

Osteoblast-derived Laminin-332 is a novel negative regulator of osteoclastogenesis in bone microenvironments

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Laminin-332 (Lm-332), a major basement membrane protein, has been shown to provide a niche for some stem cells. Here, we found that Lm-332 was expressed in osteoblasts, and is implicated in the regulation of osteoclast differentiation. Immunofluorescence analysis of laminin- β 3, a unique component of Lm-332, indicated specific expression of laminin- β 3 in osteoblast-like cells localized on bone surface. RT-PCR analysis confirmed that α 3, β 3, and γ 2 chains of Lm-332 were all expressed in primary osteoblasts prepared from mouse calvaria. Lm-332 markedly inhibited osteoclastogenesis induced by receptor activator of nuclear factor kappa B (NF- κ B) ligand (RANKL) when bone marrow-derived macrophages (BMMs) were cultured on Lm-332-coated plates. Lm-332 also blocked RANKL-induced activation of mitogen-activated protein kinases (MAPKs) (ERK, JNK, and p38) and expression of NFATc1, c-Fos, and c-Jun. Lm-332 suppressed osteoclast differentiation while retaining macrophage phenotypes, including nonspecific esterase activity and gene expression of lysozyme and EGF-like module-containing mucin-like hormone receptor-like 1 (*Emr1*). Furthermore, the treatment of primary osteoblasts with osteoclastogenic factors dramatically suppressed expression of Lm-332. These findings suggest that Lm-332 produced by osteoblasts in bone tissues has a pivotal role in controlling normal bone remodeling through suppressing osteoclastogenesis.

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Maintenance of hematopoietic stem cells (HSCs) and differentiation of progenitor cells takes place in specialized bone marrow microenvironments known as ‘niches’.¹ Adhesive interaction of hematopoietic stem and progenitor cells (HSPCs) with extracellular matrix (ECM) ligands produced by non-hematopoietic stromal cells is involved in controlling homeostasis of the HSC population.² HSPCs and precursors of all blood cell lineages express adhesion receptors that enable cell–cell and cell–ECM interactions, and activate downstream signaling pathways, which mediate various cellular functions.³

Laminins, major ECM proteins mainly located in the basement membrane, are heterotrimeric glycoproteins composed of three covalently linked chains, α , β , and γ . Their unique cruciate structures contribute to the organization of the ECM especially in basement membrane.⁴ To date, 16 laminin isoforms have been identified; these are composed of various combinations of each chain (α 1–5, β 1–3, and γ 1–3).⁵

Laminin isoforms bind to various cell surface receptors, which include integrins, syndecans, and dystroglycans. Binding of laminins to their receptors transduces adhesion signaling and regulates cellular functions. Most studies on the interaction between laminins and various hematopoietic cells including macrophages⁶ have been performed using Engelbreth-Holm-Swarm (EHS) tumor-derived laminin-111 (Lm-111). Other laminin family proteins, such as Lm-311, Lm-511, and Lm-521, have also been demonstrated to be expressed in human bone marrow microenvironments.⁷ Lm-511 and Lm-521 bind to human CD34+ hematopoietic progenitor cells, and affect mitogenic activity and migration of these cells through α 6 β 1 integrin.^{7,8} In contrast, Lm-332, which consists of laminin α 3, β 3, and γ 2 chains, is known to be an important component present in skin epidermal basement membrane.⁹ Lm-332 has recently been shown to comprise a component of the ‘niche’ for a number of tissue-specific stem cells. Lm-332 is abundant beneath the

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interfollicular epidermis, where it maintains hair follicle stem cells through the action of integrin-linked kinase (ILK), which is a key regulator of the bulge ECM microenvironments.¹⁰ Lm-332 is also a component of the niche for human hepatic cancer stem cells¹¹ and for tumor cells in B-cell chronic lymphocytic leukemia.¹² Although the expression of Lm-332 has been observed in cultures of human mesenchymal stem cells¹³ and bone marrow stromal cells,¹⁴ it is unclear whether Lm-332 is expressed in bone tissues or bone marrow tissues *in vivo*.

Osteoclasts, multinuclear giant cells specialized in bone resorption, are derived from HSCs, and in normal osteoclastogenesis, differentiate from a common precursor of macrophages and osteoclasts. Their differentiation is basically supported by two essential cytokines, receptor activator of NF- κ B ligand (RANKL), and macrophage colony-stimulating factor (M-CSF)^{15–17} produced by cells in the osteoblast lineage.¹⁸ Intracellular signaling after binding of RANKL to its receptor RANK has been extensively studied. In brief, TNF receptor-associated factor 6 (TRAF6) activates downstream signaling, including MAPK pathways, c-Jun-N-terminal kinase (JNK), and p38MAPK. Subsequently, this signaling activates transcription factors such as c-Fos and c-Jun, components of activator protein-1 (AP-1),^{19,20} and NFATc1,²¹ which induce the expression of genes required for osteoclastogenesis. In addition to RANKL signaling, cell adhesion signaling through ECM proteins contributes to fine regulation of osteoclast differentiation.²² Of the receptors for ECM proteins, integrins, composed of heterodimeric adhesion molecules with α and β subunits, have been extensively studied. Integrin α v β 3 expressed in osteoclasts recognizes the Arg-Gly-Asp (RGD) consensus sequence of ECM proteins, such as vitronectin and osteopontin,^{22–24} which is thought to enable osteoclasts to bind bone surface and to perform bone resorption. Osteoclast precursors circulate throughout the vascular system. We have previously found that Lm-111, a component of the basement membrane of blood vessels, suppresses osteoclastogenesis. We have postulated that the differentiation of osteoclasts is negatively regulated by Lm-111 localized in the basement membrane of blood vessels present throughout the vascular system.²⁵

Here we investigated the expression of Lm-332 in bone tissues, and we examined the role of Lm-332 in the regulation of osteoclast differentiation. We found that Lm-332 is expressed in osteoblasts, and has an important regulatory role in osteoclastogenesis. Our findings suggest the presence of a novel mechanism operating through Lm-332 that controls normal bone remodeling.

