EPLINβ Is Involved in the Assembly of Cadherin-catenin Complexes in Osteoblasts and Affects Bone Formation

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Epithelial protein lost in neoplasm (EPLIN) is an actin-associated cytoskeletal protein that plays an important role in epithelial cell adhesion. EPLIN has two isoforms: EPLINα and EPLINβ. In this study, we investigated the role of EPLINβ in osteoblasts using EPLINβ-deficient (EPLINβGT/GT) mice. The skeletal phenotype of EPLINβGT/GT mice is indistinguishable from the wildtype (WT), but bone properties and strength were significantly decreased compared with WT littermates. Histomorphological analysis revealed altered organization of bone spicules and osteoblast cell arrangement, and decreased alkaline phosphatase activity in EPLINβGT/GT mouse bones. Transmission electron microscopy revealed wider intercellular spaces between osteoblasts in EPLINβGT/GT mice, suggesting aberrant cell adhesion. In EPLINβGT/GT osteoblasts, α- and β-catenins and F-actin were observed at the cell membrane, but OB-cadherin was localized at the perinuclear region, indicating that cadherin-catenin complexes were not formed. EPLINβ knockdown in MC3T3-e1 osteoblast cells showed similar results as in calvaria cell cultures. Bone formation markers, such as RUNX2, Osterix, ALP, and Col1a1 mRNA were reduced in EPLINβ knockdown cells, suggesting an important role for EPLINβ in osteoblast formation. In conclusion, we propose that EPLINβ is involved in the assembly of cadherin-catenin complexes in osteoblasts and affects bone formation.

Key words: LIMA1, EPLINβ, OB-cadherin, cell adhesion, osteoblast

I. Introduction

Intercellular interaction and communication are important in multicellular organisms to ensure functional development and differentiation [14, 17]. Osteoblasts are mononuclear, non-terminally differentiated and specialized cells that are arranged in a single layer adherent to periosteal or endosteal bone surfaces. The alignment and interaction of osteoblasts are important events for bone formation and mineralization. It has been reported that gap junctions, tight junctions, and adherens junctions are involved in intercellular communication of osteoblast lineages [2, 4, 37]. Adherens junctions are intercellular structures that allow homophilic, calcium-dependent cell-cell adhesion via cadherins, which are transmembrane proteins containing Ca2+-dependent homophilic adhesion receptors that play important roles in cell recognition and cell fate during development [21, 40]. It has been reported that some cadherin family members, such as OB-cadherin

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osteoblasts (known as cadherin-11), play an important role in the regulation of osteoblast differentiation and osteoid matrix mineralization in osteoblasts [12, 34, 36].

We developed an efficient screening system for identifying novel genes involved in bone metabolism using mutant mouse strains established by an exchangeable gene trap method based on the Exchangeable Gene Trap Clones (EGTC) database [26]. As a result of the screening, we selected epithelial protein lost in neoplasm (EPLIN) as a target molecule. EPLIN, which is a product of the LIMA1 gene, is a cytoskeletal protein that was initially identified as the product of a gene transcriptionally downregulated in oral cancer cell lines [5]. EPLIN is known to bind to actin fibers via α-catenin and link the cadherin-catenin complex to F-actin, thus actively stabilizing the actin bundles and playing important roles in cell adhesion and the formation of adherence junctions to bind cells [13, 29]. These functions of EPLIN have been demonstrated in epithelial, endothelial, and cancer cells [7, 38]. However, the role of EPLIN in osteoblast function has not yet been clarified.

EPLIN exists in two isoforms: EPLINα, which consists of 600 amino acids, and EPLINβ, which has 759 amino acids [6, 30]. The EPLIN gene consists of 11 exons and has distinct promoters for the two isoforms. EPLINβ mRNA consists of all 11 exons, while EPLINα mRNA consists of exons 4–11, and the expression of the two isoforms is regulated independently. EPLINα is a cytoskeletal protein whose expression is often lost in cancerous cells, including metastatic cells [23, 30]. Recently, EPLINβ has been reported to play an important role as a controller of stress fiber formation and stability in endothelial cells [38]. However, the isoform-specific role of EPLINβ in bone structure and function has remained unclear.

