Human Immunodeficiency Virus Type I Enhancer Binding Protein 3 is Essential for the Expression of Asparagine-linked Glycosylation 2 in the Regulation of Osteoblast and Chondrocyte Differentiation

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Running title: Hivep3-dependent Alg2 expression inhibits osteogenesis

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Keywords: bone morphogenetic protein (BMP); human immunodeficiency virus type I enhancer binding protein 3 (Hivep3); asparagine-linked glycosylation 2 (Alg2); osteogenesis; chondrogenesis; Runx2; ER stress
**Background:** The mechanisms by which Hivep3 regulates the osteochondrogenesis remain elusive.

**Results:** Knockdown of Hivep3 downregulated Alg2 expression. Alg2 suppressed osteoblast differentiation by inhibiting the activity of Runx2. Alg2-silencing suppressed the expression of Creb3l2 and chondrogenesis.

**Conclusion:** Alg2 may be a modulator of osteochondrogenesis.

**Significance:** This is the first report to describe the association of an Alg gene with osteochondrogenesis.

**ABSTRACT**

Human immunodeficiency virus type I enhancer binding protein 3 (Hivep3) suppresses osteoblast differentiation by inducing proteasomal degradation of the osteogenesis master regulator Runx2. In this study, we tested the possibility of cooperation of Hivep1, Hivep2, and Hivep3 in osteoblast and/or chondrocyte differentiation. Microarray analyses with ST-2 bone stroma cells demonstrated that expression of any known osteochondrogenesis-related genes was not commonly affected by the 3 Hivep siRNAs. Only Hivep3 siRNA promoted osteoblast differentiation in ST-2 cells, whereas all 3 siRNAs cooperatively suppressed differentiation in ATDC5 chondrocytes. We further used microarray analysis to identify genes commonly downregulated in both MC3T3-E1 osteoblasts and ST-2 cells upon knockdown of Hivep3 and identified asparagine-linked glycosylation 2 (Alg2), which encodes a mannosyltransferase residing on the endoplasmic reticulum (ER). The Hivep3 siRNA-mediated promotion of osteoblast differentiation was negated by forced Alg2 expression. Alg2 suppressed osteoblast differentiation and bone formation in cultured calvarial bone. Alg2 was immunoprecipitated with Runx2, while combined transfection of Runx2 and Alg2 interfered with Runx2 nuclear localization, which resulted in suppression of Runx2 activity. Chondrocyte differentiation was promoted by Hivep3 overexpression, in concert with increased expression of Creb3l2, whose gene product is the ER stress transducer crucial for chondrogenesis. Alg2 silencing suppressed Creb3l2 expression and chondrogenesis of ATDC5 cells, whereas infection of Alg2-expressing virus promoted chondrocyte maturation in cultured cartilage rudiments. Thus, Alg2, as a downstream mediator of Hivep3, suppresses osteogenesis, whereas it promotes chondrogenesis. To our knowledge, this study is the first to link a mannosyltransferase gene to osteochondrogenesis.
In skeletal development and bone remodeling, osteoblasts play major roles not only in bone formation but also in inducing the differentiation of bone-resorbing osteoclasts (1,2). Runx2 is a critical transcription factor in osteoblast differentiation, as evidenced by Runx2-knockout mice, which exhibit a complete lack of both intramembranous and endochondral ossification due to the absence of osteoblasts (3). Cleidocranial dysplasia, a human autosomal dominant inherited disorder of bone development, is characterized by hypoplasia of clavicles and abnormalities in cranial and facial bones and is caused by mutations in the Runx2 gene (4,5). Some genes, e.g., LDL receptor-related protein 5 (Lrp5), sclerostin (Sost), and human immunodeficiency virus type 1 enhancer binding protein 3 (Hivep3), have been found to control osteoblast function in the adult human and/or mouse during postnatal skeletal remodeling (6-10).

Hivep3, also known as Schnurri-3, Zas3, and Krc, is a member of 3 mammalian homologs of the Hivep/Schnurri family of large zinc finger proteins. Hivep proteins have been studied for their roles in the regulation of an assortment of genes, including those encoding collagen type IIA, αA-crystallin, β interferon, and HIV genes (11). Hivep2 can indirectly interact with the peroxisome proliferator activated receptor γ2 (Pparg2) promoter to promote adipogenesis, through binding to Smad1, an intracellular mediator of bone morphogenetic protein (BMP) signaling. Hivep2 can also dock to CCAAT/enhancer binding protein α (C/EBPα) to interact with a CCAAT site on the distal part of the Pparg gene (12). Mice lacking Hivep3 demonstrate adult-onset osteosclerosis with increased bone volume due to enhanced osteoblast activity (10). Hivep3 promotes proteosomal degradation of the Runx2 protein through recruitment of the E3 ubiquitin ligase Wwp1 to Runx2 (10). A D-domain motif within Hivep3 mediates the interaction with and inhibition of ERK mitogen-activated protein kinase (MAPK), thereby inhibiting Wnt/Lrp5 signaling through regulation of the activity of a downstream mediator glycogen synthase kinase 3-β (GSK3β). This interaction results in the suppression of subsequent osteoblast differentiation (13). On the other hand, Hivep3 indirectly promotes osteoclastogenesis by promoting osteoblastic expression of receptor activator of nuclear factor-κB ligand (RANKL), a crucial factor for osteoclast differentiation (14). Hivep3 also cell-autonomously promotes osteoclastogenesis by inducing the expression of NFATc1, a master transcription factor in osteoclast differentiation, by interacting with TRAF6 to enhance its activity while forming a complex with
c-jun to activate the NFATc1 promoter (15). Thus, Hivep3 controls both bone formation and resorption at multiple steps to maintain normal bone mass. However, whether Hivep3 controls gene expression in osteoblasts to regulate osteoblast activity is unclear.