MATERIALS AND METHODS

Animals

C57BL/6 mice were obtained from Kyudo (Tosu, Japan). Animal care and experiments were conducted in accordance with guidelines and procedures approved by the Institutional

Animal Care and Use Committee of Kyushu University (protocol number: A27-179-0, A27-179-1).

Immunohistochemical Analysis

Femurs were excised from 5- to 6-week-old male C57BL/6 mice and immersed in 10% formalin neutral buffer solution for 24 h and demineralized in 10% (w/v) ethylene diamine tetraacetic acid disodium salt (EDTA-2Na; Dojindo) at room temperature for a week. The samples were dehydrated with graded ethanol and embedded in paraffin. For immunohistochemistry, the tissue sections of femurs were deparaffinized, rehydrated, and soaked in HistoVT One antigen retrieval solution (Nacalai, Kyoto, Japan). After blocking with Blocking One Histo (Nacalai), the sections were incubated with rabbit polyclonal antibody to laminin β 3 (GeneTex, Irvine, CA, USA) at 4 °C overnight in a humidified chamber, followed by three washes with TBS-Tween (TBST). The sections were further incubated with goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) and counterstained with DAPI for nuclei. The sections were mounted with Fluoromount/Plus (Diagnostic Biosystems, Pleasanton, CA, USA). Images were acquired on a Carl Zeiss fluorescent microscope using the Apotome function and ZEN software (Carl Zeiss, Jena, Germany).

Preparation and Culture of Osteoblasts

Primary osteoblasts were prepared from calvariae of 3–5-day-old C57BL/6 mice by five sequential digestions using 0.1% collagenase I (Wako Pure Chemicals). The cells were grown for 21 days in α -MEM containing 10% FBS in the presence of β -glycerophosphate and ascorbic acid (Wako Pure Chemicals). Alkaline phosphatase (ALP) activity was examined using TRAP/ALP Staining kit (Wako Pure Chemicals) according to the protocol provided in the kit. For 1,25(OH)₂D₃ or PTH stimulation, primary osteoblasts were treated with 10 nM of 1,25(OH)₂D₃ (Cayman, Ann Arbor, MI, USA) or 100 ng/ml of human PTH (1–31) (Bachem Bioscience, King of Prussia, PA, USA) as indicated in each experiment.

Immunofluorescence Analysis

Immunofluorescence analysis was performed as described previously.²⁶ Calvarial osteoblasts prepared from 3- to 5-day-old C57BL/6 mice were grown for 21 days on 35 mm glass bottom dishes (Matsunami Glass, Osaka, Japan) in α -MEM containing 10% FBS in the presence of β -glycerophosphate and ascorbic acid. After fixation with 10% formalin neutral buffer solution for 10 min, the cells were washed with TBST and blocked with Blocking One Histo (Nacalai) for 10 min. The cells were incubated with rabbit polyclonal antibody to laminin β 3 overnight at 4 °C. After washing with TBS, 0.1% Tween, cells were incubated with anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes) at room temperature for 1 h. The cells were then counterstained with DAPI for 5 min. Cells were imaged using BZ-8100 fluorescence microscope (Keyence). Non-immune

rabbit IgG (DAKO, Carpinteria, CA, USA) was used as the negative control in place of a primary antibody.

Cell Culture

Murine osteoclast precursor cell line, RAW264 D clone (RAW-D)^{27,28} was maintained in α -minimal essential medium (α -MEM) (Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Life Technologies) supplemented with 100 U/ml of penicillin and 100 mg/ml of streptomycin at 37 °C in a 95% air/5% CO₂ atmosphere. The human recombinant Laminin-5 (Lm-332) was obtained from Oriental Yeast (Tokyo, Japan). The human Lm-511-E8 was obtained from Nippi (Tokyo, Japan). Culture plates were coated with each ECM protein overnight at 4 °C and washed twice with PBS before use.

Preparation of BMMs and *In Vitro* Osteoclastogenesis

The femurs and tibiae were excised from 5–6-week-old male C57BL/6 mice. Bone marrow cells were cultured for 24 h in α -MEM containing 10% FBS in the presence of M-CSF (20 ng/ml). Non-adherent cells were collected and seeded in a 100 mm dish with α -MEM containing 10% FBS and 20 ng/ml M-CSF (Peprotech, London, UK). After 72 h, adherent cells were used as BMMs. For osteoclastogenesis, BMMs were further cultured in the presence of 20 ng/ml M-CSF and

50 ng/ml RANKL (Peprotech) for 4–5 days, and RAW-D cells were cultured in the presence of 50 ng/ml RANKL for 3 days. BMMs were plated onto 96-well plates and cultured in the presence of M-CSF and RANKL for up to 5 days. The cells were fixed and stained using the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma, Saint-Louis, MO, USA). The numbers of TRAP-positive multinuclear osteoclasts (with more than three nuclei) per well were counted.

Actin Ring Staining

BMMs were cultured in 96-well plates in the presence of M-CSF and RANKL for 4–5 days. The cells were then fixed with 10% formalin neutral buffer solution (WAKO, Osaka, Japan) and stained with fluorescein-labeled phalloidin (Life Technologies) for F-actin and with 4',6-diamidino-2-phenylindole (DAPI; Dojindo Kumamoto, Japan) for nuclei. Images were captured using a BZ-8100 fluorescence microscope (Keyence, Osaka, Japan).

