*, 100 μm. LV-infected bones were treated with 300 ng/ml of BMP-2 for 3 days. Alcian blue/alizarin red staining was performed. The width of fontanelle (between *asterisks*) was measured (*G*). *Scale bar*, 2 mm. *, $p < 0.05$; **, $p < 0.01$.

Atf4 also known as CCAAT/enhancer-binding protein homologous protein (Chop), was indeed mildly up-regulated by siAlg2, but only in BMP-2-treated cells (Fig. 6*A*). The expression of a target of the Atf6 pathway, heat shock protein 5 (*Hspa5*), also known as Bip, was not altered by loss of Alg2 (Fig. 6*A*). These data suggest that ER stress is not substantially accelerated by loss of Alg2. To identify other mechanisms by which siAlg2 promotes osteoblast differentiation, we next evaluated if BMP signaling was increased by Alg2 knockdown, by assessing the expression of the representative direct target genes of the BMP-Smad pathway, *Id1* and *Smad6* (31, 32). We found no change in the level of *Id1* or *Smad6* upon Alg2 silencing (Fig. 6*B*).

Alg2 Interferes with the Transcriptional Activity and Nuclear Localization of Runx2—To test whether Alg2 interferes with Runx2 activity, we analyzed a Runx2-binding 6×OSE2 luciferase reporter. We found that Alg2 dose-dependently suppressed the Runx2-induced elevation of luciferase activity in COS-7 cells, whereas it showed no effect on the activity of 6×OSE2 reporter with Runx2-binding site mutation, suggesting that the inhibitory effect was Runx2-dependent (Fig. 6*C*). A similar result was obtained in ST-2 cells where Alg2 reduced Runx2 activity with an efficiency comparable with that of Hivep3 (Fig. 6*D*). To investigate the mechanism by which Alg2 suppresses Runx2 activity, we examined the impact of siAlg2 on the expression of an inhibitor and an activator of Runx2. Hairy/enhancer-of-split related with YRPW motif 1 (*Hey1*) is a transcriptional repressor that binds to Runx2 and suppresses its transcriptional activity (33). Contrary to our hypothesis, the expression of *Hey1* was not decreased by Alg2 knockdown; rather, it increased in a statistically significant manner (Fig. 6*E*). The expression of hairy and enhancer of split 1 (*Hes1*), which forms a complex with Runx2 to promote Runx2-dependent transcription (34, 35), was found to be unchanged by Alg2 siRNA (Fig. 6*E*). We next investigated the possibility of whether Alg2 forms a complex with Runx2 to interfere with its localization, because targeting of Runx2 to subnuclear foci, the nuclear matrix, is crucial for the bone-specific transcription of Runx2