In the present study, we first investigated the role of EPLINβ in bone formation and cell adhesion using WT and EPLINβGT/GT mice. Bone morphological changes were demonstrated by hematoxylin and eosin (HE) staining, alkaline phosphatase (ALP) staining, and transmission electron microscopy, whereas the functional activity of osteoblast cells was evaluated by ALP assay and staining. The role of EPLINβ in cell adhesion was examined by immunofluorescence in osteoblast cells. Finally, the in vivo experimental findings were confirmed by EPLINβ knockdown experiments in vitro.

II. Materials and Methods

Animals and tissue preparation

All experiments using mice were performed with the approval of the Animal Care and Use Committee and the Genetic Modification Safety Committee of Kumamoto University and the University of Miyazaki in accordance with the institutional Guidelines for Animal Experiments and Safety Management Rules for Genetic Modification. We generated EPLINβGT/GT mice to investigate the role of EPLINβ in bone metabolism. For HE and ALP stainings, 11-day-old WT and EPLINβGT/GT mouse femoral bones were used.

Western blot analysis

Cell lysates containing 20 μg of protein were mixed with loading solution [250 mM Tris/HCl (pH 6.8), 0.5 M sucrose, 5 mM EDTA, 0.006% bromophenol blue, 2-mercaptoethanol, and 4% SDS], boiled for 5 min, and separated by SDS-PAGE with an 8% gradient gel according to previous reports [8, 28]. Samples were electrophoretically transferred onto iBlot® Gel Transfer Device, Thermo Fisher Scientific Waltham, MA, USA) for dry protein transfer and then washed with double-distilled water. The membranes were blocked with 10% Block Ace (UKB80®, Yukijirushi, Hokkaido, Japan) at room temperature for 1 hr. Membranes were then incubated overnight at 4°C with rabbit polyclonal EPLIN antibody (EPLIN antibody NB100-2305, Novus Biologicals, Cambridge, UK) and beta-actin (β-actin, polyclonal antibody 20536-1-AP, Proteintech, Chicago, IL, USA), diluted 1:2,500 and 1:1,600 with Can Get Signal® Immunoreaction Enhancer Solution 1 (NKB-101, Toyobo, Osaka Japan), respectively. Membranes were then reacted with HRP-goat anti-rabbit IgG antibody (DK-2600, Dako, Glostrup, Denmark) diluted 1:1,000 with Can Get Signal® Immunoreaction Enhancer Solution 2 (NKB-201, Toyobo) for 1 hr, prior to being washed three times for 5 min each with PBST/0.05% Triton X-100 buffer. The bands were then visualized with 3,3’-diaminobenzidine (DAB), 1 M PB, 1% NiSO4, 1% CoCl2, and 30% H2O2 [8].

Micro-computed tomography

To analyze the bone structure, soft tissues surrounding the femur were removed prior to micro-computed tomography (μCT) scanning, which was performed using a μCT system (ScanXmate-L090H; Comscantecno, Kanagawa, Japan) as described previously [11, 15, 26]. Bone morphometric analysis was performed on the trabecular bone of the distal femur at 16 weeks after birth (n = 5). The measurement area was set 0.2 mm from the growth plate to 2 mm into the proximal area. For the cortical bone analysis, the mid-point of the femur was scanned. Data from the μCT scans were analyzed and calculated using a three-dimensional image analyzing system (TRI/3D-BON; Ratoc System Engineering Co. Ltd., Tokyo, Japan). Bone morphometry analysis included trabecular bone mineral content (T.BMC), trabecular bone mineral density Tb.BMD, trabecular bone volume/total volume (BV/TV), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), and trabecular separation (Tb.SP). For the cortical bone analysis, cortical bone volume (Ct.V) and cortical bone thickness (Ct.Th) were included.

Biomechanical strength analysis

To determine bone strength, a three-point bending test (EZ-test S; Shimadzu Co., Kyoto, Japan) was conducted on
the excised femur as described previously [15, 26]. The span of the two support points was 6 mm. The deformation rate was 1.0-mm/min. Maximal load (N), displacement (mm), and work to failure (mJ) were obtained from the load-deformation curves.