In contrast to Hivep3-knockout mice, mice lacking Hivep2 exhibited decreased cortical bone volume and increased cancellous bone mass (16), suggesting different roles for Hivep2 and Hivep3 in the skeleton. Combined ablation of Hivep2 and Hivep3 in mice resulted in synergistically increased trabecular bone volume, demonstrating a redundancy between the 2 proteins in the regulation of postnatal bone mass (17). Interestingly, in the double-knockout mice, the growth plate cartilage of the long bones was uncoupled with bone phenotype, with significantly delayed maturation of chondrocytes resulting in chondrodysplasia (17), suggesting a role for Hivep proteins in the promotion of chondrocyte differentiation. However, the mechanism by which Hivep proteins affect chondrogenesis remains unknown. In addition, to date, no information has been reported on the possible role of Hivep1 in osteogenesis and/or chondrogenesis.

In this study, in vitro analysis showed that, among the 3 Hivep proteins, only Hivep3 was inhibitory and that the others promoted osteoblast differentiation. In contrast, all 3 Hivep genes seemed to support chondrocyte differentiation in BMP-2-induced ATDC5 cells, suggesting their redundancy in chondrogenesis. We found that asparagine-linked glycosylation 2 (Alg2) is commonly downregulated in BMP-2-induced osteoblast differentiation in both MC3T3-E1 and ST-2 cells. Alg2 inhibited Runx2 activity without altering its protein level, resulting in suppression of osteoblast differentiation. Interestingly, in chondrogenesis of ATDC5 cells, Hivep3 induced the expression of cAMP responsive element binding protein 3-like 2 (Creb3l2), an endoplasmic reticulum (ER) stress transducer crucial for chondrogenesis (18), suggesting a possible role for Hivep3 in physiological mild ER stress. Alg2 was also decreased by Hivep3 knockdown in ATDC5 chondrocytes, while silencing of Alg2 suppressed the expression of Creb3l2 and chondrogenesis. To our knowledge, this study is the first to show a linkage between an asparagine-linked glycosylation mannosyltransferase gene and osteochondrogenesis.

**EXPERIMENTAL PROCEDURES**

*Cell culture and differentiation—The mouse carvarial bone-derived osteoblast cell line, MC3T3-E1 (clone 4) and the mouse chondrogenic fibroblast cell line C3H10T1/2 were obtained from the ATCC. The mouse bone marrow stromal cell line ST-2 and the mouse chondrogenic cell line*
ATDC5 were obtained from the RIKEN BioResource Center. MC3T3-E1 cells were cultured in minimum essential media (MEM) alpha (Invitrogen) containing 10% fetal bovine serum (FBS). ST-2 cells were cultured in RPMI1640 medium (Sigma) containing 10% FBS. ATDC5 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 (1:1) (Invitrogen) containing 5% FBS. C3H10T1/2 cells were cultured in basal medium Eagle (Sigma) with 2 mM L-glutamine and 10% FBS. COS-7 cells were purchased from RIKEN BioResource Center and maintained in DMEM supplemented with 10% FBS. All cell culture medium contained 100 U/ml penicillin G and 100 µg/ml streptomycin. Cell differentiation was induced by the addition of recombinant human BMP-2 (Peprotech) at a concentration of 300 ng/ml. Micromass culture of ATDC5 cells was performed as previously described (19) to accelerate the maturation of chondrocyte differentiation.

Alkaline phosphatase (ALP) and alcian blue staining—The activity of ALP secreted into the extracellular matrix (ECM) of cultured cells was visualized with an ALP staining kit (85L-3R, Sigma). Cartilaginous glycosaminoglycans produced in the ECM by cultured cells were stained with alcian blue 8GX (Sigma).

RNA interference—Dharmacon siRNA ON-TARGETplus SMARTpool, a mixture of 4 independent siRNAs against mouse *Hivep1*, *Hivep2*, *Hivep3*, and *Alg2*, and the control reagent were purchased from Thermo Scientific. siRNAs were transfected into cells using Lipofectamine RNAiMax (Invitrogen).

Real-time quantitative PCR—Cells were lysed with TRIzol reagent (Invitrogen) to purify RNA, and 1 µg of total RNA was subjected to reverse transcription with the Verso cDNA Kit (Thermo Scientific). The relative amounts of the gene transcripts were determined by real-time quantitative PCR (qPCR) using SYBR premix Ex Taq II (Takara) and the Thermal Cycler Dice TP850 system (Takara). PCR reactions were performed in duplicate per sample, and the measured expression level of each gene was normalized to that of *Hprt1*. The sequence information for the primers used is listed in Supplemental Table 1. All primer sets are for mouse genes, except for the m/h*Hivep3* primer set, which can be used to amplify both the mouse and human *Hivep3* genes. For evaluation of the tissue distribution of the *Hivep* genes and *Alg2* in vivo, tissues were harvested from 3-month-old mice, and mRNA was purified with TRIzol reagent before subjecting samples to qRT-PCR.

Microarray analysis—Cells transfected with siRNA overnight were further incubated with BMP-2 for 2 d before being lysed with TRIzol reagent for mRNA purification. mRNA samples
were cleaned up using the RNeasy MinElute Cleanup Kit (Qiagen) and analyzed on a Mouse Gene 2.0 ST Array (Affymetrix) by Bio Matrix Research.

