Ameloblastin regulates osteogenic differentiation by inhibiting Src kinase via crosstalk between integrin β1 and CD63

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ABSTRACT

Ameloblastin, the most abundant non-amelogenin enamel matrix protein, plays a role in ameloblast differentiation. Here we found that ameloblastin was expressed in osteosarcoma cells; to explore the potential functions of ameloblastin in osteoblasts, we investigated whether this protein is involved in osteogenic differentiation and bone formation on the premise that CD63, a member of the transmembrane-4 glycoprotein superfamily, interacts with integrins in the presence of ameloblastin. Ameloblastin bound to CD63 and promoted CD63 binding to integrin β1. The interaction between CD63 and integrin β1 induced Src kinase inactivation via the binding of CD63 to Src. The reduction of Src activity and osteogenic differentiation mediated by ameloblastin was abrogated by treatment with anti-CD63 antibody and overexpression of constitutive active Src, respectively. Moreover, amelobastin upregulated the formation of stress-fibre and focal adhesions and downregulated cell migration in association with RhoA regulation via Src activity. Therefore, our results suggest that ameloblastin is expressed in osteoblasts and functions as a promoting factor for osteogenic differentiation via a novel pathway through the interaction between CD63 and integrin β1.

INTRODUCTION

Ameloblastin (AMBN), also known as sheathlin or amelin, is the most abundant non-amelogenin enamel matrix protein (6, 9, 19) and a member of the secretory calcium-binding phosphoprotein (SCPP) gene cluster of evolutionary-related molecules that regulate skeletal mineralization (18). Further, AMBN induces cell attachment, proliferation and differentiation of periodontal ligament cells *in vitro* (46). In AMBN-null mice, ameloblasts are detached from the matrix, lose cell polarity and resume proliferation. However, protein expression was not completely inactivated and trancated RNA missing a portion of exon 5 and 6 is still translated in AMBN KO mice (42). Therefore, it is conceivable that exon 5 and 6 of AMBN plays a role in ameloblast differentiation (11, 42). In this mouse model, structural change was shown in the alveolar bone (42): the alveolar bone exhibited more porosity in truncated AMBN-expressing mice than in wild-type mice. The changes in alveolar bone in $AMBN^{\Delta 5\text{-}6}$ mice cannot be directly related to the protein, as they could arise from other factors such as changes in occlusal forces in teeth without enamel (42). On the other hand, it has recently been reported that AMBN is expressed in osteoblasts during craniofacial development (35). Although AMBN may play a significant role not only in tooth development but also in bone formation, it is still unclear the role of AMBN in bone formation.

AMBN has been shown to interact with CD63 via a yeast two-hybrid assay (41). CD63 is a member of the transmembrane-4 glycoprotein superfamily, also known as the tetraspanin family (36, 44). Most of these proteins are cell-surface proteins that are characterized by the presence of four hydrophobic domains and two extracellular domains (36, 44). CD63 mediates signal transduction events in the regulation of cell survival, development, activation, growth and motility (17, 21, 44). In particular, cell-surface CD63 is known to complex with integrins (3) and is involved in the control of integrin outside-in signalling

activity (13, 17).

Integrins are heterodimeric adhesion receptors for extracellular matrix proteins consisting of α- and β-subunits. Osteoblast differentiation is induced by the aggregation of cell-surface α2β1-integrin with matrix type I collagen (16, 38, 43), an event triggered by the stimulation of integrins through the association of their cytoplasmic domain with focal adhesion kinase, Src and other cytosolic non-receptor tyrosine kinases. Src is a non-receptor tyrosine kinase found in a wide variety of tissues (31) and has two important phosphorylation sites in Tyr at 416 and 527 (32). Src phosphorylated at Tyr527 interacts with the Src SH2 domain at the intramolecular level and exhibits a kinase constitutively negative. On the other hand, Tyr416 of Src is present within a kinase domain and phosphorylation of Tyr416 augments kinase activity. Src-deficient mice have an osteopetrotic phenotype (34) and the reduction of Src activity stimulates osteoblast differentiation and bone formation (22). In this study, to explore further the potential functions of AMBN in osteoblasts, we investigated whether this protein is involved in osteogenic differentiation and bone formation according to the hypothesis that CD63 interacts with integrins in the presence of AMBN.

MATERIAL AND METHODS

Reagents and antibodies. Monensin for inhibiting secretion was obtained from Sigma (St. Louis, MO, USA). Src-Y527F mutant vector was donated by Addgene, Inc. (Cambridge, MA, USA). For detecting endogenous AMBN by immunohistochemistry, an anti-AMBN polyclonal antibody (W59), which was generated in rabbits by immunization with synthetic peptides (EHETQQYEYS) corresponding to residues 93 to 102 of human AMBN with amino acid sequence homology to mice and rat, was used. Commercial antibodies were purchased from the following suppliers: anti-FLAG monoclonal antibody (M2) and anti-β-actin monoclonal antibody from Sigma; anti-Src polyclonal antibody and polyclonal antibody

specific to phospho-Tyr416 Src from Cell Signaling Technology (Danvers, MA, USA); anti-integrin β1 (M-106) polyclonal antibody and anti-CD63 (MX-49.129.5) monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CD63 blocking antibody (H-193) was purchased from Santa Cruz Biotechnology, and Integrin ß1 blocking antibody (MAB2253Z) was purchased from Chemicon (Temecula, CA, USA).

Cell culture of fetal rat calvaria cells. Tweny-one-day-old fetal Wistar rat calvaria cells were isolated by sequential collagenase digestion, which resulted in five cell fractions. Briefly, calvariae were dissected free from loosely adherent connective tissues, minced, and sequentially digested in collagenase (type I; Sigma-Aldrich Corp., St. Louis, MO) solution. Cells from the last four of five digestion fractions were separately grown in αMEM supplemented with 10% fetal bovine serum (Cansera, Etobicoke, Canada) and antibiotics. After 24 h, cells were trypsinized, pooled, and grown in 24-well plates or 35-mm dishes ($0.3x10^4$ cells/cm^2) in the same medium supplemented additionally with 50 μg/ml ascorbic acid. All cultures were maintained at 37 ºC in a humidified atmosphere with 5% CO_2, and medium was changed every second or third day.

Cell lines and tissue samples. Human osteosarcoma cell lines (NOS-1, SaOS-2, MG63 and HOS) were provided by Cell Bank, RIKEN BioResource Center (Ibaraki, Japan). They were maintained in RPMI-1640 or Dulbecco's modified Eagle's Medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA) and 100 U/ml penicillin-streptomycin (Invitrogen) under 5% CO_2 in air at 37°C. Tissue samples of osteosarcoma were retrieved from the Surgical Pathology Registry of Hiroshima University Hospital, after approval by the Ethical Committee of Hiroshima University Hospital. Six osteosarcoma tissue samples were used for the immunohistochemical analysis.

Immunohistochemical staining. Immunohistochemical detection of AMBN in the tissue samples was performed on 4.5 μm sections mounted on silicon-coated glass slides, by using a streptavidin-biotin peroxidase technique as described previously (40). For the immunohistochemical study, the anti-AMBN polyclonal antibody (W59) was used.

Immunofluorescence. NOS-1 and SaOS-2 cells as well as AMBN-overexpressing SaOS-2 cells were seeded on cover slips and grown to approximately 80% confluence. Before fixing, monensin was added to the NOS-1 cells at a final concentration of 5 μM for 4 h. The cells were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed thrice with ice-cold PBS and then permeabilized in 0.1% Triton X-100 in PBS for 15 min at room temperature. After rinsing three times with PBS, the cover slips were incubated with the primary antibodies in PBS. We used anti-AMBN polyclonal antibody (W59), anti-phospho-Tyr antibody (4G10; Millipore, Billerica, MA, USA) and Alexa Fluor 594 phalloidin (Molecular Probes, Eugene, OR, USA) as the primary antibodies and anti-rabbit Alexa 488 (Molecular Probes) as the secondary antibody. DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. Immunostaining of the cell preparations was recorded by using an epifluorescence Zeiss Axioplan 2 microscope (Zeiss, Inc., Thornwood, NY, USA) attached to a CCD camera.