Transmission electron microscopy

The femur tissue was dissected from 9-day-old WT and EPLINβGT/GT mice and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and 2% osmium tetroxide in 0.1 M cacodylate buffer. Bone specimens were dehydrated in a graded ethanol series and embedded in epoxy resin. The specimens were cut into 90- to 95-mm-thick sections, stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope (TEM) Hitachi 7100 (Hitachi, Tokyo, Japan) [15].

Immunofluorescence staining

Cells cultured on glass coverslips were rinsed several times in Ca²⁺- and Mg²⁺-containing PBS, fixed in 4% paraformaldehyde (PFA) for 10 min at 37°C, and permeabilized in 4% PFA containing 0.1% Triton X-100 for 1 min at room temperature. After blocking in 4% Block Ace solution (Yukijirushi Inc., Sapporo, Japan), the cells were incubated for 1 hr separately with primary antibodies: OB-cadherin (#4442; Cell Signaling Technology, Beverly, MA, USA) at 1:100 dilution; and EPLIN (16639-1-AP; Proteintech, Rosemont, IL, USA), β-catenin (H102 sc-7199; Santa Cruz, Dallas, TX, USA) at 1:100 dilution; and α-catenin (anti-α1 catenin antibody EP1793Y; Abcam, Cambridge, UK) all at a dilution of 1:50. After washing, bound antibodies were detected using Alexa Fluor 488-labeled anti-rabbit secondary antibody (Thermo Fisher Scientific) for 30 min. Then, F-actin (Phalloidin, Thermo Scientific) was stained in all slides. Cell nuclei were stained with DAPI (SlowFade Gold Antifade Mountant with DAPI; Thermo Fisher Scientific). As a negative control, normal rabbit IgG was used at the same concentration instead of the primary antibody in each experiment. Microphotos were taken using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

Calvaria cell cultures

Osteoblast-enriched calvaria cultures were prepared from 3- to 4-day-old WT and EPLINβGT/GT mice by sequential collagenase digestion as previously described [31, 33]. The calvaria was digested with 0.1% collagenase (Wako Pure Chemical Industries, Osaka, Japan) and 0.2% dispase (Gibco, Thermo Fisher Scientific) at 37°C for 10 min. The solution was moved to a fresh sterile tube (fraction 1). This procedure was repeated five more times with fresh solution (fractions 2–4). Fractions 2–4 were then combined, and the cells were pelleted by centrifugation at 15,000 × g for 5 min. The isolated cells were grown in alpha-modified Eagle’s medium (α-MEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS). The medium was changed every three days, and the cell culture supernatant was collected 24 hr after the last medium change. A total of 8 × 10⁶ cells/ml were plated and maintained in α-MEM supplemented with 10% FBS, 100 ng/ml bone morphogenetic protein (BMP)-2 (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate for six weeks. Cells were then maintained in α-MEM with 10% FBS, and the medium was changed twice a week.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from the MC3T3-e1 osteoblastic cell line using the ReliaPrep RNA Tissue Miniprep System (Promega) and reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Gene expression levels were analyzed by the ΔΔCT method using the SYBR Green and Step One Plus PCR system (Thermo Fischer Scientific). All samples were assayed in triplicate, and the CT values were averaged. GAPDH was used as the internal control. The CT values of the target genes were compared with control siRNA-transfected cells at day one. Primer sequences EPLINα F-5’-GACTTAAGCTTTACAGCGTGTTG-3’ and R-5’-CAGCAGGAGATGCTGGT-3’, EPLINβ F-5’-TCTAGTCAGCCCCAGGTGTTCTC-3’ and R-5’-TCAATGACAGGGAAGTCCACTC-3’, RUNX2 F-5’-TACAAACCATCCCAGTCCCTGT-3’ and R-5’-AGTGTCCTTAACACAGCTCCAGCA-3’, Osterix F-5’-ATGGCTCTCTCCTCTGTTG-3’ and R-5’-TGAAACCTCAGCTATG-3’, ALP F-5’-CCAACCACTTTTGTGGCCAGAGA-3’ and R-5’-GGCTACATTGGTGTTGAGCTTT-3’, OB-Cadherin F-5’-TGAAAGATAGAGGCCGCCAAT-3’ and R-5’-CCCAAGACATGGAGGGCTCAT-3’, and Col1a1 F-5’-GGCTACATTGGTGTTGAGCTTT-3’, OB Cadherin F-5’-TGAAAGATAGAGGCCGCCAAT-3’ and R-5’-CCCAAGACATGGAGGGCTCAT-3’, and Col1a1 F-5’-GGCTACATTGGTGTTGAGCTTT-3’, OB Cadherin F-5’-TGAAAGATAGAGGCCGCCAAT-3’ and R-5’-CCCAAGACATGGAGGGCTCAT-3’, and Col1a1 F-5’-GGCTACATTGGTGTTGAGCTTT-3’, OB Cadherin F-5’-TGAAAGATAGAGGCCGCCAAT-3’ and R-5’-CCCAAGACATGGAGGGCTCAT-3’, and Col1a1 F-5’-GGCTACATTGGTGTTGAGCTTT-3’, OB Cadherin F-5’-TGAAAGATAGAGGCCGCCAAT-3’ and R-5’-CCCAAGACATGGAGGGCTCAT-3’, and Col1a1 F-5’-GGCTACATTGGTGTTGAGCTTT-3’, OB Cadherin F-5’-TGAAAGATAGAGGCCGCCAAT-3’ and R-5’-CCCAAGACATGGAGGGCTCAT-3’, and Col1a1 F-5’-GGCTACATTGGTGTTGAGCTTT-3’.