**Plasmids, adenovirus, and lentivirus**—The mouse Hivep3 expression plasmid, pEF-Shn3, was a kind gift from Dr. Laurie Glimcher (Harvard Medical School). The human HIVEP3 expression plasmid pFN21A-HIVEP3 was obtained from Kazusa DNA Research Institute. The mouse type II Runx2 expression plasmid was a kind gift from Dr. Toshihisa Komori (Nagasaki University). The FLAG-Runx2-def expression plasmid has been described in our previous study (20). Mouse Alg2 or Runx2 cDNA was cloned from ST-2 cells by using a RT-PCR-based technique; subcloned into the entry vector, pENTR; and further transferred into the C-terminally V5-tagged expression vector, pEF-DEST51 (Invitrogen). For overexpression assays, cells were transfected with expression vectors using FuGENE 6 (Roche) or Lipofectamine 2000 (Invitrogen). Cells transiently expressing the transgenes were selected and enriched by incubation with G418 disulfate (Nacalai Tesque) at a concentration of 250 µg/ml for 3–7 d. To generate adenovirus-carrying Alg2 cDNA, the Alg2 gene in the pENTR-Alg2 vector was transferred into the C-terminally V5-tagged adenovirus expression vector pAd/CMV/V5-DEST by LR recombination (Invitrogen) and was further transfected into the adenovirus-producing cell line 293A according to the manufacturer’s protocol. The pAd/CMV/V5-GW/lacZ adenovirus expression vector was used to generate a control adenovirus. For generation of lentivirus carrying the Alg2 gene, pENTR-Alg2 and pENTR-5'EF1αP were subjected to LR recombination with pLenti6.4/R4R2/V5-DEST (Invitrogen) to generate a lentiviral vector expressing C-terminally V5-tagged Alg2 from the EF1α promoter. The lentiviral expression vector or pLenti6/V5/GW-lacZ control vector was transfected into 293FT cells to generate the lentivirus. Virus infection into ST-2 cells was performed at a multiplicity of infection (MOI) of 100. Cells infected with the lentivirus were selected by treatment with Blasticidin S HCL (Invitrogen) at a concentration of 2.5 µg/ml. These experiments were approved by the Kagoshima University safety control committee for gene-recombination techniques (no. 22053).

**Embryonic bone organ culture**—Carvarial bone and metatarsal bone (cartilage) rudiments were harvested from C57BL/6J mouse embryos at 17.5 days post-coitum (E17.5) and cultured in MEM alpha or DMEM/Ham’s F-12 (1:1), respectively, supplemented with 10% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin, as described (21). The bone rudiments were incubated in virus
solution overnight for infection of adenovirus or lentivirus. Cultured bones and cartilages were fixed in 96% ethanol, and stained with 0.015% alcian blue 8GX in a mixture solution of 96%-ethanol/acetic acid (4:1) for 1 day, followed by a dehydration step in 100% ethanol. Dehydrated bones were immersed briefly in 1% potassium hydroxide (KOH), followed by staining in 0.001% alizarin red S (Sigma) in 1% KOH for 1 day. Images were captured with stereomicroscope M165FC (Leica). The animal experiments were approved by the Institutional Animal Care and Use Committee of Kagoshima University (no. MD12137).

**Immunoprecipitation (IP) and immunoblotting (IB)**—For IP assays, COS-7 cells were transfected with Alg2-V5 and/or FLAG-Runx2 plasmids and were lysed in M-PER lysis buffer (Thermo Scientific) supplemented with aprotinin and phenylmethylsulfonyl fluoride (PMSF). The lysate was immunoprecipitated with anti-FLAG M2 Agarose Affinity Gel (A2220, Sigma) and the M2 antibody-bound protein complex was eluted by incubation with 3xFLAG Peptide (F4799, Sigma), according to the manufacturer’s protocol. For IB assays, cells were lysed in either M-PER or NE-PER lysis buffer (Thermo Scientific) supplemented with aprotinin and PMSF, or directly with 1× sodium dodecyl sulfate (SDS) sample buffer. SDS-polyacrylamide gel electrophoresis (PAGE), membrane transfer, and chemiluminescence were performed using a standard protocol. The blots were incubated with primary antibodies against Alg2 (1:1000; LS-C81338, Lifespan Biosciences), Runx2 (1:200; M-70, sc-10758, Santa Cruz), Runx2 (1:1000; 8G5, MBL), Sp7 (1:1000, ab22552, Abcam), Ibsp (1:1000, LS-C190916, Lifespan Biosciences), type II collagen (1:1000, LS-C175971, Lifespan Biosciences), Creb3l2 (1:1000, ab76856, Abcam), V5 (1:5000; R960-25, Invitrogen), FLAG (1:1000; M2, F1804, Sigma), and tubulin (1:1000; DM1A, T9026, Sigma) and with horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies (1:10,000) (Cell Signaling). Signals were detected using the LAS 4000 mini image analyzer (Fujifilm).

**Immunofluorescence (IF)**—For IF assays, cells transfected with Runx2 and/or Alg2-V5 expression plasmids were fixed with 4% paraformaldehyde in PBS for 30 min and treated with 0.2% Triton X-100. CAS block (Zymed) was used for blocking. Cells were incubated with anti-Runx2 (1:100; 8G5, MBL), Alexa Fluor 568 rabbit anti-mouse IgG (1:1000; A11061,
Invitrogen), and anti-V5-FITC (1:500; R619-25, Invitrogen) antibodies. Nuclei were stained with Hoechst dye (Invitrogen). Confocal fluorescent imaging was performed and analyzed using a laser scanning microscope system (LSM 700, Zeiss). For confirmation of the efficiency of virus infection in cultured bones, formalin-fixed mouse E17.5 embryo calvariae or metatarsal bones were embedded in paraffin blocks, which were sliced at a 4-µm thickness. The antigen was retrieved by the Liberate Antibody Binding (L.A.B.) solution (Polysciences). A CAS block was used for blocking. Bone sections were incubated with anti-V5-FITC antibody. Images were captured with microscope AX80 and digital camera DP70 (Olympus).

Luciferase assay—COS-7 cells or ST-2 cells were seeded in triplicate in 24-well plates and transiently transfected with the 6xOSE2 luciferase reporter plasmid (a kind gift from Dr. Toshihisa Komori), the mutant 6xOSE2 luciferase reporter plasmid (a kind gift from Dr. Gerard Karsenty, Columbia University Medical Center), the pGL4.75hRlucCMV Renilla vector (Promega), and expression vectors for Runx2, Alg2, or Hивep3. Dual luciferase assays were performed as described earlier (20) by using the GloMax 96 microplate luminometer (Promega).