Generation of AMBN-overexpressing SaOS-2 cells. Human AMBN cDNA was isolated from the cDNA of NOS-1 cells by RT-PCR using sense and antisense primers. The cDNA was then subcloned by insertion into the *Eco*RI/*Bam*HI restriction site of pcDNA3.1, and the vector was modified by insertion of a FLAG-tag sequence behind the signal peptide sequence using a mutagenesis kit (Stratagene, La Jolla, CA, USA). The AMBN-pcDNA3.1 plasmid or

the vector alone was introduced into SaOS-2 cells, and stable clones were obtained by G418 selection (500 μg/ml, Invitrogen) in the culture medium. Cell transfection was performed by using FuGENE 6HD (Roche, Basel, Switzerland) according to the manufacturer's instruction. Conditioned media from control and AMBN overexpressing cells was collected after 48 h of plating.

Silencing by siRNA. Logarithmically growing NOS-1 cells were seeded at a density of 10^5 cells/dish (6 cm) and transfected with oligos twice (at 24 and 48 h after replating) by using Oligofectamine (Invitrogen). Forty-eight hours and ninety-six hours after the last transfection, the cells were prepared and analysed by real-time RT-PCR and mineralization assay, respectively. The siRNA is a 19-bp duplex oligoribonucleotide with a sense strand corresponding to nucleotides 1205–1223 of the human AMBN mRNA sequence: 5'-ugaccacauccguggauuu-3'. A scrambled sequence without significant homology to rat, mouse or human gene sequences was used as a control.

RT-PCR and real-time RT-PCR analysis. Total RNA was isolated from cultures of confluent cells by using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The preparations were quantified, and their purity was determined by standard spectrophotometric methods. cDNA was synthesized from 1 μg total RNA according to the ReverTra Dash (Toyobo Biochemicals, Tokyo, Japan). The RT-PCR oligonucleotide primers for human, mouse or rat AMBN, CD63, runt-related transcription factor 2 (RUNX2), bone morphogenetic protein 2 (BMP2), alkaline phosphatase (ALP), type 1 collagen (COL I), bone sialoprotein (BSP), osteocalcin (OCN) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 1. Aliquots of total cDNA were amplified with 1.25 U rTaq-DNA polymerase (Qiagen) in a PC701 thermal cycler

(Astec, Fukuoka, Japan) for 25-30 cycles after denaturation for 30 s at 94°C, annealing for 30 s at 60°C and extension for 1 min at 72°C in the case of all the primers. The amplified products were resolved on 1.5% agarose/TAE gels (Nacalai Tesque, Inc., Kyoto, Japan), electrophoresed at 100 mV and visualized by ethidium-bromide staining. For real-time PCR, aliquots of total cDNA were amplified with Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Data acquisition and analysis were performed with a Step One Real Time PCR System using Step One Software V 2.1 (Applied Biosystems).

Generation of recombinant human AMBN. The full-length human AMBN cDNA-inserted FLAG-tag sequence was subcloned into pEU vector (CellFree Sciences, Yokohama, Japan), which was then transcribed into mRNA. The total mRNA product was translated by using WEPRO 3240 according to the manufacturer's instructions (CellFree Sciences).

Mineralization assay. Mineral nodule formation was detected by the Dahl method for calcium (5). Cells were placed in a 24-well or 6-well plate at a density of subconfluence per well and cultured in medium supplemented with 10% FBS, 50 μg/ml ascorbic acid and 5 mM sodium β-glycerophosphate at 37°C for a week. The cells were fixed in 70% ethanol for at least 1 h and then stained with alizarin red S (ALZ). After mineral deposits were stained and photographed, the staining was destained with 10% (w/v) cetylpyridinium chloride (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and the concentrations were determined by the absorbance measured at 562 nm on a multiplate reader.

Western blot and immunoprecipitation analyses. Western blotting was carried out as described previously (40). Thirty micrograms of protein was subjected to 10% polyacrylamide gel electrophoresis followed by electroblotting onto a nitrocellulose filter. For

detection of the immunocomplex, the ECL western blotting detection system (Amersham) was used. The immunoprecipitates were subjected to immunoblot analysis. For detecting CD63–integrin interaction, cell lysates were obtained by lysing the cell monolayer with 1% Brij 96 lysis buffer containing 1% Brij 96, 25 mM Hepes (pH 7.5), 150 mM NaCl and 5 mM $MgCl_2$.

RESULTS

AMBN expression in osteosarcoma samples and cell lines. To determine the expression of AMBN during proliferation-differentiation-maturation sequence of osteogenic cells *in vitro*, rat embryonic calvalia cell populations were obtained and examined for AMBN expression by RT-PCR analysis (Fig. 1A). Under growth conditions, AMBN mRNA was detected in exponentially growing cells, but its expression was clearly downregulated when cells reached confluence (7 day) (Fig. 1A). At early osteogenic differentiation to late maturation stages, the osteoblast markers ALP, a relatively early marker of osteoblast differentiation and OCN, later markers of osteoblast differentiation were upregulated (Fig. 1A). These findings suggest that AMBN may be involved in early stage of osteogenesis.

Osteosarcoma cell lines are often used for studying the mechanisms that control osteoblast-specific gene expression. To investigate the detailed role of AMBN in osteogenesis, we used osteosarcoma cells. We found that osteosarcoma cell line, NOS-1 cells expressed AMBN mRNA (Fig. 1B). NOS-1 cells also showed the expression of RUNX2, ALP, COL I, BSP and OCN mRNA (Fig. 1B). In NOS-1 cells, the expression of AMBN in the cytoplasm by immunofluorescence analysis in the presence of monensin for inhibiting the secretion (Fig. 1C). We also examined the immunohistochemical expression of AMBN in six paraffin-embedded osteosarcoma samples. We checked the specificity of AMBN antibody in ameloblasts of a 7-day-old mouse. The sequence specificity of AMBN antibody used in this

study was conserved between mice and humans. The AMBN antibody specifically recognized AMBN in ameloblasts (Fig. 1D). By using this antibody, we found the expression of AMBN in two of the samples (Fig. 1D). Therefore, we demonstrated that the osteosarcoma cells expressed AMBN.

Involvement of AMBN in osteogenic differentiation and mineralization. To explore the involvement of AMBN in osteoblastic activity, we examined osteogenic differentiation and mineralization in NOS-1 cells treated with AMBN siRNA. In the AMBN siRNA-treated cells, AMBN mRNA expression was reduced (Fig. 2A). Interestingly, remarkable downregulation of ALP, COL I and BSP mRNA was observed in AMBN-knockdown cells in comparison with the control cells (Fig. 2A). Moreover, when the control and AMBN-knockdown cells were cultured for 4 days in the presence of osteogenic media containing β-glycerophosphate and ascorbic acid after transfection with AMBN siRNA for 48 h, matrix mineralization was found to be decreased in the AMBN-knockdown cells (Fig. 2B).

To elucidate the detailed role of AMBN in mineralization, we examined ectopic overexpression of AMBN by transfection of the pcDNA3.1-AMBN vector with insertion of FLAG-tag sequences behind signal peptide sequences into SaOS-2 cells without AMBN expression (Fig. 3A). We obtained SaOS-2 cells with stable AMBN overexpression (Fig. 3B). In the conditioned media from AMBN-overexpressing SaOS-2 cells, several bands were detected by Western blotting using anti-FLAG antibody, indicating that AMBN might have been modified after secretion (Fig. 3C). As expected, the upregulation of RUNX2 and BSP mRNA expression (Fig. 3D) and promotion of matrix mineralization were observed in the AMBN-overexpressing SaOS-2 cells (Fig. 3E). We also confirmed that treatment with recombinant human FLAG-AMBN enhanced matrix mineralization in the SaOS-2 cells, as seen in AMBN overexpression (Fig. 3F and 3G).