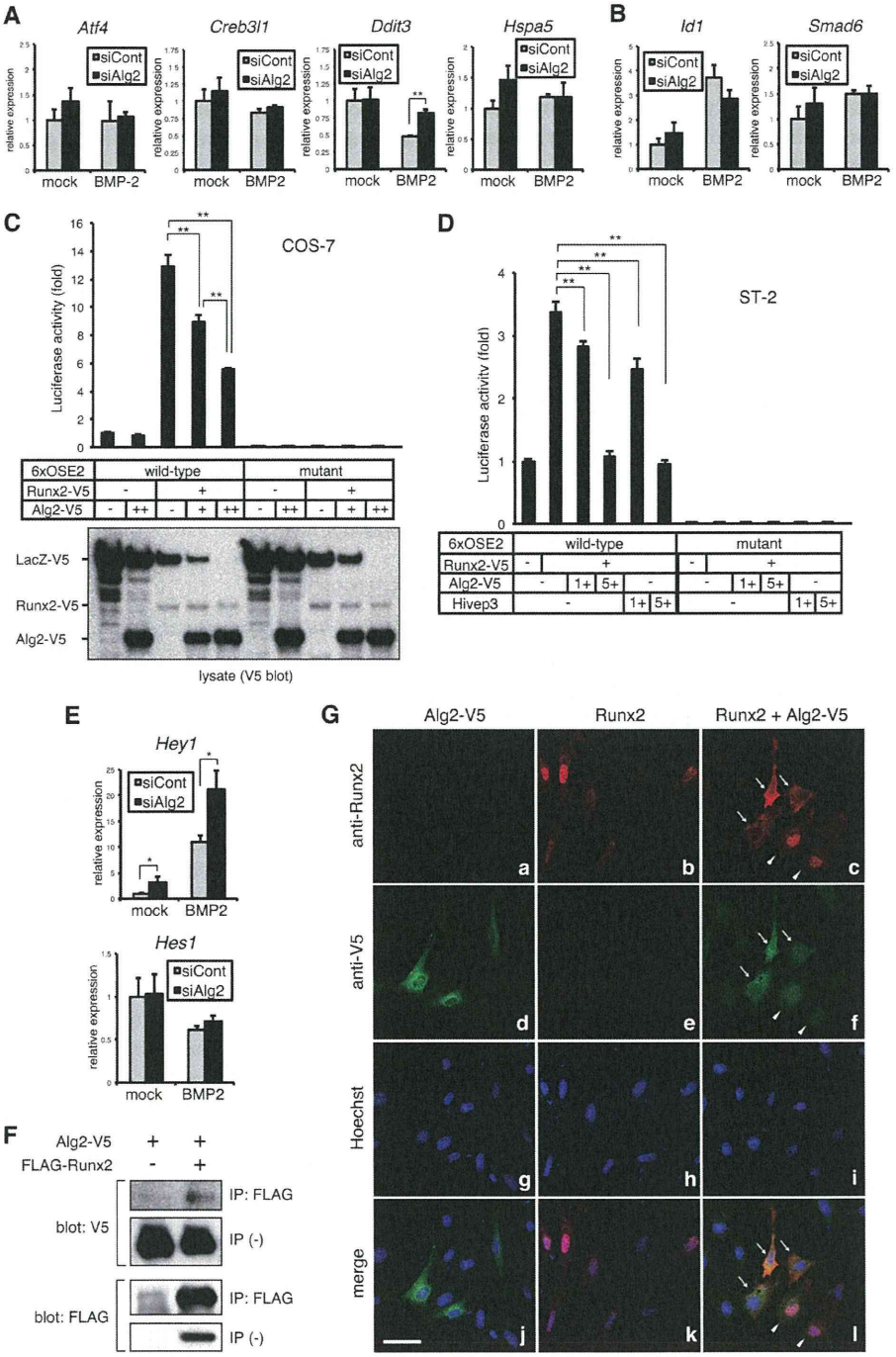


FIGURE 6. **Alg2 suppresses the transcriptional activity of Runx2.** *A* and *B*, ST-2 cells were transfected with siRNA for *Alg2* and treated with or without BMP-2 (300 ng/ml) for 3 days. Expression of indicated genes was analyzed by qRT-PCR. *C* and *D*, a luciferase reporter, 6×OSE2 luc or a Runx2-binding sequence mutant 6×OSE2 luc, and a Runx2 expression vector were transfected with or without Alg2 or Hivep3 expression plasmid into COS-7 cells (*C*) or ST-2 cells (*D*) and examined by a luciferase assay. Protein expression from the transgenes was confirmed by anti-V5 immunoblotting (*C*). *E*, ST-2 cells were transfected with siRNA for *Alg2* and treated with or without BMP-2 (300 ng/ml) for 3 days. Expression of *Hey1* and *Hes1* was analyzed by qRT-PCR. *F*, COS-7 cells were transfected with a V5-tagged Alg2 expression plasmid with or without a FLAG-tagged Runx2 vector. The cell lysate was subjected to immunoprecipitation (*IP*) with an anti-FLAG antibody and subsequent immunoblotting with an anti-V5 or anti-FLAG antibody. *G*, ST-2 cells were transfected with a V5-tagged Alg2 plasmid and/or a Runx2 expression vector. Immunofluorescence was examined with anti-Runx2 or anti-V5 antibodies. Nuclei were stained with Hoechst dye. *Scale bar*, 50 μm. *, $p < 0.05$; **, $p < 0.01$.