Statistical analysis—The data in this study have been expressed as mean ± SD values of 3 independent experiments. Statistical comparisons between the different treatments were performed using an unpaired Student’s t-test in which P < 0.05 was considered significant and P < 0.01 was considered highly significant.

RESULTS

Loss of Hивep1 or Hивep2 suppresses osteoblast differentiation in ST-2 cells, in contrast to Hивep3 silencing, whereas all 3 Hивep siRNAs inhibited chondrogenesis—The precise roles of Hивep1 and Hивep2 in osteoblast differentiation are unclear. In a tissue cDNA panel from a 3-month-old adult mouse, all the Hивep genes showed a relatively specific expression pattern with high expression being observed in the lung, spleen, articular cartilage, and bone (Fig. 1, A-C). However, the in vitro results for the osteochondrogenic cell lines ST-2, MC3T3-E1, ATDC5, and C3H10T1/2 showed that the expression profiles were completely different between the Hивep genes, with Hивep1 being expressed ubiquitously, Hивep2 abundant in ST-2 and ATDC5, and Hивep3 prominent in MC3T3-E1 osteoblasts (Fig. 1D). If the Hивep genes cooperate in osteoblast differentiation, they should regulate some common sets of genes. To test this hypothesis, ST-2 cells transfected with siRNA for Hивep1, Hивep2, or Hивep3 were analyzed by a microarray assay (supplemental table 2, 3, or 4, respectively),
and the results were compared. The expression of 5 genes decreased in all the 3 knockdown experiments (Table 1A), while 6 genes, 4 genes, or 3 genes were downregulated in common by siHivep1 and siHivep2 (Table 1B), siHivep1 and siHivep3 (Table 1C), or siHivep2 and siHivep3 (Table 1D), respectively, although no trend was observed in the purified genes. Moreover, none of the purified genes was reported to correlate with differentiation of osteoblasts or chondrocytes. To investigate the roles and possible synergism of the Hivep genes in osteoblast differentiation, the expression of the 3 Hivep genes was knocked down in ST-2 cells, alone or in combination (Fig. 1E). Although combined genetic ablation of the Hivep2 and Hivep3 genes in mice resulted in synergistically increased bone formation and bone volume (17), siRNA-mediated silencing of Hivep2 in BMP-2-stimulated ST-2 cells decreased the expression and activity of ALP (Fig. 1E, compare lanes 2 and 4), whereas loss of Hivep3 alone enhanced the osteoblast differentiation (Fig. 1E, compare lanes 2 and 5). Interestingly, combined transfection of siHivep2 with siHivep3 negated the enhancement of ALP production by Hivep3-knockdown (Fig. 1E, compare lanes 2, 5, and 8). Similar to siHivep2, Hivep1 siRNA inhibited ALP activity; however, there was no synergistic or additive effect on combined knockdown of Hivep1 and Hivep2. These results suggest that, in ST-2 bone marrow stromal cells, the cell-autonomous actions of Hivep genes are diverse and show no cooperation in osteoblast differentiation and that Hivep1 and Hivep2 promote the counteraction of the suppressive effect of Hivep3. In contrast, in a siRNA-mediated knockdown assay in ATDC5 chondrocytes, siHivep1, siHivep2, and siHivep3 all decreased the BMP-2-induced expression of the chondrocyte-specific type XI collagen gene (Col11a2) (Fig. 1F). A similar result was observed in another chondrocytic cell line, C3H10T1/2, where knockdown of both Hivep2 and Hivep3 decreased the level of a chondrocyte marker, type II collagen gene (Col2a1) (Fig. 1G). In both experiments in chondrogenic cells, the combined loss of the Hivep genes showed some additive effects.

Hivep3 suppresses osteoblast differentiation in vitro—Although the mRNA level of Runx2 was comparable between wild-type and Hivep3 knockout cells, the protein levels of Runx2, as well as the mRNA levels of the early osteoblast differentiation markers osterix (Sp7), alkaline phosphatase (Alpl), activating transcription factor 4 (Atf4), and bone sialoprotein (Ibsp) and of the late maturation marker osteocalcin (Bglap2) increased in knockout osteoblasts (10). We first checked if this effect could be reproduced via siRNA-mediated knockdown in osteoblastic cell
lines. We used the mouse bone marrow stromal cell line ST-2 as a model for premature osteoblast progenitors and MC3T3-E1 mouse carvaria-derived osteoblasts as mature osteoblasts. In both MC3T3-E1 and ST-2 cells, approximately 50% knockdown was achieved by transfection of siHivep3 (Fig. 2, A and C). As expected, Hivep3 silencing did not have any effect on the mRNA expression of Runx2 (Fig. 2, A and C). In ST-2 cells treated with cycloheximide to block de novo synthesis of Runx2 protein, the protein level of Runx2 decreased in a time-dependent manner (Fig. 2B), whereas the protein expression was maintained in siHivep3-transfected cells. Moreover, combined induction of BMP-2 and siHivep3 in ST-2 cells increased Runx2 protein in a time-dependent fashion (Fig. 2B). Therefore, siRNA-mediated silencing of Hivep3 stabilized Runx2 protein. As a result, expression of Sp7, Ibsp, and Bglap2 was upregulated in MC3T3-E1 and ST-2 cells (Fig. 2, A and C). In addition, the expression of an osteocyte marker, dentin matrix protein 1 (Dmp1), was elevated by Hivep3 knockdown in MC3T3-E1 osteoblasts (Fig. 2A). The siHivep3-mediated increase of osteoblastic differentiation in ST-2 cells was confirmed by immunoblotting against Sp7 and Ibsp (Fig. 2D) or alkaline phosphatase (ALP) staining (Fig. 2E). We introduced the human HIVEP3 gene in ST-2 cells through transfection and confirmed the transgene expression by qRT-PCR (Fig. 2F). HIVEP3 suppressed BMP-2-induced osteoblast differentiation (Fig. 2, F and G) and protein expression of Runx2 (Fig. 2G). Interestingly, the mRNA expression of Runx2 decreased following transfection with HIVEP3. As HIVEP3 destabilizes Runx2 protein, this result is likely due to loss of auto-induction of Runx2 (22), that endogenous Runx2 mRNA expression increased in Runx2 transgenic mice (23). In both cases of knockdown and overexpression of Hivep3, expression of Atf4 did not change (Fig. 2, C and F), although it increased in Hivep3 knockout osteoblasts (10).