Regulation of Src kinase activity via interaction between integrin and CD63. We next investigated the mechanism of AMBN for promoting osteogenic differentiation and mineralization. On the basis of our hypothesis that CD63 interacts with integrins in the presence of AMBN, we first examined the expression of CD63 and integrin β1 in the osteosarcoma cell lines. These cell lines expressed CD63, and especially, the SaOS-2 cells expressed CD63 strongly (Fig. 4A). The CD63 protein, being heavily glycosylated, exhibited diffuse distribution on SDS-PAGE, as previously reported (17, 36). To determine the interaction between CD63 and AMBN, we performed immunoprecipitation using anti-FLAG and anti-CD63 antibodies in FLAG-AMBN-overexpressing SaOS-2 cells. As shown in Fig. 4B, ectopic AMBN bound to CD63 in the AMBN-overexpressing cells. Then, to examine the intracellular signalling event in the AMBN-overexpressing cells, we examined the Src activity in control and AMBN-overexpressing cells by using phospho-Tyr416 Src antibody. Tyr416 of Src is present within a kinase domain and phosphorylation of Tyr416 augments the kinase activity. Interestingly, the level of phospho-Tyr416 Src was remarkably low in the AMBN-overexpressing cells compared with the control cells (Fig. 4C). To know the cell adhesion-dependent autophosphorylation of Src Tyr416 in AMBN-overexpressing cells, we examined the phosphorylation levels of Src at Tyr416 in control and AMBN-overexpressing cells at 0, 30 and 60 min after attachment on COL I-coated dishes. The phosphorylation levels of Src were found to be as low in these AMBN-overexpressing cells as in those under the normal condition (Fig. 4C). We also examined the expression of the phosphorylation levels of Src at Tyr416 in AMBN siRNA transfected NOS-1 cells. AMBN knockdown upregulated the phosphorylation level of Tyr416 of Src (Fig. 4D). To investigate the crosstalk among integrin β1, CD63 and Src, we performed immunoprecipitation with anti-CD63 antibody in control and AMBN-overexpressing cells after 48 h of attachment on COL I-coated dishes. Only in the

AMBN-overexpressing cells, CD63 bound to integrin β1 and Src (Fig. 4E). These findings suggested that the interaction between CD63 and integrin β1 inhibited Src activity in the presence of AMBN.

To support these established roles of AMBN in regulating osteogenesis via CD63, we examined the inhibition of the osteogenic function of CD63 using CD63 blocking antibody in control and AMBN-overexpressing cells. Previous report shows that AMBN interacts with amino acids 103–205 of the human CD63 protein where is hydrophobic and accessible to the external environment, demonstrated by yeast-two hybrid assay (47). CD63 blocking antibody used in this study is raised against amino acids 45-238 mapping at human CD63 protein. Indeed, we confirmed that CD63 blocking antibody interrupted the interaction of CD63 with AMBN (data not shown). On Western blot analysis using anti-phospho-Tyr416 Src antibody, reduced phosphorylation levels of Src in AMBN-overexpressing cells were observed without anti-CD63 treatment, but pretreatment with anti-CD63 antibody induced the upregulation of the phosphorylation levels (Fig. 5A). Interestingly, pretreatment with anti-CD63 antibody blocked the promotion of mineralization in SaOS-2 cells treated with the conditioned media from AMBN-overexpressing cells (Fig. 5B). To know the involvement of integrin ß1 in the inhibition of Src activity by AMBN overexpression, we treated the integrin ß1 blocking antibody in AMBN-overexpressing cells. Treatment with integrin ß1 blocking antibody upregulated phosphorylation levels of Src at Tyr416 in AMBN-overexpressing cells (Fig. 5C). Interestingly, treatment with integrin ß1 blocking antibody decreased the expression of RUNX2, ALP, COLI, BSP and OCN mRNA (Fig. 5D).

Moreover, we examined whether Src activation inhibits the AMBN-mediated mineralization by transfection of constitutive active mutant of Src (Y527F) in AMBN-overexpressing cells. Transfection of the constitutive active mutant of Src upregulated the phosphorylation level of Src at Tyr416 (Fig. 6A). Interestingly, matrix mineralization was

inhibited by Src activation in the AMBN-overexpressing cells (Fig. 6B). Further, RUNX2, ALP and BSP were downregulated by Src activation in these cells (Fig. 6C). These findings indicated that the AMBN-CD63 axis inhibited Src activation, which in turn promoted mineralization and osteogenic differentiation.

We examined the effects of AMBN and/or CD63 in normal mouse osteoblastic cell line, MC3T3-E1. CD63 was expressed in osteosarcoma cell lines but not in normal mouse osteoblastic cell lines ST2 and MC3T3-E1 (Fig. 7A). We transiently transfected AMBN and/or CD63 into MC3T3-E1 cells (Fig. 7B). Although mineral deposit was not remarkably promoted by ectopic AMBN and AMBN/CD63 (data not shown), ectopic expression of AMBN upregulated RUNX2, BSP and OCN, and ectopic expression of AMBN/CD63 upregulated BSP (Fig. 7C).

DISCUSSION

AMBN is an ameloblast-specific glycoprotein, and *AMBN* is localized to human chromosome 4q21 within the autosomal dominant amelogenesis imperfecta locus (24), suggesting that this protein is important for enamel formation. AMBN is present in the secretory stage of enamel formation, as demonstrated by immunostaining of ameloblastin (29). It has recently been revealed that AMBN is also expressed in osteoblasts during craniofacial skeletal development and in an osteosarcoma cell line, SaOS-2, cultivated on biosilica matrices (25, 35). However, the function of AMBN in osteoblasts is unknown. Here, we found that AMBN promoted mineralization through the inhibition of Src activation in osteosarcoma. This is the first report on the function of AMBN in osteogenesis. We found an osteosarcoma cell line with high expression of AMBN. Interestingly, knockdown of AMBN inhibited the formation of mineralization deposits in this cell line. In fact, AMBN was found to induce osteogenic differentiation and promote mineralization by ectopic AMBN overexpression and

recombinant human AMBN treatment. In particular, RUNX2, ALP and BSP mRNA expressions were upregulated by AMBN overexpression and downregulated by AMBN knockdown. RUNX2, ALP and BSP are osteogenic markers, and enhance osteogenic differentiation and matrix mineralization (1, 12). Therefore, we suggest that increased expression of RUNX2, ALP and BSP by AMBN overexpression may promote osteogenic differentiation. AMBN, a 65-70 kDa protein, is rapidly processed into several lower-molecular-weight proteins ranging from 52 kDa to 13 kDa (29). AMBN is cleaved by MMP-20 (enamelysin), which is an ameloblast-specific gene (15). The cells of bone lineage don't express any traces of MMP-20 (data not shown). Defects of MMP-20 function cause autosomal-recessive amelogenesis imperfecta (27). In this study, we found that secreted AMBN protein was cleaved in the conditioned media from AMBN-overexpressing osteoblastic cells (Fig. 3C). It is likely that AMBN is cleaved by proteases other than MMP-20 in osteosarcoma cells. Moreover, osteogenic differentiation mediated by AMBN may be promoted by cleaved functional AMBN fragments in osteosarcoma.