(36). In a co-transfection experiment using COS-7 cells, Alg2 was immunoprecipitated with Runx2 (Fig. 6*F*). By an immunofluorescence assay in ST-2 cells, we found that Alg2 protein overexpressed alone was localized to the ER (Fig. 6*G*, *panel d*), whereas Runx2 overexpressed alone was stained in the nuclei (Fig. 6*G*, *panel b*). However, in cells with combined transfection of Alg2 and Runx2, in the portion of cells in which abundant expression of Alg2 was observed, Runx2 was excluded from

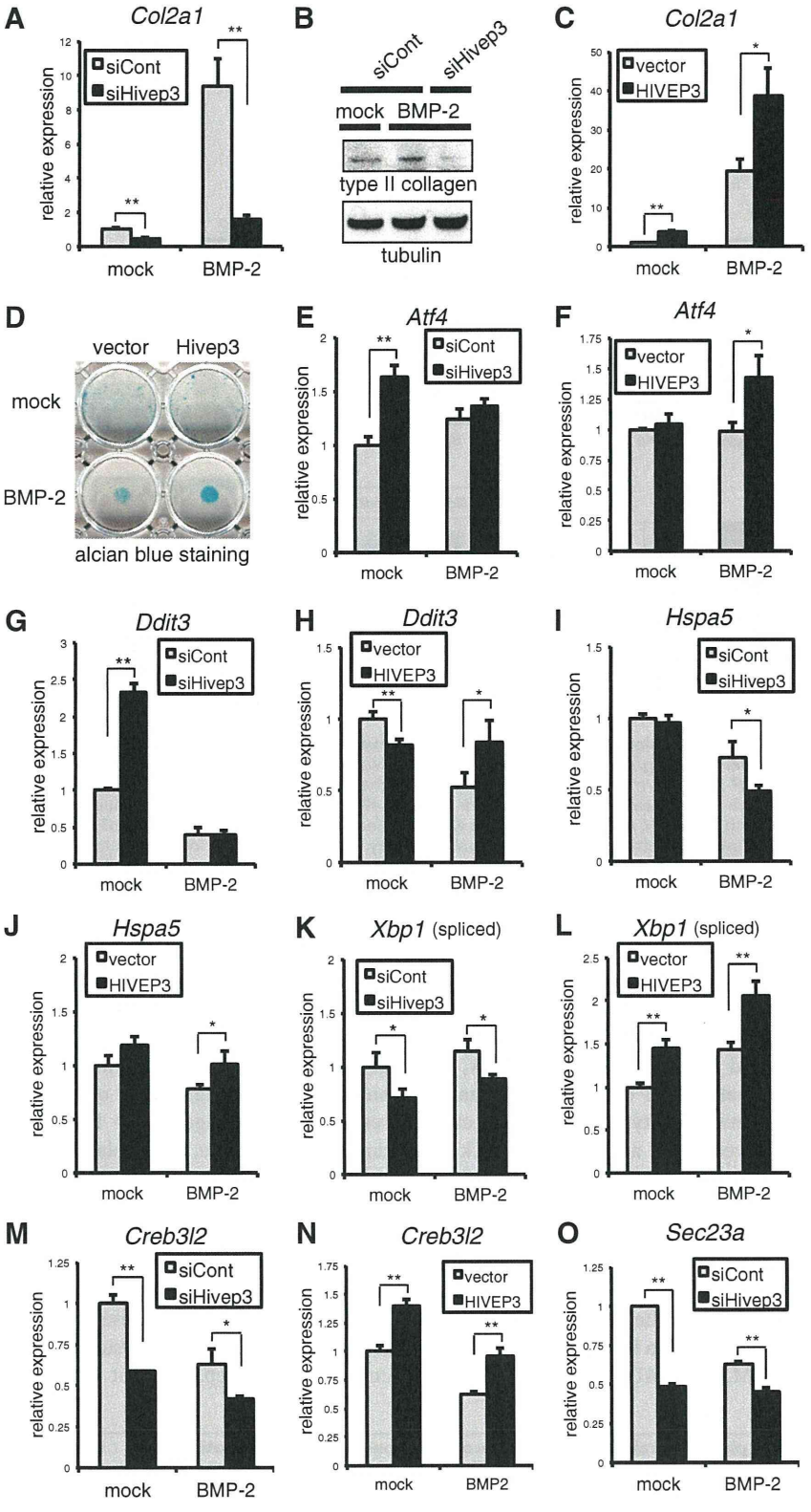
nucleus and co-localized with Alg2 in the cytoplasm (*arrows*), whereas cells with a lower level of Alg2 had a nuclear pattern of Runx2 staining (*arrowheads*) (Fig. 6*G*, *panels c, f,* and *l*). These data suggest that the interaction of Runx2 with Alg2 interfered with proper subnuclear localization of Runx2, thereby decreasing its transcriptional activity.