Reduced Alg2 gene expression following knockdown of Hivep3—We next screened for genes whose expression was commonly reduced in both ST-2 cells and MC3T3-E1 cells upon Hivep3 silencing by microarray analysis. The genes with decreased expression in MC3T3-E1 or ST-2 cells by >1.5-fold are listed in supplemental table 5 (38 genes) or supplemental table 4 (74 genes), respectively. Among these genes, only 5 were commonly downregulated by siHivep3 in the 2 cell lines (Fig. 3A). For more stringent screening, we further increased the cutoff threshold to >1.8-fold-decrease, which left 2 genes: Lypla2 and Alg2 in MC3T3-E1 cells and Alg2 and Igbp5 in ST-2 cells (Fig. 3A). Therefore, Alg2 most commonly showed decrease in expression due to
Hivep3 knockdown in both MC3T3-E1 and ST-2 cells. Asparagine-linked glycosylation (ALG) is one of the most common protein modification reactions in eukaryotic cells, as many proteins that are translocated across or integrated into the rough ER carry N-linked oligosaccharides (24). Alg2 is an α-1,3 mannosyltransferase forming a type-I transmembrane protein on the ER, with its active site being cytosolically oriented (25). To date, no information has been reported to link Alg2 to cell differentiation. We confirmed the microarray results by qRT-PCR (Fig. 3B) or immunoblotting (Fig. 3C) and verified that knockdown of Hivep3 in ST-2 cells decreased the level of Alg2 by over 50%. However, forced expression of HIVEP3 did not increase Alg2 expression (Fig. 3D). We next examined the tissue distribution of Alg2 in 3-month-old mice by qPCR analysis of a tissue cDNA panel (Fig. 3E). In tissues with low expression of Hivep3 (Fig. 1C), i.e., the heart or skin, Alg2 also showed a minimum level of expression, while both genes were highly expressed in the brain and lungs, suggesting a linkage between the levels of the 2 genes. However, there were some exceptions, e.g., Hivep3 was expressed at high levels in fat, cartilage, and bone, whereas Alg2 was detected at a moderate level in these tissues. From the osteoblastic and/or chondrocytic cell lines, MC3T3-E1 showed a significantly high level of Hivep3 expression (Fig. 1D), whereas Alg2 was detected in a relatively ubiquitous pattern (Fig. 3G). These results suggest that Hivep3 is essential but not sufficient for the expression of Alg2.

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 approximately 80% decrease in mRNA expression (Fig. 4A) and in protein level (Fig. 4B). Although silencing of Hivep3 increased the level of Runx2 protein, siAlg2 had no effect (Fig. 4C). As expected, loss of Alg2 also did not change the RNA level of Runx2 (Fig. 4D). However, Alg2 knockdown mildly enhanced Sp7 expression (Fig. 4, E and I), while it dramatically increased the expression (Fig. 4F) and activity (Fig. 4G) of ALP. A similar effect was seen on the level of Ibsp mRNA (Fig. 4H) and protein (Fig. 4I), 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. 5A), whereas it strongly suppressed the expression of Sp7, Alpl,
and Ibsp (Fig. 5B). 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. 5E). To assess the role of Alg2 in osteoblastic bone formation, we employed 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, that V5-tagged transgene product was detected by anti-V5 antibody (Fig. 5F). The rate of osteoblastic intramembranous bone formation can be examined by measuring the width of fontanelle (20). Application of BMP-2 promoted the bone formation that it significantly decreased the frontanelle width whereas combined induction of Alg2-expressing lentivirus cancelled the narrowing (Fig. 5G), 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. 6A). Atf4, a downstream target of PKR-like endoplasmic reticulum kinase (PERK) 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. 6A). 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. 6A). DNA-damage inducible transcript 3 (Ddit3), a target gene of Atf4 also known as C/EBP homologous protein (CHOP), was indeed mildly upregulated by siAlg2, but only in BMP-2-treated cells (Fig. 6A). 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. 6A). 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. 6B).

**Alg2 interferes with the transcriptional activity and nuclear localization of Runx2**—To test whether Alg2 interferes with Runx2 activity, we analyzed a Runx2-binding 6xOSE2 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 6xOSE2 reporter with Runx2-binding site mutation, suggesting that the inhibitory effect was Runx2-dependent (Fig. 6C). A similar result was obtained in ST-2 cells where Alg2 reduced Runx2 activity with an efficiency comparable to that of Hivep3 (Fig. 6D). 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. 6E). 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. 6E). 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 (36). In a co-transfection experiment using COS-7 cells, Alg2 was immunoprecipitated with Runx2 (Fig. 6F). By an immunofluorescence assay in ST-2 cells, we found that Alg2 protein overexpressed alone localized to the ER (Fig. 6G, d), while Runx2 overexpressed alone was stained in the nuclei (Fig. 6G, 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. 6G, 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. 7A) and in protein level (Fig. 7B). Conversely, HIVEP3 overexpression significantly enhanced expression of Col2a1 (Fig. 7C). 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. 7D). 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 homologue on chromosome 7 (Bhf2h7), 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. 7E), while gain of HIVEP3 enhanced the level in the presence of BMP-2 (Fig. 7F). Ddit3 showed similar expression patterns as Atf4, where its basal level was elevated by siHivep3 (Fig. 7G) while overexpression of HIVEP3 suppressed basal expression but promoted expression in the presence of BMP-2 (Fig. 7H). 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. 7O), which may be responsible for the inhibition of differentiation.