Although AMBN has been shown to interact with CD63 (41), the physiological role of this interaction is still unclear. As we found an interaction between AMBN and CD63 in osteosarcoma cells, the function of this interaction in osteogenesis was examined. In osteosarcoma cells with AMBN expression, CD63 blocking antibody suppressed mineralization. Previous studies have shown that cell-surface CD63 forms a complex with integrins (3) and that CD63 binds with Src and increases the phosphorylation of Src at Tyr416 (20). In this study, we found that CD63 bound to Src and integrin β1 in the presence of AMBN. Interestingly, AMBN stimulation inhibited Src activation, and AMBN-promoted osteogenic differentiation and mineralization was disrupted by constitutive active Src overexpression. Indeed, AMBN knockdown suppressed osteogenic differentiation through Src activation (Fig. 2A, 2B and 4D). Moreover, the treatment with CD63 blocking antibody or

integrin ß1 blocking antibody induced Src activation (Fig. 5A-D). These findings suggest that the AMBN-CD63-integrin axis may promote osteogenesis through Src inactivation (Fig. 8). Previous reports have demonstrated that Src-deficient mice develop osteopetrosis by accelerated osteogenic differentiation (22, 34) and that osteoblast differentiation mediated by the reduction of Src kinase activity is regulated by osteocalcin expression via the interaction between RUNX2 and Yes-associated protein (45). Thus, our findings provide evidence for the novel mechanism of AMBN-mediated osteogenic differentiation. Therefore, AMBN can be a therapeutic modality for bone regeneration. Osteosarcoma cells present osteoblastic differentiation and form tumoural bone in similar to osteoblasts. In the present study, CD63 was expressed in osteosarcoma cell lines but not in normal mouse osteoblastic cell lines ST2 and MC3T3-E1 (Fig. 7A). CD63 is reportedly expressed in human mesenchymal stem cells undergoing osteogenic differentiation (10). As neoplastic cells have a potential to undergo divergent differentiation, CD63 expression may be observed in osteosarcoma cells. The effects of AMBN on osteoblasts may be regulated by spatiotemporal interactions between AMBN and CD63 during bone development and bone healing process. In fact, ectopic expression of AMBN upregulated RUNX2, BSP and OCN, and ectopic expression of AMBN/CD63 upregulated BSP in normal mouse osteoblastic cell line, MC3T3-E1 (Fig. 7C). However, mineral deposit was not remarkably promoted by ectopic AMBN and AMBN/CD63 in MC3T3-E1 cells (data not shown). These findings suggest that additional factor may be required for AMBN-promoted osteogenic differentiation in normal osteoblasts.

In conclusion, our study has revealed an important role of AMBN in osteogenic differentiation via the AMBN-CD63-integrin-Src axis. Our findings may help to clarify the pathway involved in osteogenesis.

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Figure legends

FIG.1. AMBN expression in ostesarcoma cells. (A) Outline of differentiation and treatment protocols during typical time windows of rat calvaria cell cultures (left panel). Cells ($0.3x10^4$ cells/cm^2) reach confluence at approximately 7 day and subsequently initiate nodule formation. Cells are at exponential proliferation/primitive progenitor stages before confluence and at early osteogenic differentiation to late maturation stages after confluence to culture termination. Rat calvaria cells were collected at 3, 7, 10 and 14 day. Expression of AMBN, CD63, ALP and OCN mRNA was examined by RT-PCR (right panel). GAPDH expression was used as a control. (B) mRNA expression of AMBN and mineralization-related molecules RUNX2, BMP-2, ALP, COL I, BSP and OCN in osteosarcoma cell lines NOS-1, SaOS-2, MG63 and HOS by RT-PCR. GAPDH mRNA expression was used as the control. (C) Intracellular AMBN expression (green) after monensin treatment and DAPI staining for nuclei (blue) in NOS-1 cells. The merged image is also shown. (D) Immunohistochemical expression of AMBN in osteosarcoma tissues. To check the specificity of an anti-AMBN antibody, immunohistochemical expression of AMBN was examined in ameloblasts of developing teeth of a 7-day-old mouse (left panel). Arrow and arrowhead indicates ameloblastic layer of lower incisor and lower first molar, respectively. Asterisk is enamel matrix. Immunohistochemical expression of AMBN in the osteosarcoma tissues (case No.1 and No.2) (right panel).

FIG. 2. Suppression of osteogenic differentiation and mineralization by AMBN knockdown. (A) mRNA expression levels of AMBN and mineralization-related molecules in control (scramble-siRNA) and AMBN siRNA-transfected NOS-1 cells were examined by real-time RT-PCR. The data are shown as the ratio of each mRNA to GAPDH mRNA and the values are expressed as the mean ± S.D. (N = 3); $*P < 0.05$ (t-test). (B) Control and AMBN siRNA-transfected NOS-1 cells were cultured for 4 days in the absence or presence of 50

μg/ml ascorbic acid (AA) and 5 mM sodium β-glycerophosphate (βGP). ALZ staining was performed to visualize mineral deposition.

FIG. 3. AMBN enhanced osteogenic differentiation and mineralization. (A) The structure of the pcDNA3.1-AMBN vector, which was modified by insertion of a sequence encoding FLAG-tag behind the signal peptide sequence. (B) SaOS-2 cells were stably transfected with the vector for FLAG-AMBN. The transient and stable expression of AMBN in SaOS-2 cells was examined by Western blot analysis using anti-FLAG antibody. The asterisk indicates non-specific bands. (C) Ectopic expression of FLAG-AMBN in the conditioned media (CM) from stably transfected cells was examined by Western blot analysis using anti-FLAG tag antibody. Arrowhead indicates the fragment of AMBN in conditioned media. (D) The mRNA expression levels of mineralization-related molecules in control (pcDNA3.1) and AMBN-overexpressing cells were assessed by real-time RT-PCR. The data are shown as the ratio of each mRNA to GAPDH mRNA and the values are expressed as the mean ± S.D. (N = 3); $*P < 0.05$ (t-test). (E) Control and AMBN-overexpressing cells were cultured for 10 and 15 day in the presence of 50 μg/ml ascorbic acid and 5 mM sodium β-glycerophosphate. After mineral deposits were stained and photographed, the staining was destained by using 10% (w/v) cetylpyridinium chloride and the concentrations were determined by the absorbance measured at 562 nm on a multiplate reader. The picture shows mineral deposits at 15 day. The absorbance of the control cells at 10 days was defined as 1. The graph shows the relative ALZ levels. (F) Recombinant AMBN promotes mineralization in osteosarcoma cells.AMBN full-length protein including FLAG-tag (rhAMBN) were produced by Wheat Germ Cell-Free System and were examined by Western blot using anti-FLAG tag antibody. We also used control recombinant protein (rCtrl). (G) SaOS-2 cells cultured for 10 days in the presence of 50 mg/ml ascorbic acid and 5 mM sodium b-glycerophosphate and also each cells cultured on

normal dish, control recombinant protein coated dish (rCtrl) and 10 μg/ml of recombinant AMBN coated dish (rAMBN). After mineral deposits were stained and photographed, the staining was destained by 10% (w/v) cetylpyridinium chloride and the concentration were determined by absorbance measurement at 562 nm on a multiplate reader. The absorbance of control cells at 10 days is defined as 1. Graph shows relative ALZ levels.