EPLINβ knockdown experiment

The murine osteoblastic cell line MC3T3-e1 was purchased from RIKEN (RIKEN BioResource Center, Wako, Japan). For transfection, MC3T3-e1 cells were divided into two groups, including control-siRNA and EPLINβ-siRNA. Transfection was performed using Lipofectamine RNA iMAX (Invitrogen), and all transfection operations were performed in strict accordance with Lipofectamine RNA iMAX transfection instructions. Cells that were successfully transfected were prepared into cell suspensions using α-MEM with L-Glutamine and Phenol Red with 10% FBS and 100 ng/ml, 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate. They were seeded at a density of 2 × 10⁴ cells per well in 24-well plates, followed by incubation in a constant temperature incubator (5% CO₂, 37°C, 95% humidity). Then, the expression of EPLINβ was detected by quantitative real-time polymerase chain reaction (qRT-PCR) after transfection for three days.
**ALP assay and staining**

Alkaline phosphatase (ALP) activity was examined in the MC3T3-e1 osteoblastic cell line after three days of transfection with control-siRNA and EPLINβ-siRNA using a TRACP&ALP Assay Kit (Takara Bio, Japan). In bone tissue sections and cultured cells, ALP expression was examined using a TRAP/ALP staining kit (Fujifilm Wako Chemicals, Osaka, Japan) according to the manufacturer’s protocol.

**Statistical analyses**

The data were analyzed by Student’s t-test using Statistical Package for Social Sciences (version 20; IBM Corp., Armonk, NY, USA). The data in the graphs are presented as the mean ± standard deviation. P values of <0.05 were considered to indicate statistical significance.

**III. Results**

**Generation and validation of EPLINβGT/GT mice**

EPLINβ mRNA requires all 11 exons, while EPLINα mRNA requires exons 4–11. EPLINα and EPLINβ mRNA are transcribed from distinct promoters, not alternative splicing, and the promoter region of EPLINα is near upstream exon 4 [6, 10]. Two pU-21W gene trap vectors were inserted 6.5 kb upstream of exon 4 (Fig. 1A). From the position of the insertion point and the promoter region, there was possible deficiency of EPLINα, EPLINβ, or both. Western blot (WB) was performed to investigate the EPLIN expression, which is a product of the LIMA1 gene. As a result, both EPLINα and EPLINβ were expressed in WT mouse (Fig. 1B). However, only EPLINα was detected in knockout mouse bone. This result confirms that EPLINβ was deficient in EPLINβGT/GT mice.