Hivep3 Promotes the Expression of Creb3l2 and Differentiation of Chondrocytes—Next we investigated the mechanism by which Hivep3 supports chondrocyte differentiation. *Hivep3* silencing in ATDC5 chondrocytes strongly suppressed *Col2a1* in mRNA (Fig. 7*A*) and in protein levels (Fig. 7*B*). Conversely, HIVEP3 overexpression significantly enhanced expression of *Col2a1* (Fig. 7*C*). At day 21 of micromass culture, BMP-2-treated ATDC5 cells produced an abundant cartilage matrix that was stained with Alcian blue and was significantly enlarged by Hivep3 vector transfection (Fig. 7*D*). Chondrocytes secrete cartilage ECM proteins such as type II or XI collagens during differentiation, which evokes mild ER stress and induces an ER stress sensor, BBF2 human homolog on chromosome 7 (*Bbf2h7*), also known as Creb3l2. Creb3l2 plays a crucial supportive role in chondrocyte differentiation by directly inducing the expression of *Sec23a*, encoding a coat protein complex II component cargo protein responsible for the transport of secretory ECM proteins from the ER to the Golgi (18). *Atf4* expression was increased by loss of Hivep3, but only in the absence of BMP-2 (Fig. 7*E*), although gain of HIVEP3 enhanced the level in the presence of BMP-2 (Fig. 7*F*). *Ddit3* showed similar expression patterns as *Atf4*, where its basal level was elevated by siHivep3 (Fig. 7*G*), although overexpression of HIVEP3 suppressed basal expression but promoted expression in the presence of BMP-2 (Fig. 7*H*). These data indicate that the ER stress pathway of PERK-Atf4-Chop may be associated with the enhancement of BMP-2-induced differentiation by forced expression of HIVEP3. Expression of *Hspa5* was decreased or increased by knockdown or overexpression of Hivep3, respectively (Fig. 7, *I* and *J*). The expression of a spliced form of *Xbp1* was examined to monitor the inositol-requiring 1 (IRE1) pathway of ER stress and found to be suppressed or enhanced by silencing or addition of Hivep3, respectively (Fig. 7, *K* and *L*). These data suggest that Hivep3 evoked mild ER stress through the Atf6-Bip and Ire1-Xbp1 pathways. We found that *Creb3l2* expression was decreased or increased by loss or gain of Hivep3, respectively (Fig. 7, *M* and *N*). Importantly, the siHivep3-mediated reduction in *Creb3l2* expression was reflected in that of *Sec23a* (Fig. 7*O*), which may be responsible for the inhibition of differentiation.

Alg2 Is Decreased by Hivep3 Silencing in ATDC5 Chondrocytes, although Loss of Alg2 Suppresses the Expression of Creb3l2 and Chondrocyte Differentiation—We investigated whether Alg2 is a mediator of Hivep3 also in chondrocytes to affect differentiation. Indeed, knockdown of *Hivep3* strongly suppressed the expression of *Alg2* in ATDC5 chondrocytes (Fig. 8*A*). Moreover, *Alg2* siRNA inhibited the BMP-2-induced expression of type II collagen in mRNA (Fig. 8*B*) and in protein level (Fig. 8*C*). We checked whether the loss of Alg2 evoked ER stress and that the basal expression of *Atf4* (Fig. 8*D*) and *Hspa5* (Fig. 8*F*) decreased, whereas *Ddit3* expression increased in the presence of BMP-2 (Fig. 8*E*), following siAlg2 transfection. Importantly, *Creb3l2* was down-regulated by *Alg2* silencing (Fig. 8*G*), and silencing of *Alg2* or *Hivep3* both diminished the band of Creb3l2 in immunoblotting (Fig. 8*H*). Finally, we infected Alg2-expressing adenovirus in cultured mouse metatarsal cartilage, because this *ex vivo* organ culture is an excellent system to evaluate the rate of chondrocyte maturation (37). Indeed, application of BMP-2 into this cartilage culture promoted the calcification of cartilage matrix by mature hypertrophic chondrocytes (Fig. 8*J*, *1st* and *3rd lanes*). The infection efficiency of adenovirus was evaluated by immunofluorescence, and the V5-tagged transgene product was detected by anti-V5 antibody in the cartilage sample (Fig. 8*I*). Importantly, Alg2-expressing adenovirus mildly but significantly increased the zone of mature chondrocytes regardless of BMP-2 treatment (Fig. 8*J*), indicating that Alg2 promotes cartilage maturation. Taken together, these data suggest that Alg2, induced by Hivep3, is necessary for the induction of *Creb3l2* to promote chondrogenesis.