Quantitative Real-Time PCR Analysis

The mRNA expression was analyzed by quantitative real-time PCR with the Thermal Cycler Dice (TAKARA, Shiga, Japan). Total RNA was prepared using an RNeasy Mini kit (Qiagen, Valencia, CA, USA), and the cDNA was then reverse-transcribed using a SuperScript III first-strand cDNA synthesis kit (Life Technologies). PCR amplification was performed in triplicate using a KAPA SYBR FAST qPCR kit (KAPA Biosystems, Boston, MA, USA). The PCR conditions were as follows: enzyme activation at 95 °C for 30 s, 40 cycles of 5 s at 95 °C and 20 s at 60 °C. Expression was calculated using the comparative threshold cycle (CT) method.²⁹ Primer sequences used in this study are shown in Table 1.

Cell Proliferation Analysis

Viable cells were identified using the PrestoBlue fluorescent dye (Life Technologies) according to the manufacturer's instructions. BMMs and RAW-D cells were plated at 1.5×10^4 and 5×10^3 cells/well, respectively, on 96-well plates coated with/without Lm-332. BMMs and RAW-D cells were cultured for 4 and 3 days, respectively, and PrestoBlue fluorescent dye was added to each well, followed by incubation for 1 h at 37 °C. The fluorescence was read (Ex 560 nm/Em 590 nm) on a plate reader (Infinite 200; Tecan, Switzerland).

Western Blot Analysis

Western blots were performed as described previously.³⁰ Cell lysates were prepared by homogenization in cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with protease inhibitor cocktail. Nuclear and cytoplasmic proteins were extracted by NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Rockford, IL, USA). The protein concentration of the supernatants was measured using a detergent-compatible protein assay kit (Bio-Rad). The following primary antibodies were used: anti-c-Jun, anti-Lamin A/C, anti-PARP-1, anti-Tubulin, anti-phospho-p44/42

Table 1 List of primers used for real-time PCR analysis

Gene name	Forward primer	Reverse primer
<i>Trap</i>	CAGCCCTTACTACCGTTTGC	GTAGTCCTCCTTGCGTGCTG
<i>Ctsk</i>	AGGCGGCTATATGACCACTG	TCTTCAGGGCTTTCTCGTTC
<i>ATP6v0d2</i>	CACGGAGATGAGGAAGAAGC	TGGGTGACACTTGGCTAGAA
<i>Dcstamp</i>	GTATCGGCTCATCTCCTCCA	GGAACCCAAGTCTCAGACACA
<i>Itgb3</i>	ACAGAGCGTGTCCCCTAATC	GTCTTCCATCCAGGGCAATA
<i>Calcr</i>	TCTTGGTTGGAGAGGTGCTT	TCGGTTTCTTCTCTCTGGA
<i>Nfatc1</i>	GGAAGAAGATGGTGCTGTC	TTGCGAAAGGTGGTATCTC
<i>c-fos</i>	GGCAGAAGGGGCAAAGTAG	GTTGATCTGTCTCCGCTTGG
<i>Rank</i>	CCAGGAGAGGCATTATGAGC	CTTCATCCAGGTGTCCAAG
<i>Emr1</i>	TTGTACGTGCAACTCAGGACT	GATCCAGAGTGTGATGCAA
<i>Lyz</i>	ATGGAATGGCTGGCTACTATGG	ACCAGTATCGGCTATTGATCTGA
<i>Runx2</i>	AGGGACTATGGCGTCAAACA	GGCTCACGTCGCTCATCTT
<i>Ocn</i>	TAGCAGACCATGAGGACCCT	TGGACATGAAGGCTTTGTGAGA
<i>Rankl</i>	CAGCATCGCTGTCTCTGTA	CTGCGTTTTATGGAGTCTCA
<i>Opg</i>	ACCCAGAACTGGTCATCAGC	CTGCAATACACACTCATCACT
<i>Lama3</i>	CTGTGACTACTGCAATTCTGAGG	CAAGGTGAGGTGACTTGATTGT
<i>Lamb3</i>	GGCTGCCTCGAAATTACAACA	ACCTCCATGTCTTGCCAAAG
<i>Lamc2</i>	CAGACACGGGAGATTGCTACT	CCACGTTCCCAAGGGAT
<i>Actb</i>	CCCTGAAGTACCCATTGAA	GGGGTGTGAAGGTCTCAAA
<i>B2m</i>	CCTGGTCTTCTGGTGCTTG	CCGTTCTTCAGCATTTGGAT