Alg2 is decreased by Hivep3 silencing in ATDC5 chondrocytes, while 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. 8A). Moreover, Alg2 siRNA inhibited the BMP-2-induced expression of type II collagen in mRNA (Fig. 8B) and in protein level (Fig. 8C). We checked if loss of Alg2 evokes ER stress, that basal expression of Atf4 (Fig. 8D) and Hspa5 (Fig. 8F) decreased, whereas Ddit3 expression increased in the presence of BMP-2 (Fig. 8E), following siAlg2 transfection. Importantly, Creb3l2 was downregulated by Alg2 silencing (Fig. 8G), and silencing of Alg2 or
Hivep3 both diminished the band of Creb3l2 in immunoblotting (Fig. 8H). 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 matured hypertrophic chondrocytes (Fig. 8J, lanes 1 and 3). The infection efficiency of adenovirus was evaluated by immunofluorescence, that V5-tagged transgene product was detected by anti-V5 antibody in the cartilage sample (Fig. 8I). Importantly, Alg2-expressing adenovirus mildly, but significantly increased the zone of matured chondrocytes regardless of BMP-2 treatment (Fig. 8J), 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 3 Schnurri homologs in vertebrates, Hivep1, Hivep2, and Hivep3, is also similar to that of the fly Schnurri, while sharing additional features, including unusually large size (~2,500 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 downregulated 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-knockout 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.

Mutations in 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 myelination (25), with no remarkable bone phenotype. However, because the reported patient was 3 years old while Hivep3 knockout 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 is 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, 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. On the other hand, 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 osteoblast differentiation. In contrast, Hivep3 increased not only the level of Creb3l2, but also of other canonical ER stress-related genes in ATDC5 chondrocytes, while 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. reported results similar to ours where gain of Hivep2 expression enhanced osteoblast differentiation, whereas Hivep2 knockout osteoblasts failed to
support efficient osteoclastogenesis *in vitro* (16). 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-knockout mice. In contrast, we showed that each of the 3 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 3 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 knockout 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.
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FIGURE LEGENDS

TABLE1. Genes downregulated by loss of the Hivep family genes. ST-2 cells were transfected with siRNA for Hivep1, Hivep2, or Hivep3 and treated with BMP-2 (300 ng/ml) for 2 d. mRNA samples were subjected to microarray analysis. A, The expression of 5 genes was commonly decreased by all the siRNAs for the 3 Hivep genes. B, The expression of 6 genes was commonly downregulated by the Hivep1 and Hivep2 siRNAs. C, The expression of 4 genes was commonly downregulated by the Hivep1 and Hivep3 siRNAs. D, The expression of 3 genes was commonly downregulated by the Hivep2 and Hivep3 siRNAs.

FIGURE 1. All the 3 Hivep genes are expressed in bone and cartilage to support osteochondrogenesis except Hivep3, which is inhibitory for osteoblast differentiation. A-C, A tissue cDNA panel of a 3-month-old mouse was subjected to quantitative RT-PCR (qRT-PCR) for Hivep1 (A), Hivep2 (B), or Hivep3 (C). D, The expression level of Hivep1, Hivep2, or Hivep3 in the indicated cell lines was examined by qRT-PCR. E, ST-2 cells were transfected with siRNA for Hivep1, Hivep2, or Hivep3 and treated with BMP-2 (300 ng/ml) for 6 d. Expression of Alpl was analyzed by qRT-PCR, while activity of ALP on ECM
was visualized by ALP staining. F, ATDC5 cells were transfected with siRNA for Hivep1, Hivep2, and/or Hivep3 and treated with BMP-2 (300 ng/ml) for 3 d. Expression of Col11a2 was evaluated by qRT-PCR. G, C3H10T1/2 cells were transfected with siRNA for Hivep2 and/or Hivep3 and treated with BMP-2 (300 ng/ml) for 4 d. Expression of Col2a1 was analyzed by qRT-PCR.

FIGURE 2. Silencing of Hivep3 increases protein stability of Runx2 to promote BMP-2-induced osteoblast differentiation. A, MC3T3-E1 osteoblasts were transfected with siRNA for Hivep3 with or without BMP-2 treatment (300 ng/ml) for 6 d. qRT-PCR analysis was performed for the indicated genes. B, siRNA-transfected ST-2 cells were treated with 100 µg/ml of cycloheximide (CHX) with or without BMP-2 treatment (300 ng/ml) for indicated time points. Cell lysates were analyzed by immunoblotting with an anti-Runx2 antibody. Tubulin served as a loading control. C, ST-2 cells were transfected with siRNA for Hivep3 with or without BMP-2 treatment (300 ng/ml) for 3 d. The expression level of the indicated genes was examined by qRT-PCR. D, ST-2 cells were transfected with siRNA for Hivep3 with BMP-2 treatment (300 ng/ml) for 7 d. Cell lysates were analyzed by immunoblotting with indicated antibodies. Tubulin served as a loading control. E, ST-2 cells were transfected with Hivep3 siRNA and stimulated with BMP-2 (300 ng/ml) for 6 d. ALP staining was performed. F, ST-2 cells were transfected with a human HIVEP3 expression vector, and further stimulated with BMP-2 (300 ng/ml) for 4 d. The expression of indicated genes was evaluated by qRT-PCR. G, ST-2 cells transfected with a human HIVEP3 expression vector were stimulated with BMP-2 (300 ng/ml) for 5 d. Cell lysates were analyzed by immunoblotting with indicated antibodies. Tubulin served as a loading control. *P < 0.05; **P < 0.01.