DISCUSSION

Drosophila Schnurri was one of the first partners identified for BMP-specific Smads (38, 39) for positive or negative regulation of BMP signaling. The structure of three Schnurri homologs in vertebrates, Hivep1, Hivep2, and Hivep3, is also similar to that of the fly Schnurri and shares additional features, including an unusually large size (~2500 amino acids) and acidic domains. We initially hypothesized that Hivep3 may inhibit BMP signaling to suppress osteoblast differentiation; however, the expression of the direct target genes of the BMP-Smad pathway, *Id1* or *Smad6*, was not altered by *Hivep3* knockdown in osteoblasts (data not shown). In this study, we found that the *Alg2* gene was commonly down-regulated in both ST-2 and MC3T3-E1 osteoblasts by *Hivep3* knockdown. Alg2 inhibited the activity of Runx2 without affecting its protein expression unlike Hivep3. Therefore, the Hivep3-Alg2 pathway efficiently blocks Runx2-mediated transcription and subsequent osteoblast differentiation by two approaches, regulation of protein degradation and intracellular localization. In ATDC5 chondrocytes, Alg2 expression was also controlled by Hivep3 to support chondrocyte differentiation (*Col2a1* expression), but its possible actions against Runx2 in chondrocytes remain elusive. Because Runx2 is directly crucial for transcription of the chondrocyte maturation marker gene type X collagen (*Col10a1*) in promoting chondrocyte hypertrophy (40), loss of Hivep3 or Alg2 may increase expression of *Col10a1* if they target Runx2 in chondrocytes. However, because expression of *Col10a1* arises sequentially after expression of early markers such as *Col2a1* or *Col11a2*, which had diminished expression in response to transfection with siRNA for *Hivep3* or *Alg2*, we could not clearly evaluate their effect on *Col10a1* expression (data not shown). Indeed, in *Hivep2/Hivep3* double knock-out mice, hypertrophic chondrocytes as well as expression of *Col10a1* have been found to decrease in the growth plates of long bones (17). Our data and those of other researchers suggest that Runx2 is not the target of Hivep3 in chondrocytes.