FIG. 4. Src phosphorylation via the AMBN-CD63-integrin β1 axis. (A) Expression of CD63 (long and short exposure) and integrin β1 in osteosarcoma cell lines was detected by Western blot analysis. β-actin was used as a loading control. (B) Interaction between AMBN and CD63 in FLAG-AMBN-overexpressing SaOS-2 cells was examined by immunoprecipitation with anti-FLAG or anti-CD63 antibody. The arrowheads show immunoprecipitated FLAG-AMBN or CD63. (C) Phosphorylation status of Src at Tyr416 in control and FLAG-AMBN-overexpressing cells under the condition of adhesion and suspension. For suspension, the control and AMBN-overexpressing cells were detached after trypsinization and kept in suspension at 37°C for 2 h. Then, they were collected after plating on COL I-coated dishes for 0, 30 and 60 min. The cell lysates were immunoblotted with phospho-Tyr416 Src and Src antibody. β-actin expression was used as a loading control. (D) We transfected AMBN siRNA into NOS-1 cells. The cell lysates were immunoblotted with phospho-Tyr416 Src, Src and integrinß1 antibody. β-actin expression was used as a loading control. (E) Lysates of the control and AMBN-overexpressing cells were immunoprecipitated with anti-CD63 antibody and immunoblotted with anti-integrin β1 or anti-Src antibody. The arrowheads indicate immunoprecipitated integrin β1 or Src. The expressions of integrin β1 and Src were examined in whole cell lysates by Western blot analysis to show equal amounts of protein.

FIG. 5. AMBN induced osteoblast mineralization via CD63 and Src interaction. (A) After suspension culture with or without CD63 blocking antibody, lysates of control and AMBN-overexpressing cells plated on COL-1-coated dish for 60min were immunoblotted with anti-phospho-Tyr416. The asterisk indicates IgG of CD63 antibody and the arrowhead indicates Src phosphorylation at Tyr416. β-actin was used as a loading control. (B) SaOS-2 cells were cultured for 10 days after pre-suspension at 37 ℃ for 2h in the presence of 50 μg/ml ascorbic acid and 5 mM sodium ß-glycerophosphate with or without CD63 blocking antibody and conditioned medium (CM) from control or AMBN-overexpressing cells. ALZ staining was performed to visualize mineral deposition. ALZ was destained by using 10% (w/v) cetylpyridinium chloride and the concentrations were determined by measuring the absorbance at 562 nm. The values are expressed as the mean ± S.D. (N = 3); $*P < 0.05$ (t-test). (C) Integrin ß1 blocking antibody was used at a concentration of 1 μg/ml and incubated for 3h. In control and AMBN-overexpressing SaOS cells, treatments were repeated every 3h to a total of four treatments in 12h. The cell lysates were immunoblotted with phospho-Tyr416 Src, Src and FLAG antibody. β-actin expression was used as a loading control. (D) In control and AMBN-overexpressing SaOS cells after treatment with integrin ß1 blocking antibody, mRNA expression levels of AMBN, CD63, RUNX2, BSP and OCN were examined by real-time RT-PCR. The data are shown as the ratio of each mRNA to GAPDH mRNA and the values are expressed as the mean ± S.D. (N = 3).

FIG. 6. (A) SaOS-2, AMBN-overexpressing SaOS-2 and AMBN and constitutive active Src mutant (Src-Y527F) cotransfected cells were examined by Western blot analysis by using anti-phospho-Tyr416 Src and anti-Src antibodies. β-actin was used as a loading control. (B) Control and AMBN-overexpressing cells were cultured for 10 days in the presence of 50

μg/ml ascorbic acid and sodium 5 mM β-glycerophosphate after transfection with constitutive active Src mutant (Src-Y527F). Then, ALZ staining was performed to visualize mineral deposition. ALZ was destained by 10% (w/v) cetylpyridinium chloride and the concentrations were determined by measuring the absorbance at 562 nm. The values are expressed as the mean ± S.D. (N = 3); $*P < 0.05$ (t-test). (C) Control and AMBN-overexpressing cells were cultured for 4 days after transfection with constitutive active Src mutant (Src-Y527F) and the mRNA expression levels of mineralization-related molecules were assessed by real-time RT-PCR. The data are shown as the ratio of each mRNA to GAPDH mRNA. The values are expressed as the mean ± S.D. (N = 3); $*P < 0.05$ (t-test).

FIG. 7. (A) Expression of CD63 in ST-2 and MC3T3-E1 cells. CD63 expression was detected by Western blot analysis. β-actin was used as a loading control. (B) Ectopic expression of AMBN and CD63 in MC3T3-E1 cells. MC3T3-E1 cells were transiently transfected with the vector for FLAG-AMBN and/or GFP-CD63. The ectopic expression of AMBN and CD63 in MC3T3-E1 cells was examined by RT-PCR. GAPDH mRNA expression was used as the control. (C) mRNA expression levels of AMBN, CD63, RUNX2, BSP and OCN in MC3T3-E1 cells were examined by real-time RT-PCR. The data are shown as the ratio of each mRNA to GAPDH mRNA and the values are expressed as the mean ± S.D. (N = 3).

FIG. 8. Schematic of the molecular mechanism of AMBN in osteogenesis. In this model, osteoblasts cultured on COL I-coated dishes show elevation of Src phosphorylation via integrin β1 signalling in the absence of AMBN. After stimulation of AMBN, AMBN binds to CD63 and CD63 binds to integrin β1. Src binds to CD63 and is dephosphorylated. Finally, dephosphorylated Src promotes osteogenic differentiation.