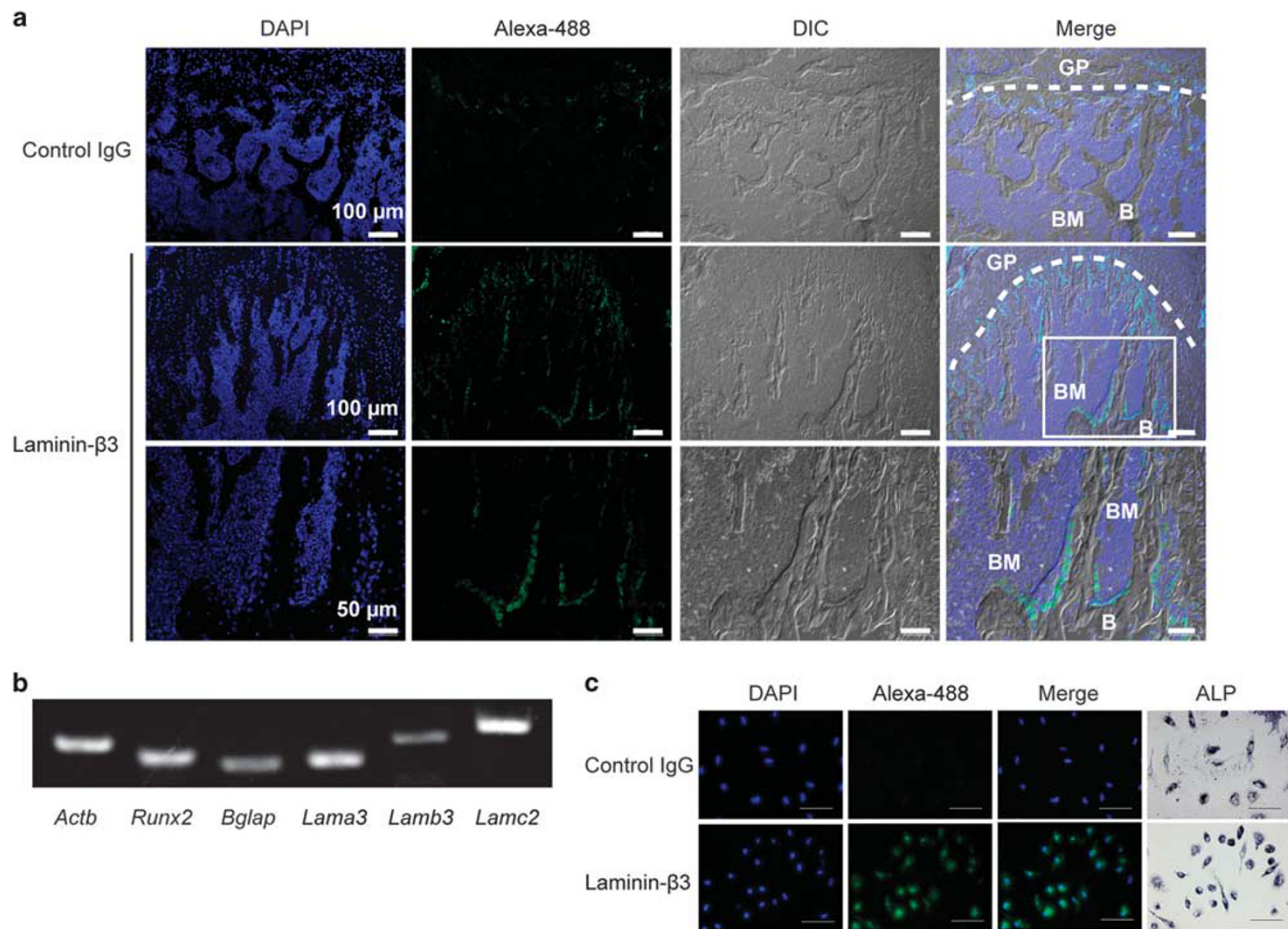


Figure 1 Expression of Lm-332 in murine osteoblasts. **(a)** Immunohistochemical analysis. Longitudinal sections of femur were prepared from 5-week-old male mice. The sections were stained with control non-immune IgG (upper panels) or anti-laminin $\beta 3$ chain antibody (IgG) (middle and lower panels) and detected with anti-rabbit IgG conjugated with Alexa Fluor 488 (green). Nuclei were stained with DAPI (blue). Data show the metaphyseal regions of distal femur. Higher magnification of rectangle region is shown in lower panels. Bars, 100 μ m (upper and middle panels); 50 (lower panels). B, bone; BM, bone marrow; GP, growth plate. Dashed lines mark growth plate. **(b)** Osteoblasts prepared from calvaria of newborn mice were cultured in 24-well plates in the presence of 2.5 mM β -glycerophosphate and 50 μ g/ml ascorbic acid for 14 days to form mature osteoblasts. RT-PCR was used to amplify the indicated mRNA transcripts from mouse osteoblasts (14 days). The *Runx2* and *osteocalcin* (*Bglap*) were used as a positive control for osteoblast maturation. β -actin (*Actb*) was amplified as a positive control. **(c)** The cells were fixed and stained for laminin- $\beta 3$ and ALP as described in the 'Materials and Methods' section.

MAPK (Thr202/Tyr204), anti-phospho-p38MAPK antibody (Thr180/Tyr182), anti-p38, anti-phospho-SAPK/JNK (Thr183/Tyr185), and anti-SAPK/JNK, (Cell Signaling Technology); and anti-NFATc1, anti-c-Fos, anti-ERK1, and HRP-conjugated polyclonal anti-Actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical Analysis

All discrete values are expressed as mean \pm standard deviation (s.d.) from at least three independent experiments. The data were analyzed by paired *t*-test for comparison between two groups or by one-way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons. $P < 0.05$ was considered as statistically significant.

RESULTS

Expression of Lm-332 in Bone Tissues

As Lm-332 is the only laminin isoform that contains the $\beta 3$ chain, we examined the localization of Lm-332 protein in bone tissues using an antibody specific for laminin $\beta 3$ chain. Laminin $\beta 3$ antibody selectively stained cuboidal osteoblast-like cells observed on surface of the bone in the metaphyseal area of the distal femur, but did not stain bone marrow (Figure 1a, lower panels). Control IgG failed to stain any component of the bone (Figure 1a, upper panels). These data suggest that Lm-332 was expressed in cells in osteoblast lineage in bone tissues. To confirm the expression of Lm-332 in osteoblasts, expression of laminin $\alpha 3$, $\beta 3$, and $\gamma 2$ chains in primary osteoblasts was examined by immunostaining and PCR analysis. Calvarial-derived primary osteoblasts were