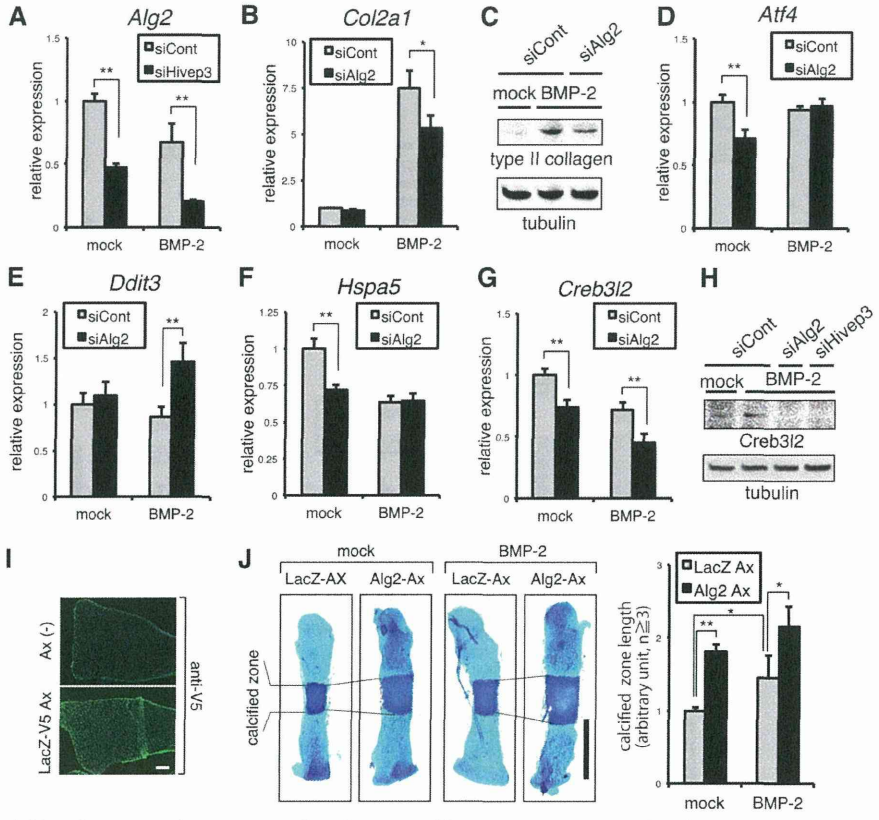


FIGURE 8. **Hivep3 is essential for Alg2 expression in ATDC5 chondrocytes, althoughAlg2 promotes chondrogenesis.** *A*, ATDC5 cells were transfected with siRNA for *Hivep3* and treated with BMP-2 (300 ng/ml) for 4 days. Expression of *Alg2* was evaluated by qRT-PCR. *B*, *D*, *E*, *F* and *G*, ATDC5 cells were transfected with siRNA for *Alg2* and treated with BMP-2 (300 ng/ml) for 4 days. The expression of the indicated genes was analyzed by qRT-PCR. *C*, ATDC5 chondrocytes were transfected with siRNA for *Alg2* with BMP-2 treatment (300 ng/ml) for 7 days. Cell lysates were analyzed by immunoblotting with an anti-type II collagen antibody. Tubulin served as a loading control. *H*, ATDC5 chondrocytes were transfected with siRNA for *Alg2* or *Hivep3* with BMP-2 treatment (300 ng/ml) for 3 days. Cell lysates were analyzed by immunoblotting with an anti-Creb3l2 antibody. Tubulin served as a loading control. *I* and *J*, metatarsal cartilages of E17.5 mouse embryos were infected with indicated adenovirus for 16 h. Immunostaining using FITC-linked anti-V5 antibody on bone coronal sections was performed at day 2 of culture (*I*). Nuclei were stained with Hoechst dye. Merged images are presented. *Scale bar*, 100 μm. Alcian blue/alizarin red staining was performed at day 3 of BMP-2 treatment (*J*). The length of calcified zone (matured hypertrophic chondrocytes) was measured. *Scale bar*, 500 μm. *, $p < 0.05$; **, $p < 0.01$.

Mutations in the *ALG2* gene cause the rarest form of congenital disorders of glycosylation (CDG) in humans, CDG type-Ii (CDG-Ii) (25). A single patient of CDG-Ii has been identified, who was only mildly affected with developmental delay, seizures, poor vision, coagulopathy, and delayed myelinization (25), with no remarkable bone phenotype. However, because the reported patient was 3 years old, and *Hivep3* knock-out mice have been reported to show adult-onset osteosclerosis (10), skeletal disorders may develop during the adulthood of the CDG-Ii patient. The glycosyltransferase enzymes of the ALG pathway use nucleotide- or dolichol-activated monosaccharides as donor substrates (41), which are biosynthesized by the cytosolic enzyme PMM2. CDG-Ia is the largest group of CDG cases with more than 800 patients who have been identified as having mutations in the *PMM2* gene (41). It is noteworthy that CDG-Ia patients show a variety of skeletal phenotypes, including osteopenia, rhizomelia, and ossification delay of bones (42). Fibrillar collagens, such as type I or type II collagens, are found predominantly in the ECM of bone or cartilage, respectively, and mature through ALG of the C-terminal pro-collagen, resulting in cleavage of the N- and C-terminal pro-peptide domains. Therefore, dysregulation of ALG may affect the maturation and function of collagens in the skeleton. Conversely, during the differentiation of osteoblasts and chondrocytes, nascent ECM protein is delivered in amounts that exceed the capacity of the ER, whose machinery processes the post-translational modifications and folding of proteins; ALG proteins play important roles for these steps. Such events of "overload" trigger mild ER stress (physiological ER stress) (43). In ST-2 cells, loss of Hivep3 did not influence mild ER stress during

FIGURE 7. **Hivep3 potentiates the physiological mild ER stress to promote chondrocyte differentiation.** *A*, *E*, *G*, *I*, *K*, *M*, and *O*, ATDC5 cells were transfected with siRNA for *Hivep3* and treated with BMP-2 (300 ng/ml) for 4 days. The expression of the indicated genes was evaluated by qRT-PCR. *B*, ATDC5 chondrocytes were transfected with siRNA for *Hivep3* with BMP-2 treatment (300 ng/ml) for 7 days. Cell lysates were analyzed by immunoblotting with an anti-type II collagen antibody. Tubulin served as a loading control. *C*, *F*, *H*, *J*, *L*, and *N*, ATDC5 cells were transfected with a human HIVEP3 expression vector and further stimulated with BMP-2 (300 ng/ml) for 4 days. The expression of the indicated genes was evaluated by qRT-PCR. *D*, ATDC5 cells were stably transfected with a pEF-Shn3 (*Hivep3*) expression vector. Micromass culture of the transfectants was treated with BMP-2 (300 ng/ml) for 21 days and stained with Alcian blue dye. *, $p < 0.05$; **, $p < 0.01$.