**Bone morphometric analysis in EPLINβGT/GT mice**

Micro-CT scans of the femurs were performed on 16-week-old WT and EPLINβGT/GT mice. Bone masses in trabecular bones of the distal femur were significantly decreased in the EPLINβGT/GT mice compared with WT mice (Fig. 2A). In biomechanical strength analysis, the maximum load was significantly decreased in EPLINβGT/GT mice compared with WT mice, confirming significant bone fragility in EPLINβGT/GT mice (Fig. 2B). Trabecular bone parameters including trabecular bone mineral content (T.BMC), trabecular bone mineral density (Tb.BMD), trabecular bone thickness (Tb.Th), and trabecular bone number (Tb.N) were significantly decreased in EPLINβGT/GT mice compared with WT littermates (Fig. 2C). Trabecular separation (Tb.Sp), which is the parameter of the cavities containing the trabecular region, showed an increasing trend in EPLINβGT/GT mice. Moreover, cortical bone thick-
ness (Ct.Th) and cortical bone volume (Ct.V) were significantly decreased in \textit{EPLIN}^{GT/GT} mice (Fig. 2D).

\textbf{Histomorphological analysis of \textit{EPLIN}^{GT/GT} mouse bones}

For detailed analysis of bone morphology, WT and \textit{EPLIN}^{GT/GT} mouse femur bones were examined by HE staining. In \textit{EPLIN}^{GT/GT} mouse bones, reduced staining intensity of the bone matrix and altered organization of bone spicules and osteoblast cell arrangement were observed compared to WT littermates (Fig. 3A). Functional activity of osteoblast cells was examined by ALP staining (Fig. 3B). In the WT mice, trabecular and cortical bone regions were intensely stained. However, in \textit{EPLIN}^{GT/GT} mice, only weak ALP staining was observed in the same regions. Next, the ultrastructure of mouse trabecular bones was examined by TEM (Fig. 3C). In WT mice, osteoblast morphology and intercellular spaces were uniform. The nuclei of osteoblast cells were localized just below the intercellular adhesion site, and intracellular organelles, such as the rough endoplasmic reticulum (rER), Golgi apparatus, and mitochondria were abundantly observed in each cell. In \textit{EPLIN}^{GT/GT} mice, osteoblast cells were localized sparsely due to altered intercellular junctions (Fig. 3C, asterisks). Moreover, osteoblast cytoplasm became thin, whereas intercellular spaces became wider, and pseudopodia-like structures were observed at the cell membranes.

\textbf{Subcellular localization of junctional proteins in osteoblasts}

The subcellular localization of EPLIN was examined by immunofluorescence in osteoblast cultures derived from WT and \textit{EPLIN}^{GT/GT} mouse calvaria. In WT osteoblasts, EPLIN was mostly co-localized with F-actin around the cell membrane (Fig. 4A). However, in \textit{EPLIN}β-depleted cells, significantly decreased expression was found, which

\begin{figure}[h]
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\caption{Bone morphometric analysis. (A) Micro-CT scans of WT and \textit{EPLIN}^{GT/GT} mouse femur bones. Bar = 1 mm. (B) Bone strength parameters were revealed, including maximal load, maximum displacement, and maximum work. Trabecular bone parameters (C), trabecular bone mineral content (T.BMC), trabecular bone mineral density Tb.BMD, trabecular bone volume/total volume (BV/TV), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), trabecular separation (Tb.Sp), cortical bone parameters (D), cortical bone volume (Cl.V), and cortical bone thickness (Cl.Th) were compared in WT and \textit{EPLIN}^{GT/GT} mouse bones. Data represent the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.}
\end{figure}
Fig. 3. Histomorphological analysis of EPLINβGT/GT mouse bones. (A) HE staining of 11-day-old WT and EPLINβGT/GT mouse femoral bones. Dashed line areas are enlarged in the inset. (B) ALP staining of WT and EPLINβGT/GT mouse femoral bones. Scale bar, 200 μm. (C) TEM of 9-day-old WT and EPLINβGT/GT mouse femoral bones. Osteoblast cell nuclei (n), cytoplasm (c), and cellular junctions (black arrow) are marked. Asterisks indicate aberrant cellular junctions between osteoblasts. Pseudopodia-like structures are present between intercellular spaces (red arrow). Bar = 5 μm.
Expression of junctional proteins in osteoblast-enriched calvaria cultures. Osteoblast cells was prepared from WT and \textit{EPLIN}{\beta^{GT/GT}} mice. The expressions of EPLIN (A), OB-cadherin (B), α-catenin (C), and β-catenin (D) were examined by immunofluorescence. Arrows indicate the perinuclear localization of OB-cadherin. In each specimen, cellular cytoskeletons were stained by F-actin (red staining). Cell nuclei were counterstained by DAPI. Microphotos represent three independent experimental results. Bar = 50 μm.