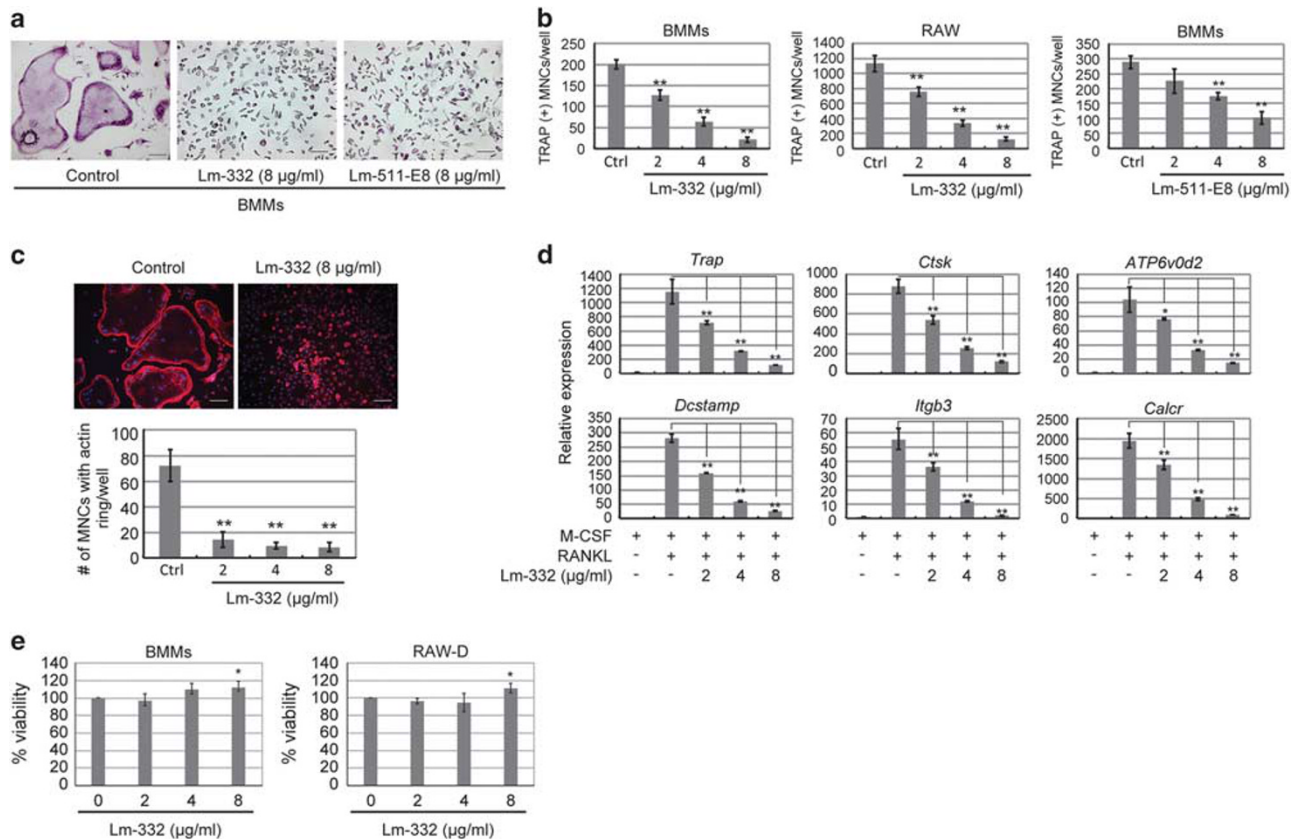


Figure 2 Inhibition of osteoclastogenesis by Laminin-332. BMMs were cultured in 96-well plates coated with indicated concentrations of Lm-332 or Lm-511. BMMs and RAW cells were differentiated into osteoclasts in the presence of 20 ng/ml M-CSF and 50 ng/ml RANKL for 4–5 days or 50 ng/ml RANKL for 3 days. The cells were fixed and stained for TRAP (**a**), and TRAP-positive multinuclear cells with three or more nuclei were counted (**b**). The cells were stained for F-actin using Alexa fluor 647-conjugated phalloidin (red) to visualize actin rings and DAPI (blue) for nuclei. The number of MNCs exhibiting actin rings was quantified (**c**); bars, \pm s.d. $**P < 0.01$ by ANOVA with Tukey's test. mRNA expression of osteoclast differentiation-related molecules was analyzed by quantitative real-time PCR. For each target gene, the expression was normalized to β -2-microglobulin (*B2m*), and the expression level is shown relative to the control (designated as 1) for comparison (**d**); bars, \pm s.d. $**P < 0.01$ by ANOVA with Tukey's test. BMMs and RAW-D cells (5×10^3 cells/well) were cultured in 96-well plates coated with indicated concentrations of Lm-332. Viable cells were identified by PrestoBlue fluorescent dye as described in the 'Materials and Methods' section (**e**); bars, \pm s.d. $*P < 0.05$ by ANOVA with Tukey's test.

cultured in the presence of β -glycerolphosphate and ascorbic acid for 14 days to form mature osteoblasts, which secrete calcified matrices. PCR analysis of mature osteoblasts confirmed the expression of osteoblast markers including Runt-related transcription factor 2 (*Runx2*) and osteocalcin (*Bglap*). All three Lm-332 chain genes (laminin α 3; *Lama3*, laminin β 3; *Lamb3*, laminin γ 2; *Lamc2*; Figure 1b). Laminin β 3 chain was detected in the cytoplasm of mature (ALP-positive) osteoblasts by immunofluorescence (Figure 1c). These data indicate that Lm-332 is likely produced by osteoblasts in bone tissues.

Lm-332 Blocks Osteoclastogenesis in The Presence of RANKL

To evaluate the role of Lm-332 produced by osteoblasts in bone, we examined the effect of Lm-332 on osteoclastogenesis. Primary BMMs and osteoclast precursor cell line RAW-D cells were cultured on plastic cell culture plates coated with

various concentrations (2–8 µg/ml) of Lm-332 in the presence of RANKL. Tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) were generated from BMMs and RAW-D cells in response to RANKL, whereas Lm-332 significantly inhibited the formation of TRAP-positive MNCs in a dose-dependent manner (Figure 2a and b). In addition, osteoclastogenesis was inhibited by Lm-511-E8, a truncated laminin composed of the C-terminal regions of the α , β , and γ chains of Lm-511, and having the same affinity for integrin α 6 β 1 as Lm-332 (Figure 2a and b). We next examined the effect of Lm-332 on actin cytoskeleton organization. MNCs having typical actin rings were observed in control BMMs in response to RANKL; however, Lm-332 suppressed the formation of MNCs with actin rings (Figure 2c). To further examine the inhibitory effect of Lm-332 on osteoclastogenesis, we investigated the expression of osteoclast marker genes, TRAP, Cathepsin K (*Ctsk*), ATPase, H⁺ transporting, lysosomal V0 subunit D2 (*ATP6v0d2*),