osteoblast differentiation. In contrast, Hivep3 increased not only the level of *Creb3l2*, but also of other canonical ER stress-related genes in ATDC5 chondrocytes, although loss of *Alg2* decreased the expression of *Creb3l2*. These results suggest that the Hivep3-Alg2 pathway is important for physiological ER stress in chondrocytes.

Although Hivep2 and Hivep3 showed cooperative roles in decreasing bone formation and bone volume *in vivo* (17), Saita *et al.* (16) reported results similar to ours where gain of Hivep2 expression enhanced osteoblast differentiation, and Hivep2 knock-out osteoblasts failed to support efficient osteoclastogenesis *in vitro*. In addition, they showed that bone formation, as well as bone resorption, decreased in the bones of Hivep2-null mice, resulting in osteopenia due to the low turnover of bone remodeling. Recently, Hivep3 was also found to promote osteoclastogenesis (14, 15). Taken together, the defects in osteoclastogenesis induced by loss of the Hivep genes seem to be mainly responsible for the additively increased bone volume in *Hivep2*/*Hivep3* double knock-out mice. In contrast, we showed that each of the three Hivep genes was essential for chondrogenesis *in vitro*, suggesting that the chondrodysplasia observed following combined ablation of Hivep2 and Hivep3 in mice (17) was a result of the loss of the cell autonomous action of the Hivep proteins. Although we found the physiological ER stress and the level of *Creb3l2* to be increased by the Hivep3-Alg2 axis, the precise molecular mechanisms are unclear. Moreover, it is still unclear why the three Hivep genes exhibit diverse actions in osteogenesis. Because expression of *Alg2* was not altered by loss of Hivep1 or Hivep2, other molecular targets downstream of Hivep1 or Hivep2 should be evaluated. Although Hivep proteins are known to act as transcription factors as well as scaffolds, they may harbor some other unknown properties, as most of the area of the large proteins has not been classified into any known functional domains.

In conclusion, Alg2 is a downstream mediator of Hivep3 in osteoblasts and chondrocytes. In addition to initiation of Runx2 protein degradation, Hivep3 interfered with the function of Runx2 via Alg2-mediated disturbance of localization and activity. In ATDC5 chondrocytes, Hivep3 and Alg2 enhanced mild ER stress to promote differentiation. Thus, our results are the first to link the ALG gene to differentiation of skeletal cells. Future studies on mice with knock-out of ALG genes, as well as detailed clinical research with corresponding CDG patients, may provide more information regarding the roles of ALG proteins in osteogenesis, chondrogenesis, and bone remodeling.

Acknowledgments—The pEF-Shn3(Hivep3) expression vector was kindly provided by Dr. Laurie Glimcher. The mouse type II Runx2 expression plasmid and 6×OSE2 luciferase plasmid were kind gifts from Dr. Toshihisa Komori. The mutant 6×OSE2 luciferase plasmid was a kind gift from Dr. Gerard Karsenty. We gratefully acknowledge the technical assistance of Hui Gao.

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Cell Biology:

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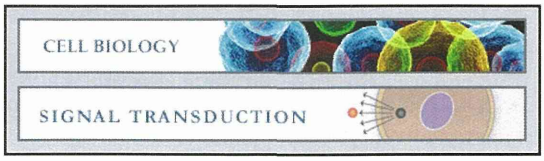

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J. Biol. Chem. 2014, 289:8135-8150.
doi: 10.1074/jbc.M113.509331 originally published online February 6, 2014

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 12, pp. 8135–8150, March 21, 2014

Bone Morphogenic Protein (BMP) Signaling Up-regulates Neutral Sphingomyelinase 2 to Suppress Chondrocyte Maturation via the Akt Protein Signaling Pathway as a Negative Feedback Mechanism*[S]

Received for publication, August 9, 2013, and in revised form, January 8, 2014 Published, JBC Papers in Press, February 6, 2014, DOI 10.1074/jbc.M113.509331

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Background: It is not clear how BMP-induced chondrocyte maturation is cell-autonomously terminated.
Results: BMP-2 induced the ceramide-generating enzyme neutral sphingomyelinase 2 (nSMase2) in chondrocytes, whereas silencing of nSMase2 enhanced maturation in an Akt signaling-dependent manner.
Conclusion: nSMase2 signaling regulates BMP-induced chondrocyte maturation as a negative feedback mechanism.
Significance: This study elucidated the novel link between BMP and lipid signaling in chondrogenesis.