FIGURE 3. Expression of the Alg2 gene is reduced upon knockdown of Hivep3 in osteoblastic cells. A, siRNA for Hivep3 was transfected into MC3T3-E1 or ST-2 cells prior to treatment with BMP-2 (300 ng/ml) for 2 d and analysis by microarray. A list of 5 genes with decreased signal intensity, in common between MC3T3-E1 and ST-2 cells, is presented. B, ST-2 cells were transfected with siRNA for Hivep3 with or without BMP-2 treatment (300 ng/ml) for 3 d. The expression level of Alg2 was examined by qRT-PCR. C, ST-2 cells were transfected with siRNA for Hivep3 with BMP-2 treatment (300 ng/ml) for 7 d. Cell lysates were analyzed by immunoblotting with an anti-Alg2 antibody. Tubulin served as a loading control. D, ST-2 cells were transfected with a human HIVEP3 expression vector to be stimulated with BMP-2 (300 ng/ml) for 4 d. The expression of Alg2 was evaluated by qRT-PCR. E, A tissue cDNA panel of
a 3-month-old mouse was subjected to real-time PCR for Alg2. *G* Expression level of Alg2 in the indicated cell lines was examined by a qRT-PCR assay. **P < 0.01.

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 d. 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 d. 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 d. 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 d. 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 d. 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 d. Cell lysates were analyzed by immunoblotting with indicated antibodies. **P < 0.01.

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 d 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 indicated siRNA and stimulated with BMP-2 (300 ng/ml) for 4 d before analyzed 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 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 d. 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.
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 (300ng/ml) for 3 d. Expression of indicated genes was analyzed by qRT-PCR. C and D, A luciferase reporter, 6xOSE2 luc or a Runx2-binding sequence mutant 6xOSE2 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 (300ng/ml) for 3 d. 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.

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 d. 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 d. 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 d. 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 d and stained with alcian blue dye. *P < 0.05; **P < 0.01.

FIGURE 8. Hivep3 is essential for Alg2 expression in ATDC5 chondrocytes, while Alg2 promotes chondrogenesis. A, ATDC5 cells were transfected with siRNA for Hivep3 and treated with BMP-2 (300 ng/ml) for 4 d. 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 d. 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 d. 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 d. 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.
| Unique Sorted Transcript ClusterID | siCont | siHivep1 | siHivep2 | siHivep3 | gene symbol | gene description |
|----------------------------------|--------|----------|----------|----------|-------------|-----------------|
| 173055520                        | 21.529846 | 11.831301 | 9.75173 | 7.9562707 | Ear1        | eosinophil-associated, ribonuclease A family, member 1 |
| 17467150                         | 24.856544 | 14.449716 | 11.51435 | 15.580148 | Vmn1r18     | vomeronasal 1 receptor 18 |
| 17444697                         | 31.70125 | 19.534565 | 21.051311 | 17.505947 | Cyp3a59     | cytochrome P450, subfamily 3A, polypeptide 59 |
| 17245120                         | 44.998398 | 29.873207 | 26.863188 | 29.873207 | 1700030020Rk | RIKEN cDNA 1700030020 gene |
| 17481207                         | 17.905426 | 10.739602 | 10.739602 | 10.603919 | Olf608      | olfactory receptor 608 |
| 17278822                         | 65.41499 | 39.391872 | 41.367386 |         | Mir679      | microRNA 679 |
| 17302598                         | 42.895184 | 26.84824  | 22.885633 |         | Gm6280      | predicted gene 6280 |
| 17344852                         | 19.322012 | 12.227982 | 11.5472975 |         | Olfr97      | olfactory receptor 97 |
| 17509018                         | 24.977375 | 16.012548 | 11.412771 |         | Adam34      | a disintegrin and metalloproteinase domain 34 |
| 17495404                         | 82.13324 | 52.764555 | 42.29805 |         | Rps13       | ribosomal protein S13 |
| 17541719                         | 21.694231 | 14.250495 | 14.178923 |         | Mir450-2    | microRNA 450-2 |
| 17356739                         | 63.32018 | 36.357323 | 32.77719 |         | Mir194-2    | microRNA 194-2 |
| 17268088                         | 22.22885 | 13.294091 | 13.626566 |         | Gm11543     | predicted gene 11543 |
| 17324996                         | 19.940779 | 12.472511 | 12.472511 |         | Mir1947     | microRNA 1947 |
| 17516159                         | 30.844234 | 20.207043 | 19.56977 |         | Olf251/Olf900 | olfactory receptor 251 / olfactory receptor 900 |
| 17395379                         | 248.2335 | 129.81422 | 142.53452 |         | LOC100504873 | zinc finger protein 14-like |
| 17320035                         | 82.69005 | 51.436905 | 49.273067 |         | Mir1249     | microRNA 1249 |
| 17366886                         | 162.14816 | 104.84351 | 90.903786 |         | Mir467e     | microRNA 467e |
Imamura et al. Fig.2

**A**

| MC3T3-E1 | Hivep3 | Runx2 | lbsp | Bglap2 | Dmp1 |
|----------|--------|-------|------|--------|------|
|          | siCont | siHivep3 | siCont | siHivep3 | siCont | siHivep3 |
| mock     | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) |
| BMP-2    | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) |

**B**

| ST-2 | mock | BMP-2 |
|------|------|-------|
|      | siCont | siHivep3 | siCont | siHivep3 |
| CHX 0 | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) |
| 4    | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) |
| 8    | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) |

Runx2 and tubulin

**C**

| ST-2 | Hivep3 | Runx2 | Sp7 | lbsp | Atf4 |
|------|--------|-------|-----|------|------|
|      | siCont | siHivep3 | siCont | siHivep3 | siCont | siHivep3 |
| mock     | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) |
| BMP-2    | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) | ![](mock) | ![](BMP-2) |