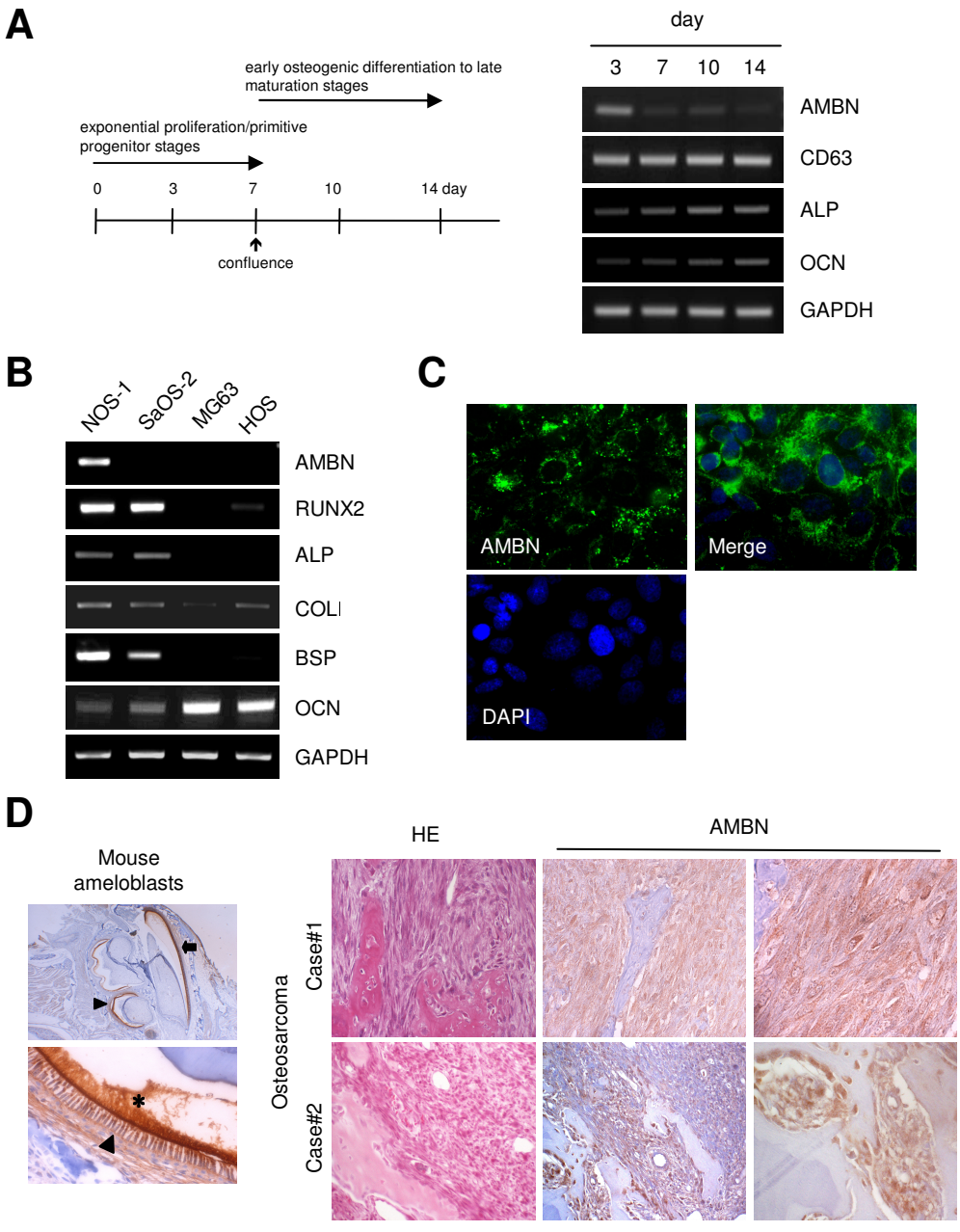
Table 1

Table 1. Primers used for real time RT-PCR

primer		Sequence	primer		Sequence
Human AMBN	F	CCTTGCAGGAAGGAGAACTG	Mouse AMBN	F	TTCTTGCTTTCCCCAATGAC
	R	CTGGGAGTGATGGACCTTGT		R	GGTGCACTTTGTTTCCAGGT
Human RUNX2	F	TTACTTACACCCCGCCAGTC	Mouse CD63	F	GCCATTGCTGGCTATGTGTT
	R	TATGGAGTGCTGCTGGTCTG		R	AGAATCGGGGACTCTGTCCT
Human ALP	F	CCTCCTCGGAAGACACTCTG	Mouse Runx2	F	CGCATTCCTCATCCCAGTAT
	R	GCAGTGAAGGGGCTTCTTGTC		R	TAAAGGTGGCTGGGTAGTGC
Human COLⅠ	F	GTGCTAAAGGTGCCAATGGT	Mouse BSP	F	ACCGGCCACGCTACTTTCTTT
	R	ACCAGGTTCACCGCTGTTAC		R	GACCGCCAGCTCGTTTTCA
Human BSP	F	AACCTACAACCCCACCACAA	Mouse OCN	F	AAGCAGGAGGGCAATAAGGT
	R	AGGTTCCCCGTTCTCACTTT		R	AGCTGCTGTGACATCCATAC
Human OCN	F	GACTGTGACGAGTTGGCTGA	Mouse ALP	F	TCCCTACCGACCCTGTTCTGA
	R	CTGGAGAGGAGCAGAACTGG		R	TGGACCTCTCCCTTGAGTGT
Human CD63	F	TCCCTTCCATGTCGAAGAAC	Rat CD63	F	TGTTGCCTGTGGTCATCATT
	R	GGCAAAGACAATTCCCAAAA		R	TGGCCGTTTTGTTGTCTGTA
Human GAPDH	F	GCATCCTGGGCTACACTGAG	Rat ALP	F	AGGCAGGATTGACCACGG
(Mouse/Rat)	R	TCCACCACCCTGTTGCTGTA		R	TGTAGTTCTGCTCATGGA
			Rat OCN	F	CAGCCCCCTACCCAGAT
				R	TGTGCCGTCCATACTTTC

Ambn : ameloblastin, Runx2 : runt-related transcription factor 2, ALP : alkaline phosphatase, COLⅠ: type Ⅰ collagen, BSP : bone sialoprotein, OCN : osteocalcin, GAPDH : glyceraldehyde-3-phosphate dehydrogenase

Figure 1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7
10
14 day
confluence
day
3
7
10
14
AMBN
CD63
ALP
OCN
GAPDH
B
NOS-1
SaOS-2
MG63
HOS
AMBN
RUNX2
ALP
COLI
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HE
AMBN
Osteosarcoma
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Case#2

Figure 2

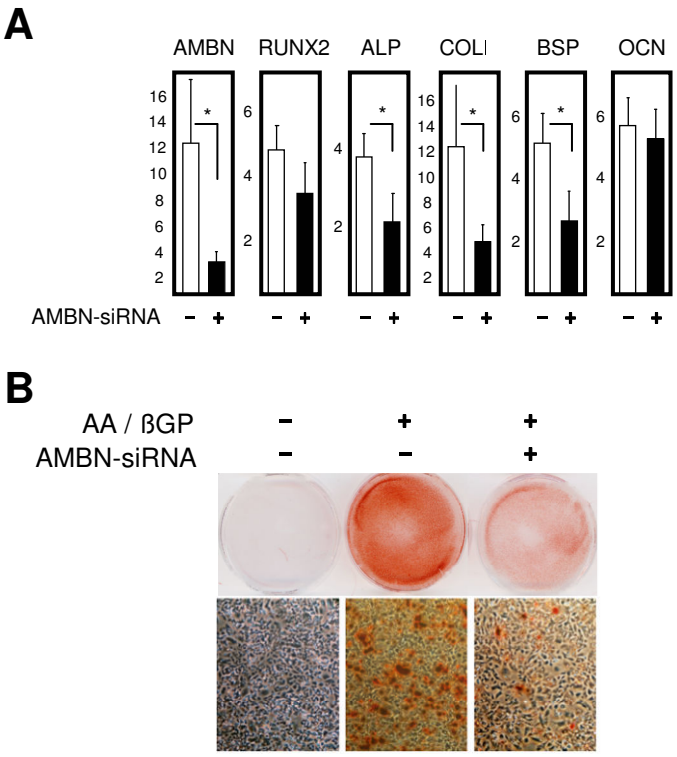
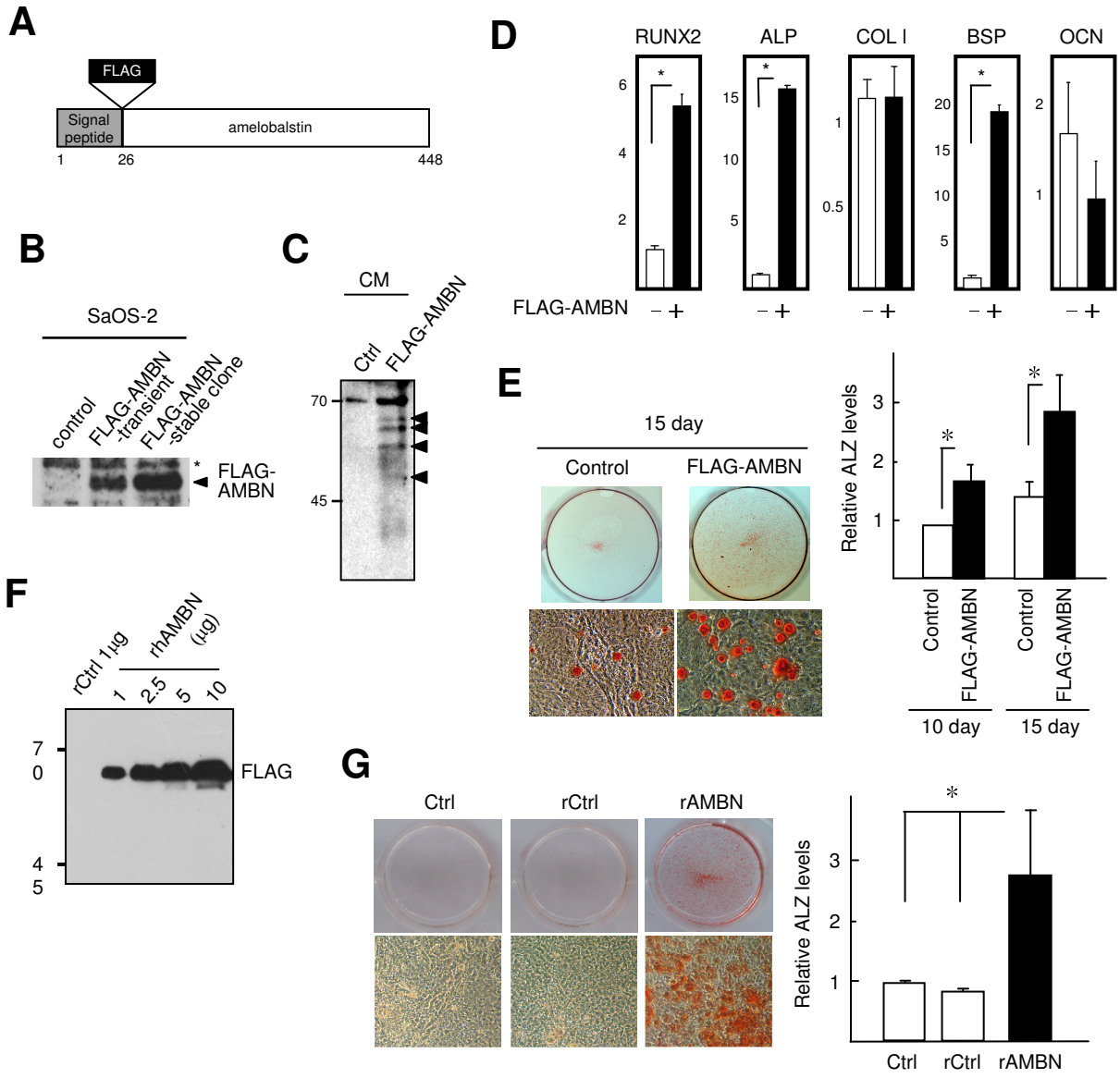