Although bone morphogenic protein (BMP) signaling promotes chondrogenesis, it is not clear whether BMP-induced chondrocyte maturation is cell-autonomously terminated. Loss of function of *Smpd3* in mice results in an increase in mature hypertrophic chondrocytes. Here, we report that in chondrocytes the Runx2-dependent expression of *Smpd3* was increased by BMP-2 stimulation. Neutral sphingomyelinase 2 (nSMase2), encoded by the *Smpd3* gene, was detected both in prehypertrophic and hypertrophic chondrocytes of mouse embryo bone cartilage. An siRNA for *Smpd3*, as well as the nSMase inhibitor GW4869, significantly enhanced BMP-2-induced differentiation and maturation of chondrocytes. Conversely, overexpression of *Smpd3* or C_2-ceramide, which mimics the function of nSMase2, inhibited chondrogenesis. Upon induction of *Smpd3* siRNA or GW4869, phosphorylation of both Akt and S6 proteins was increased. The accelerated chondrogenesis induced by *Smpd3* silencing was negated by application of the Akt inhibitor MK2206 or the mammalian target of rapamycin inhibitor rapamycin. Importantly, in mouse bone culture, GW4869 treatment significantly promoted BMP-2-induced hypertrophic maturation and calcification of chondrocytes, which subsequently was eliminated by C_2-ceramide. *Smpd3* knockdown decreased the apoptosis of terminally matured ATDC5 chondrocytes, probably as a result of decreased ceramide production. In addition, we found that expression of hyaluronan synthase 2 (*Has2*) was elevated by a loss of *Smpd3*, which was restored by MK2206. Indeed, expression of Has2 protein decreased in nSMase2-positive hypertrophic chondrocytes in the bones of mouse embryos. Our data suggest that the *Smpd3*/nSMase2-ceramide-Akt signaling axis negatively regulates BMP-induced chondrocyte maturation and Has2 expression to control the rate of endochondral ossification as a negative feedback mechanism.

Over 95% of bone formation during the embryonic and developmental stages is achieved through endochondral ossification. This process is primed by the condensation of mesenchymal progenitor cells expressing the chondrogenic master regulator Sox9 (1), after which cells further differentiate into proliferating chondrocytes that are able to express a specific marker, *Col2a1*, encoding type II collagen (2). These chondrocytes then mature to hypertrophic chondrocytes, which eventually mineralize the surrounding cartilage matrix to be replaced by bone-forming osteoblasts (3). The maturation of chondrocytes into hypertrophic chondrocytes, which are able to express the type X collagen-encoding gene *Col10a1*, is mainly governed by the Runx2 transcription factor (4, 5). The rate of proliferation and differentiation of chondrocytes *in vivo* is tightly regulated by a signaling network between Indian hedgehog, parathyroid hormone-related protein, fibroblast growth factor (FGF), and bone morphogenetic protein (BMP)[3] signaling (6).

BMPs belong to the transforming growth factor-β (TGF-β) family, which transduces signals through type II and type I receptors to activate receptor-regulated Smads. Upon ligand binding, BMP type I receptors phosphorylate Smad1/5/8 in the cytoplasm. Phosphorylated Smads form a trimeric complex

* This work was supported by Japan Society for the Promotion of Science KAKENHI Grants 25462376 (to H. K.), 23592221 (to S. M.), and 23592222 (to Y. I.), a grant from the Hip Joint Foundation of Japan (to S. M.), and a grant from the Cell Science Research Foundation (to S. M.).
[S] This article contains supplemental Table S1.
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[3] The abbreviations used are: BMP, bone morphogenic protein; nSMase2, neutral sphingomyelinase 2; MSC, mesenchymal stem/stroma cell; CHX, cycloheximide; RTK, receptor tyrosine kinase; OA, osteoarthritis; ITS, insulin/transferrin/selenium; PP2A, protein phosphatase 2A; rpS6, ribosomal protein S6.