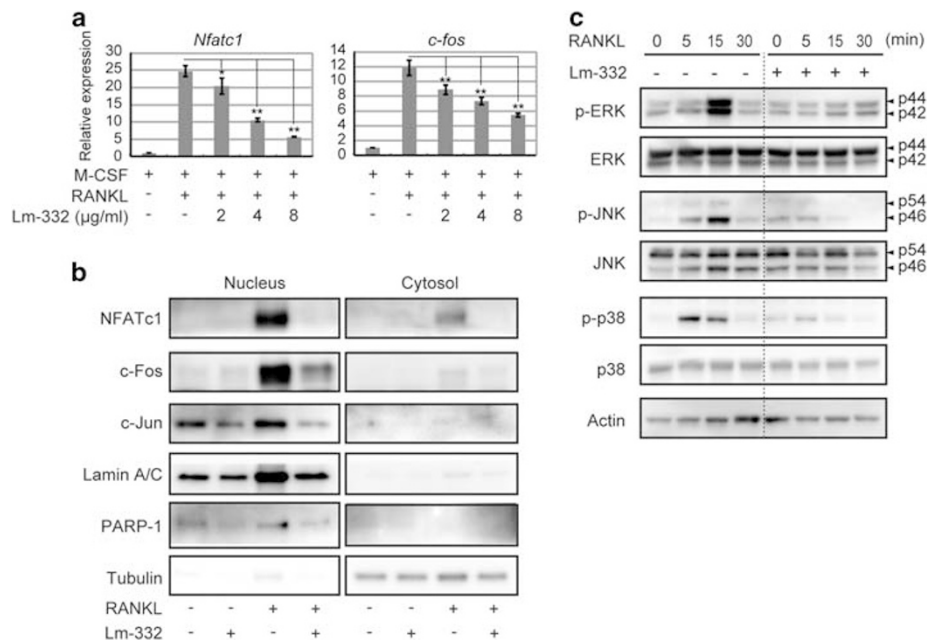


Figure 3 Lm-332 suppresses induction and nuclear localization of AP-1 and NFATc1 and blocks activation of MAPKs. **(a)** BMMs were cultured for osteoclastogenesis (24 h) in 96-well plates coated with the indicated concentrations of Lm-332. mRNA expression of *Nfatc1* and *c-fos* was analyzed by quantitative real-time PCR. For each target gene, the expression was normalized to *B2m*, and the expression level is shown relative to the control (designated as 1) for comparison; bars, \pm s.d. * $P < 0.05$. ** $P < 0.01$ by ANOVA with Tukey's test. **(b)** BMMs were cultured for 24 h with M-CSF and RANKL in 96-well plates coated with Lm-332 (8 μ g/ml) as indicated. Nuclear and cytosolic fractions were extracted and translocation of NFATc1, c-Fos, and c-Jun were analyzed by western blotting. Purity of nuclear or cytosol fractions were evaluated by analyzing Lamin A/C, PARP-1, and Tubulin. **(c)** BMMs were starved in α -MEM supplemented with 0.1% FBS for 5 h in Lm-332-coated (8 μ g/ml) six-well plates, and stimulated with RANKL (300 ng/ml) for indicated times. Lysates were prepared and subjected to western blot analysis with the indicated antibodies. Actin was used as an internal control.

Dendritic cell-specific transmembrane protein (*Dcstamp*), Integrin $\beta 3$ (*Itgb3*), and Calcitonin receptor (*Calcr*). RANKL induced expression of osteoclast marker gene mRNA in BMMs cultured on Lm-332-non-coated culture plates (non-coated plates); whereas, coating plates with Lm-332 significantly suppressed the capacity of RANKL to induce expression of these genes (Figure 2d). Lm-332 did not exhibit any cytotoxicity against BMMs and RAW-D cells, but rather slightly promoted proliferation of both BMMs and RAW-D cells (Figure 2e), indicating that Lm-332 markedly suppressed osteoclastogenesis without diminishing cell viability.

Lm-332 Suppresses Induction and Nuclear Localization of AP-1 and NFATc1 in Response to RANKL

We next examined whether Lm-332 affected the expression in osteoclasts of two fundamental transcription factors downstream of RANKL signaling, AP-1 and NFATc1. Marked increases in *Nfatc1* and *c-fos* mRNA levels were observed in BMMs cultured on non-coated plates 1 day after stimulation with RANKL; whereas, on Lm-332-coated plates their induction was suppressed in a dose-dependent manner (Figure 3a). Induction and nuclear localization of NFATc1, c-Fos, and c-Jun proteins in response to RANKL was potently blocked by Lm-332 in BMMs (Figure 3b).

Lm-332 Blocks RANKL-Induced Activation of MAPK Pathways

RANKL stimulation triggers formation of a RANK-TRAF6 complex, which results in the activation of various downstream molecules, including MAPKs.³¹ We next investigated whether Lm-332 affected MAPK signaling pathways in response to RANKL. BMMs were starved and then stimulated with RANKL. RANKL-induced phosphorylation of ERK, JNK, and p38MAPK was observed within 5 to 15 min in BMMs cultured on non-coated plates; whereas, the RANKL-induced phosphorylation of these MAPKs was markedly suppressed on Lm-332-coated plates (Figure 3c). These data suggested that Lm-332 blocked early signaling events induced by RANKL, such as activation of MAPKs.