Figure 3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70
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D
RUNX2
ALP
COL I
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OCN
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E
15 day
Control
FLAG-AMBN
Relative ALZ levels
10 day
15 day
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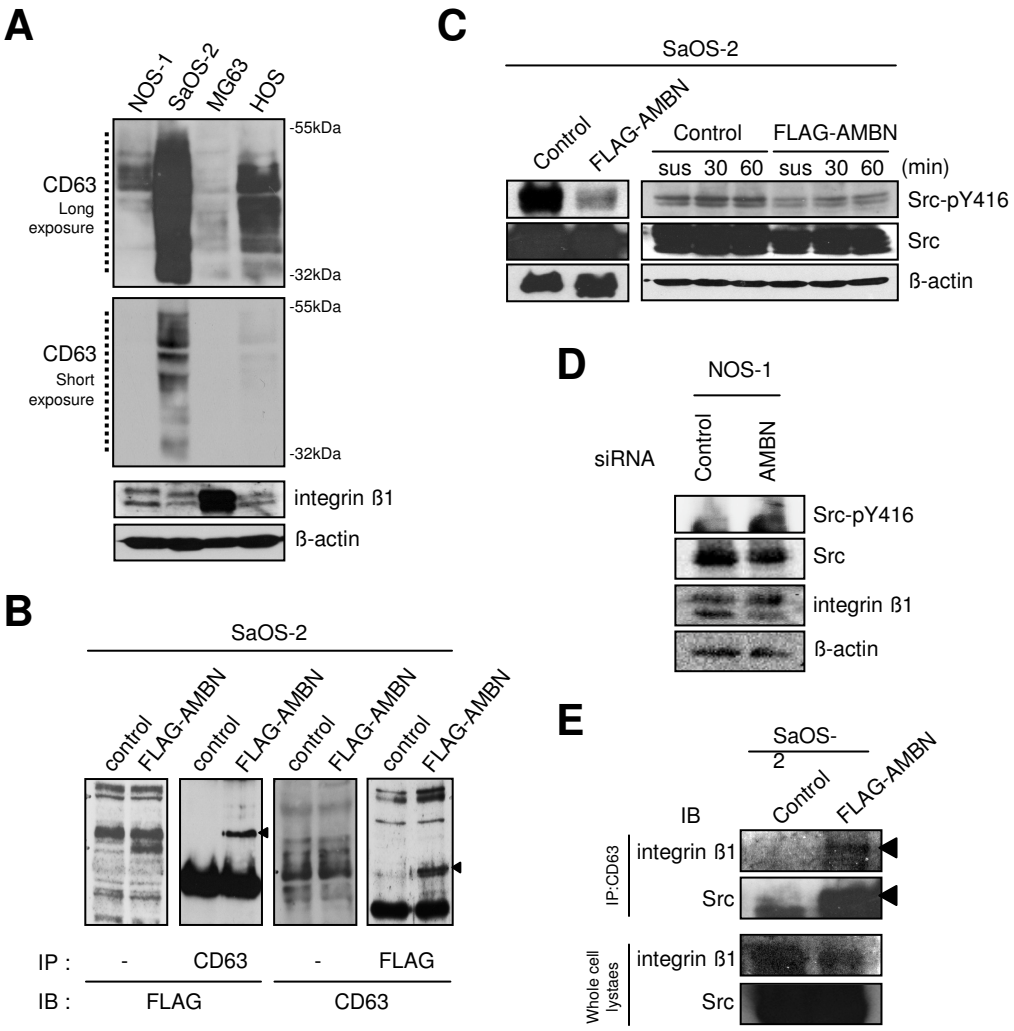
rAMBN
Relative ALZ levels