**D**

| siCont | siHivep3 |
|--------|----------|
| mock BMP-2 | ![](Sp7) | ![](lbsp) | ![](BMP-2) |
| tubulin | ![](ALP staining) |

**E**

| siCont | siHivep3 |
|--------|----------|
| mock BMP-2 | ![](Sp7) | ![](lbsp) | ![](BMP-2) |
| ALP staining | ![](tubulin) |

**F**

| m/hHivep3 | Runx2 | Sp7 | Alpl | lbsp | Atf4 |
|-----------|-------|-----|------|------|------|
| vector | ![](miRNA) | ![](vector) | ![](miRNA) | ![](vector) | ![](miRNA) |
| HIVEP3 | ![](miRNA) | ![](vector) | ![](miRNA) | ![](vector) | ![](miRNA) |

| mock BMP-2 | ![](Runx2) | ![](Sp7) | ![](Alpl) | ![](lbsp) | ![](Atf4) |
| tubulin | ![](tubulin) |

**G**

| vector | HIVEP3 |
|--------|--------|
| mock BMP-2 | ![](Runx2) | ![](lbsp) | ![](tubulin) | ![](tubulin) | ![](tubulin) |

| HIVEP3 | vector |
|--------|--------|
| mock BMP-2 | ![](Runx2) | ![](lbsp) | ![](tubulin) | ![](tubulin) | ![](tubulin) |
|gene symbol| gene description                      | siCont  | siHivep3 | fold decrease |
|------------|--------------------------------------|---------|----------|--------------|
|Lypla2      | lysophospholipase 2                  | 855.5786| 385.5512 | 2.219106146  |
|**Alg2**    | asparagine-linked glycosylation 2     | 1875.0286| 875.0924 | 2.142663563  |
|Igfbp5      | insulin-like growth factor binding protein 5 | 3540.0374| 2207.5708| 1.60358952  |
|Ankfy1      | ankyrin repeat and FYVE domain containing 1 | 1102.1857| 719.4798 | 1.531920285  |
|Gpr133      | G protein-coupled receptor 133       | 545.9816| 362.3016 | 1.50698317  |

|gene symbol| gene description                      | siCont  | siHivep3 | fold decrease |
|------------|--------------------------------------|---------|----------|--------------|
|**Alg2**    | asparagine-linked glycosylation 2     | 568.0273| 314.0774 | 1.808558464  |
|Igfbp5      | insulin-like growth factor binding protein 5 | 179.3131| 99.2304 | 1.807038429  |
|Lypla2      | lysophospholipase 2                  | 586.0297| 349.8216 | 1.675224247  |
|Ankfy1      | ankyrin repeat and FYVE domain containing 1 | 1006.6627| 650.5681 | 1.547359454  |
|Gpr133      | G protein-coupled receptor 133       | 93.8123 | 61.8311  | 1.517233593  |

**B**

![Bar graph showing relative expression of Alg2 between mock BMP-2 and BMP-2 conditions.](image)

**C**

![Western blot showing Alg2 and tubulin expression.](image)

**D**

![Bar graph showing relative expression of Alg2 in vector and HIVEP3 conditions.](image)

**E**

![Bar graph showing relative expression of Alg2 across various tissues.](image)

**F**

![Bar graph showing relative expression of Alg2 across different cell lines.](image)
Imamura et al. Fig. 5

(A) Relative expression of Runx2, V5(Alg2), and tubulin in mock BMP-2 or Alg2-V5 transduced cells. 

(B) Relative expression of Sp7, Alpl, and lbsp in mock BMP-2 or Alg2-Ax transduced cells. 

(C) Relative expression of Alg2 and V5 in mock BMP-2 or Alg2-V5 transduced cells. 

(D) Relative expression of Alg2 in mock BMP-2 or Alg2-V5 transduced cells. 

(E) Relative expression of lbsp in mock BMP-2 or Alg2-V5 transduced cells. 

(F) Relative expression of Alg2-V5 and tubulin in mock BMP-2 or Alg2-V5 transduced cells. 

(G) Fontanelle width in mock BMP-2 or BMP-2 transduced cells. 

* p < 0.05, ** p < 0.01, n.s. = not significant.
**Fig. 7**

A. **Col2a1**

B. **siCont**  **siHivep3**

C. **Col2a1**

D. **vector**  **Hivep3**

E. **Atf4**

F. **Atf4**

G. **Ddit3**

H. **Ddit3**

I. **Hspa5**

J. **Hspa5**

K. **Xbp1 (spliced)**

L. **Xbp1 (spliced)**

M. **Creb3l2**

N. **Creb3l2**

O. **Sec23a**

**clf2a1**

**siCont**  **siHivep3**

**type II collagen**  **tubulin**

**alcian blue staining**

**relative expression**

**mock**  **BMP-2**

**vector**  **HIVEP3**

**relative expression**

**mock**  **BMP-2**

**relative expression**

**mock**  **BMP-2**

**relative expression**

**mock**  **BMP-2**

**relative expression**

**mock**  **BMP-2**

**relative expression**

**mock**  **BMP-2**

**relative expression**

**mock**  **BMP-2**

**relative expression**

**mock**  **BMP-2**

**relative expression**
Human Immunodeficiency Virus Type I Enhancer Binding Protein 3 is Essential for the Expression of Asparagine-linked Glycosylation 2 in the Regulation of Osteoblast and Chondrocyte Differentiation

Katsuyuki Imamura, Shingo Maeda, Ichiro Kawamura, Kanehiro Matsuyama, Naohiro Shinohara, Yuhei Yahiro, Satoshi Nagano, Takao Setoguchi, Masahiro Yokouchi, Yasuhiro Ishidou and Setsuro Komiya

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