Lm-332 Attenuates *Rank* Expression and Retains a Macrophage Phenotype Even in the Presence of RANKL

Adhesion signaling mediated by $\alpha_v\beta_3$ integrin upregulates *Rank* expression, which is essential for RANKL-induced osteoclast differentiation.^{32,33} Laminins are also important transducers of adhesion signals through their receptors, including integrins, in many types of cells. We speculated that Lm-332 might affect *Rank* mRNA expression in osteoclast precursors. To determine this, the expression levels of *Rank* mRNA in BMMs and RAW-D cells were analyzed. We found that BMMs and RAW-D cells cultured on

non-coated plates exhibited a marked increase in *Rank* mRNA levels in response to RANKL, whereas *Rank* mRNA expression was significantly suppressed on Lm-332-coated plates in a dose-dependent manner (Figure 4a). When BMMs were stimulated with RANKL under the non-coated condition, the expression level of *Rank* mRNA peaked at 2 days and remained high through day 4 (Figure 4b). However, BMMs cultured on Lm-332-coated plates failed to induce *Rank* mRNA in response to RANKL (Figure 4b).

To determine whether Lm-332 affected commitment to osteoclast differentiation in response to RANKL, we examined the macrophage markers nonspecific esterase (NSE) activity and expression of lysozyme (*Lyz*) and *Emr1* (Figure 5). When BMMs were cultured on non-coated plates, the proportion of NSE-positive cells was ~80% before RANKL stimulation (day 0); the proportion of NSE-positive cells had significantly decreased (to ~20%) by day 4 after treatment with RANKL, and was accompanied by formation of osteoclasts (Figure 5a, left panel). In contrast, BMMs cultured on Lm-332-coated plates retained NSE activity at day 4 as compared with BMMs before RANKL stimulation (day 0). BMMs cultured on non-coated plates stimulated with RANKL formed osteoclasts and exhibited decreased expression of macrophage marker genes, such as *Lyz* and *Emr1*. In contrast, on day 4, the expression of

Lyz and *Emr1* was not affected when BMMs were cultured on Lm-332-coated plates even in the presence of RANKL (Figure 5b). These results suggested that Lm-332 selectively blocked osteoclastogenesis without affecting macrophage differentiation from bipotential precursors.

Osteoclastogenic Factors Suppress Lm-332 Expression in Osteoblasts

RANKL expression is known to be induced by bone resorbing factors, such as $1\alpha,25$ -dihydroxyvitamin D₃ ($1,25(\text{OH})_2\text{D}_3$) or parathyroid hormone (PTH).^{34,35} In light of the inhibitory effect of Lm-332 on osteoclastogenesis and the expression of Lm-332 in osteoblasts, we analyzed expression of three components of Lm-332 in osteoblasts after treatment with $1,25(\text{OH})_2\text{D}_3$ or PTH. Expression of all three laminin-332 chains (*Lama3*, *Lamb3*, and *Lamc2*) was markedly suppressed after treatment of osteoblasts with $1,25(\text{OH})_2\text{D}_3$ (Figure 6a). Similarly, the expression of all three laminin-332 chains (*Lama3*, *Lamb3*, and *Lamc2*) was also suppressed by PTH treatment (Figure 6b). These data indicated that the osteoclastogenic factors that act by inducing RANKL expression efficiently suppressed production of Lm-332, a key negative regulator of osteoclast differentiation.

DISCUSSION

We provide evidence that laminin $\beta 3$, a specific chain of Lm-332, is present in osteoblast-like cells localized on the bone surface. In our *in vitro* studies, we confirmed that Lm-332 was expressed in primary osteoblasts. We also obtained lines of evidence indicating that Lm-332 had the ability to suppress osteoclastogenesis. In normal osteoclastogenesis, osteoclasts are formed on bone surface. Lm-332 produced from osteoblasts is thought to contribute to a fine modulation of osteoclastogenesis in normal bone remodeling.

We have clarified that both Lm-332 and Lm-511-E8 exert an inhibitory effect on osteoclastogenesis. Among the subunits of laminin, the C-terminal of laminin α chain is utilized for binding to integrins.⁵ Lm-332 preferentially binds to integrin $\alpha 3\beta 1$ and/or $\alpha 6\beta 1$, and proteolytically processed $\alpha 3$ laminin G (LG) domains bind to integrin $\alpha 6\beta 4$.³⁶ Lm-511-E8 is a truncated protein composed of the C-terminal regions of the α , β , and γ chains of Lm-511 that retain the full capability and high affinity for binding to integrin $\alpha 6\beta 1$.^{37,38} Suppression of osteoclastogenesis induced by Lm-332 or Lm-511 is thought to be mediated through integrin $\alpha 6\beta 1$. We have confirmed the expression of integrin $\alpha 6$ and $\beta 1$ in BMMs (data not shown).

Lm-332 blocked RANKL-induced phosphorylation of MAPKs (ERK, JNK, and p38) in BMMs as the early event. Lm-332 suppressed RANKL-induced expression of *NFATc1* and *c-fos* and blocked nuclear localization of these transcription factors. These events are considered to be the main mechanism for inhibition of osteoclastogenesis. It will be of interest to elucidate the types of intracellular signaling that follow binding of Lm-332 to the receptor expressed on