Fig. 4. The Role of EPLIN{\beta} in Bone Formation
Expression of junctional proteins in the MC3T3-e1 osteoblastic cell line. Control-siRNA and EPLINβ-siRNA were transfected into MC3T3-e1 osteoblastic cells. The expressions of EPLIN (A), OB-cadherin (B), α-catenin (C), and β-catenin (D) were examined by immunofluorescence. Arrows indicate the perinuclear localization of OB-cadherin. In each specimen, cellular cytoskeletons were stained by F-actin (red staining). Cell nuclei were counterstained by DAPI. Microphotos represent three independent experimental results. Bar = 50 μm.

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indicates the remaining EPLINα expression in osteoblasts. Next, we examined the localization of junctional proteins. In WT osteoblasts, OB-cadherin was localized at the cell membrane (Fig. 4B). Surprisingly, OB-cadherin had its localization changed to the perinuclear region in EPLINβ\textsuperscript{GT/GT} osteoblasts (arrows). There was no change in the expressions of α-catenin and β-catenin in EPLINβ\textsuperscript{GT/GT} osteoblasts (Fig. 4C, D).

To confirm these in vivo experimental findings, we performed EPLINβ knockdown experiments using the MC3T3-e1 osteoblastic cell line (Fig. 5A). In control siRNA transfected cells, EPLIN expression was mainly co-localized with F-actin in cell membranes. Successful EPLINβ knockdown was confirmed in EPLINβ siRNA-transfected cells. The localization of OB-cadherin was also changed to the perinuclear region in EPLINβ knockdown cells (arrows in Fig. 5B). The expression of α-catenin and β-catenin was not changed in EPLINβ knockdown cells (Fig. 5C, D).

The effects of EPLINβ knockdown in osteoblast cells
Knockdown efficiency was confirmed in control and EPLINβ siRNA-transfected cells using WB analysis (Fig. 6A). In EPLINβ-transfected cells, the successful knockdown of EPLINβ was confirmed, whereas EPLINα was intact (Fig. 6B). qPCR analysis also confirmed these findings of no changes in EPLINα mRNA expression and significantly decreased expression of EPLINβ mRNA in EPLINβ knockdown cells. Next, we examined the expressions of genes important for bone formation and function. In EPLINβ knockdown cells, the expressions of Runx2, Osterix, ALP, OB-cadherin, and Col1a1 mRNAs were significantly decreased at days 1 and 3. In EPLINβ knockdown cells, the ALP activity was significantly decreased comparing to control siRNA-transfected cells (Fig. 6C). Moreover, a significant decrease in ALP staining was confirmed in EPLINβ knockdown cells (Fig. 6D).
IV. Discussion

This is the first study to investigate the role of EPLINβ in mouse bones. The main finding in this study is the important role of EPLINβ in the assembly of cadherin-catenin complexes in osteoblasts. In the absence of EPLINβ, aberrant cadherin-catenin complexes induced abnormal cellular junctions that resulted in altered bone formation.

We designed a unique screening system to detect genes that are important for bone metabolism, and EPLINβ was found as a result [26]. In this study, WB revealed the presence of EPLINα and absence of EPLINβ, indicating the successful generation of EPLINβGT/GT mice. Morphological abnormalities were found in EPLINβGT/GT mouse bones, while EPLINα expression was intact. These results suggest that EPLINα and EPLINβ have distinct functional roles in mouse bones, and EPLINα does not compensate for the absence of EPLINβ.