Figure 4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ß-actin
B
SaOS-2
control
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control
FLAG-AMBN
control
FLAG-AMBN
control
FLAG-AMBN
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CD63
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FLAG
IB :
FLAG
CD63
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SaOS-2
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Src
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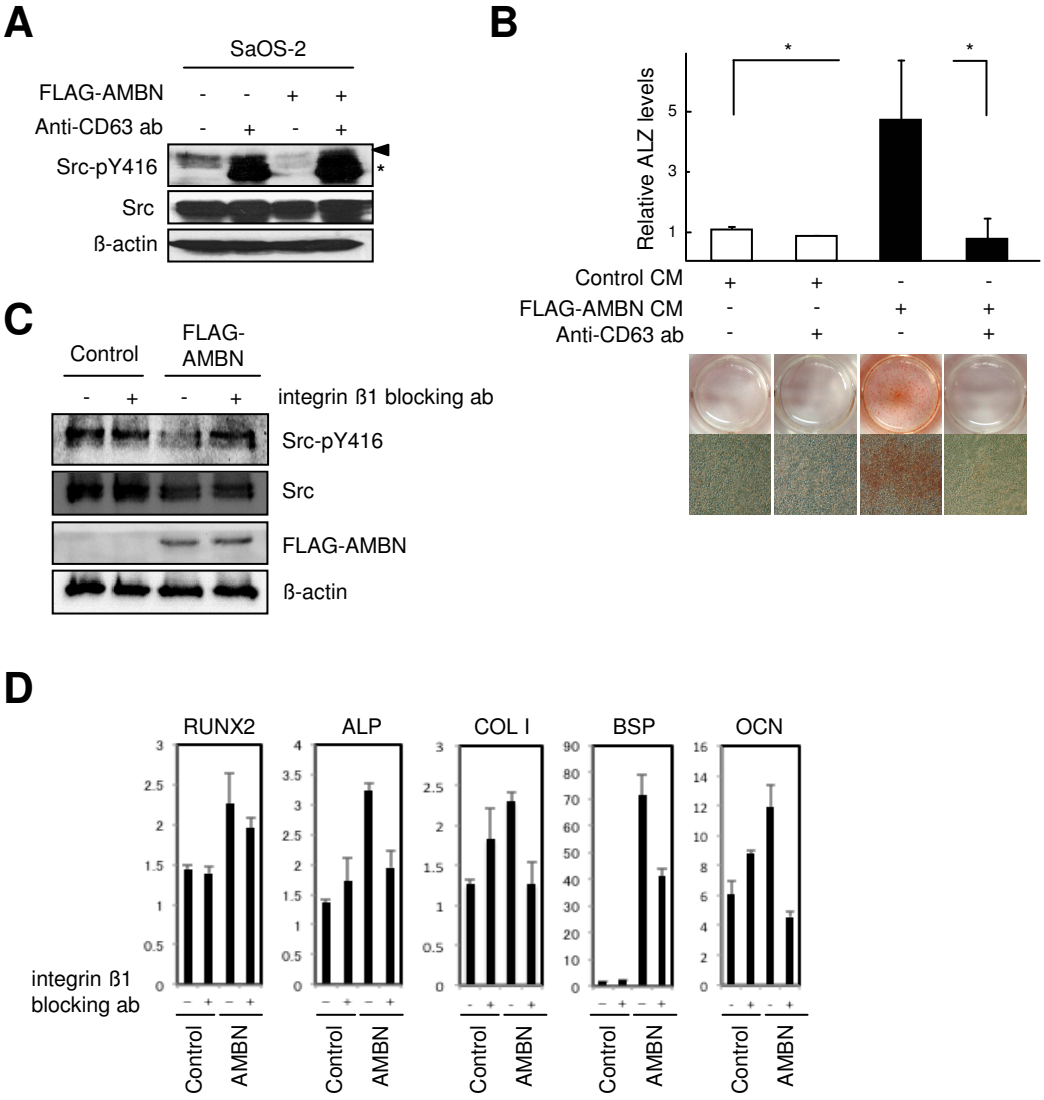
Figure 5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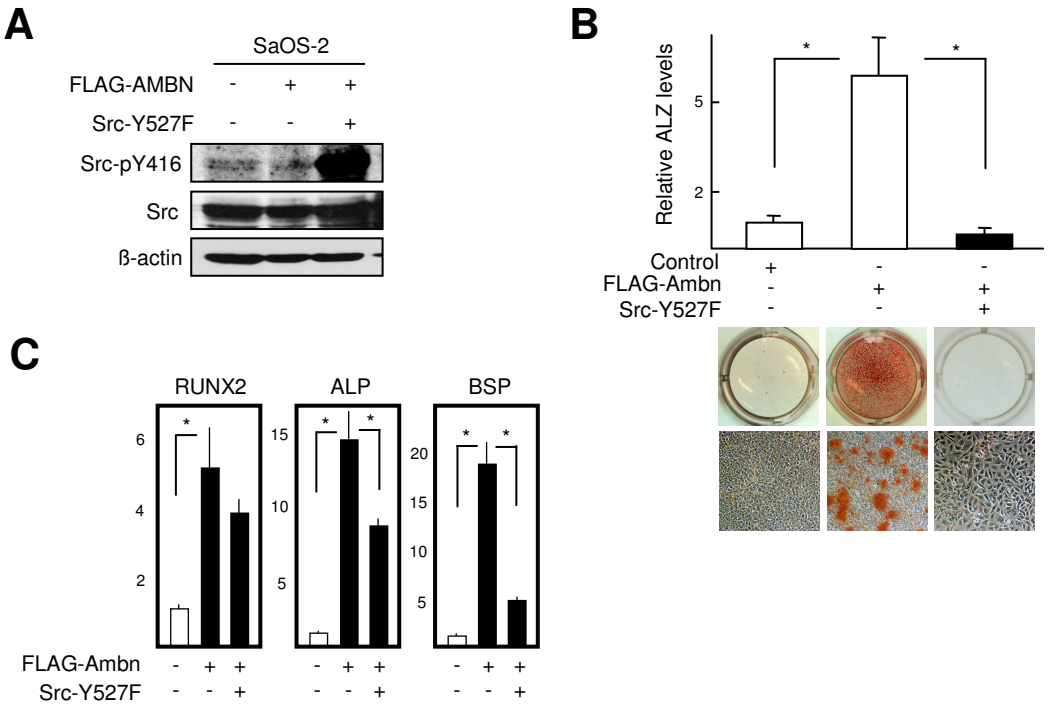
AMBN

Figure 6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Relative ALZ levels
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2
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Control + - -
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C
RUNX2
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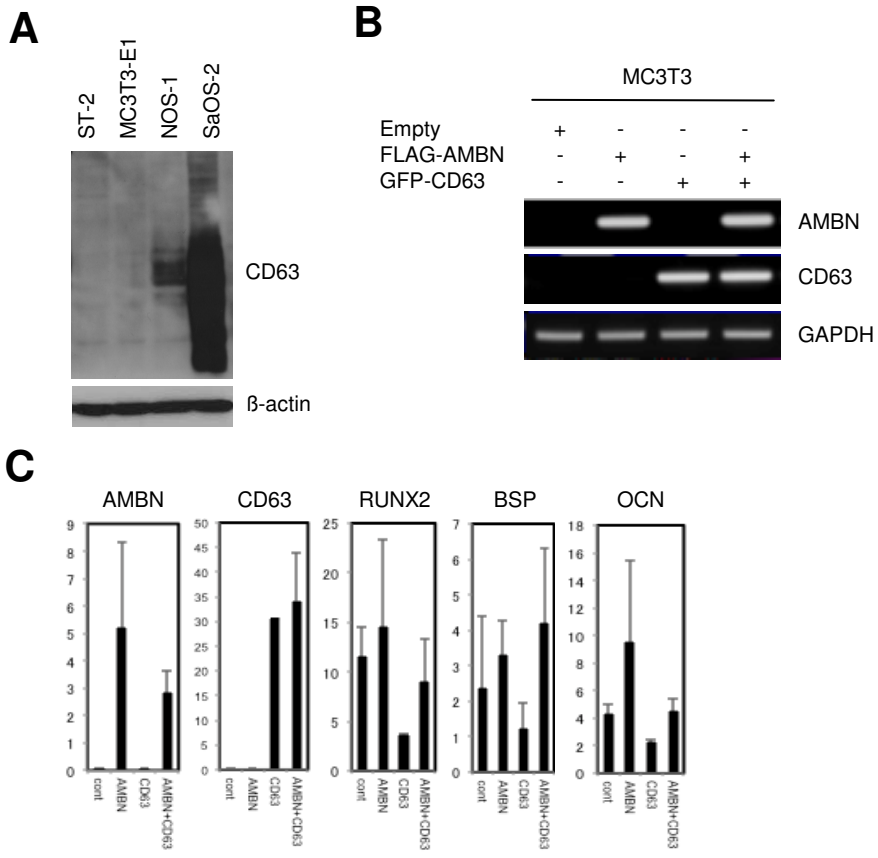
Figure 7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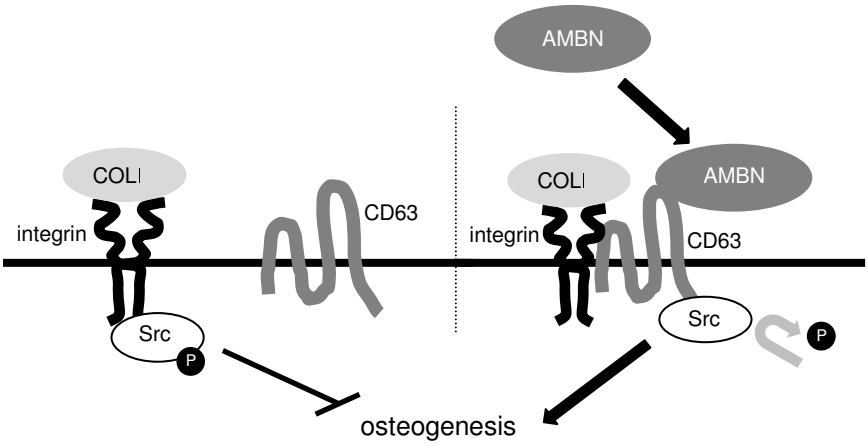
BSP
OCN
cont
AMBN
CD63
AMBN+CD63

Figure 8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CD63
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COLI
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CD63
Src
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osteogenesis