EPLINβ-deficient mice were not obviously different from their WT littermates, but the bone mass and strength of the long bones were significantly decreased in comparison. The reason for this difference was examined by histomorphological analysis, and reduced staining intensity of the bone matrix and altered organization of bone spicules and osteoblast cell arrangement were detected. These findings are expected, given that EPLIN is involved in cell adhesion and stabilizes the cytoskeleton by linking to both cadherin-catenin complexes and actin fibers [1, 29]. Ultrastructural analysis revealed aberrant cell adhesion and different sizes of osteoblasts in EPLINβ-deficient mice. Abundant Golgi apparatuses and rER were found in WT osteoblasts, whereas fewer organelles and thin cytoplasm were observed in EPLINβ-deficient osteoblasts, indicating immature differentiation. These results suggest that the differentiation of EPLINβGT/GT osteoblasts is delayed compared to WT osteoblasts.

Adhesion factors such as cadherin and members of the integrin family affect bone formation [12, 18, 25, 36]. In osteoblasts, OB-cadherin, N-cadherin, and P-cadherin are expressed at the cell membrane, with the major molecules being OB- and N-cadherin given their expression level [24]. The phenotypes of mice deficient in each cadherin are different. N-cadherin-null mutant mice are lethal at embryonic day 10 [20, 35], but OB-cadherin−/− mice have an indistinct phenotype, with decreased bone volume of the long bone metaphysis [25]. Our results also revealed that OB-cadherin was localized at the perinuclear region, but not in the cell membrane, in EPLINβGT/GT osteoblasts. The skeletal phenotype of EPLINβGT/GT mice is similar to that of OB-cadherin−/− mice, but not N-cadherin-null mice. N-cadherin is expressed constitutively, whereas OB-cadherin is expressed selectively in preosteoblasts [24]. Moreover, the expression of OB-cadherin is regulated by semaphorin 4D, which is a signal ligand on osteoclast surfaces [32]. Semaphorin 4D stimulation decreases the expression of OB-cadherin at the cell membrane in osteoblasts and inhibits bone formation. It has been revealed that intercellular adhesion affects signal transduction to the nucleus [16, 19]. Cadherin is synthesized as pro-cadherin and associates with catenins, then proteolytically processed to create the mature form in the ER or Golgi apparatus [9, 41]. However, cadherin transport has been reported to be non-one-way to the cell membrane. Surface E-cadherins are endocytosed, during which they are either recycled to the cell surface or targeted for degradation [3]. In the present study, cadherin-catenin complexes were not assembled appropriately, and OB-cadherin had perinuclear localization in EPLINβ-deficient osteoblasts. These facts suggest that EPLINβ is involved in bone formation by regulating the distribution of OB-cadherin. Further experiments will be needed to determine how EPLIN is involved in regulating cadherin distribution.

In contrast, the localizations of α- and β-catenins were normal in both WT and EPLINβ-deficient osteoblasts. The gene expressions essential for bone formation and function were significantly decreased in EPLINβ knockdown cells. These results indicate the importance of EPLINβ for bone formation. Decreased ALP activity in both in vivo and in vitro experiments suggests impaired functional activity in EPLINβ-deficient osteoblasts. However, bone and other organs are closely influenced by each other [39, 27], and since EPLINβGT/GT mice are systemically deficient in this gene, the possibility of secondary effects cannot be ruled out. Further investigation is needed to clarify the role of EPLINβ.

In environments such as gravity and muscle tension, bone has trabeculae formed in response to its stress. Under these conditions, osteoblasts cooperate with osteoclasts to form and maintain their structure. Recently, EPLINβ has been reported to be the control of stress fiber formation and stability in endothelial cell in aorta but not vena cava [38]. Therefore, stress-related EPLINβ may be important in osteoblasts [22]. Despite our data showing that EPLINα was expressed in EPLINβGT/GT mice, EPLINβGT/GT osteoblasts exhibited impaired cell differentiation and intercellular adhesion suggest that EPLINβ is an important isoform in osteoblasts.

In summary, we demonstrated the role of EPLINβ in osteoblast cell adhesion and bone formation. EPLINβ deficiency affected subcellular localization of OB-cadherin, which is an important component of the cadherin-catenin complex.

V. Conflict of Interest

The authors declare that no conflicts of interest exist.

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