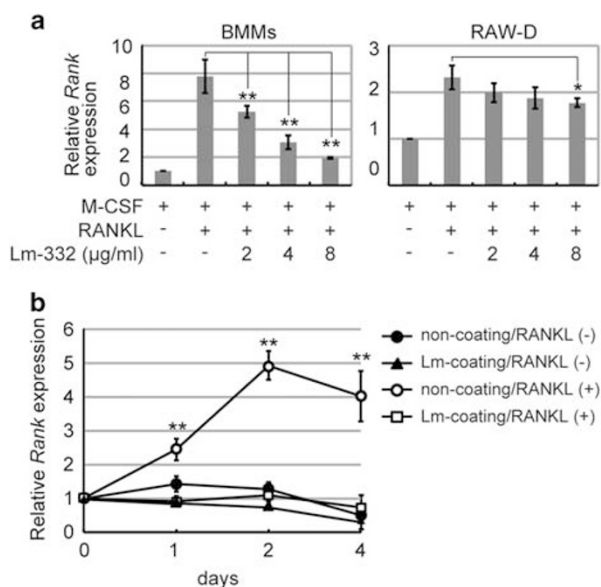


Figure 4 Lm-332 attenuates RANK expression after RANKL stimulation. BMMs and RAW cells were cultured in plates coated with the indicated concentrations of Lm-332 in the presence of M-CSF (20 ng/ml) and RANKL (50 ng/ml) for 4 days. (a) mRNA expression of *Rank* was analyzed by quantitative real-time PCR. The expression was normalized to *B2m*, and the expression level is shown relative to the control (designated as 1) for comparison; bars, \pm s.d. $^{*}P < 0.05$. $^{**}P < 0.01$ by ANOVA with Tukey's test. (b) At each time point, mRNA expression of *Rank* was analyzed. Expression was normalized to *B2m*, and the expression level is shown relative to the day 0 level (designated as 1) for comparison. $^{**}P < 0.01$ compared to BMMs cultured in Lm-332-coated plates in the presence of RANKL by ANOVA with Tukey's test.

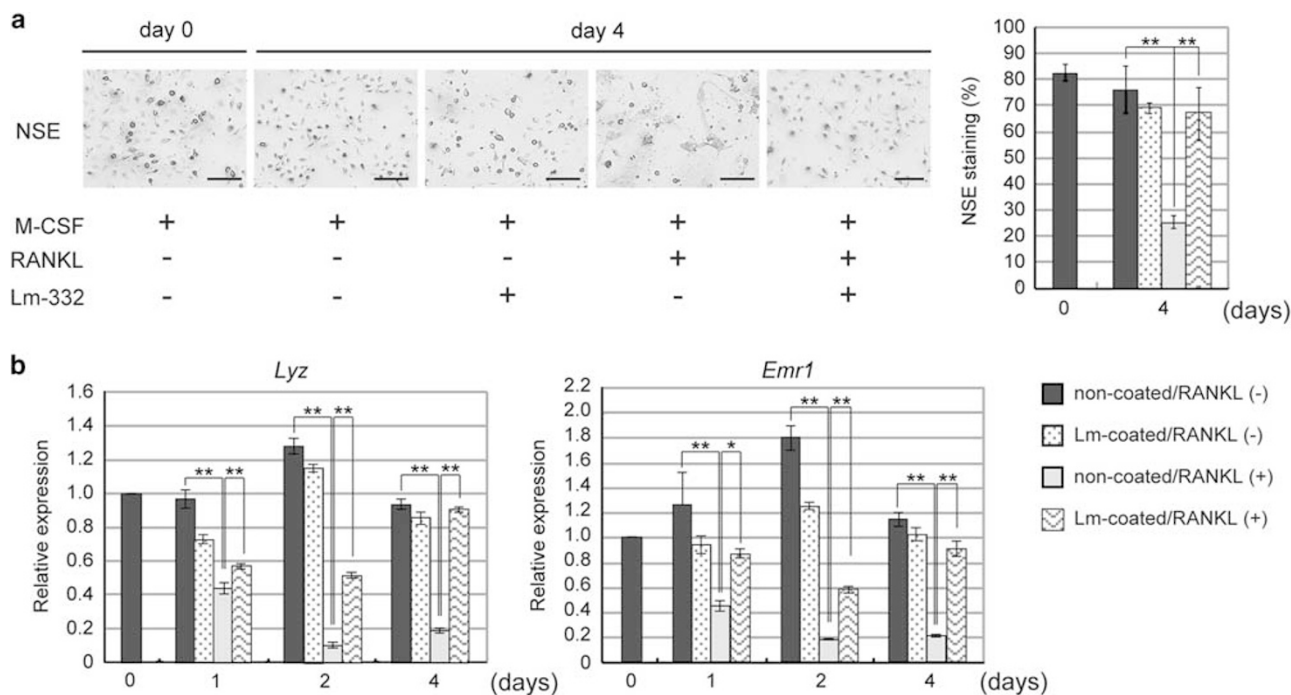


Figure 5 BMMs cultured in Lm-332-coated plates retain macrophage characteristics in the presence of RANKL. **(a)** Nonspecific esterase activity was determined using the α -Naphthyl Acetate Esterase Kit (Sigma) according to the manufacturer's instructions. BMMs exhibit activity of the macrophage-specific enzyme α -NSE, which is detectable by characteristic black cytoplasmic staining (left); bars, 50 μ m. The proportion of NSE-positive cells to total cells at day 4 was quantified (right); bars, \pm s.d. $^{**}P < 0.01$ by ANOVA with Tukey's test. **(b)** BMMs were cultured in the presence of M-CSF (20 ng/ml) and RANKL (50 ng/ml) in Lm-332-coated (2–8 μ g/ml) or non-coated plates for 1, 2, or 4 days. mRNA expression of macrophage markers (*Lyz* and *Emr1*) was analyzed by quantitative real-time PCR. For each target gene, the expression was normalized to *B2m*, and the expression level is shown relative to the control (designated as 1) for comparison; bars, \pm s.d. $^{**}P < 0.01$ by ANOVA with Tukey's test.

osteoclast precursors. Lm-332 also blocked the induction of RANK in BMMs stimulated with RANKL, suggesting that an adhesion signal through Lm-332 might affect RANK induction. Mochizuki *et al.* reported that a cell adhesion signal, possibly through $\alpha v \beta 3$ integrin, is essential for RANK induction in osteoclast precursors during RANKL-induced osteoclastogenesis.³³ Interestingly, they showed that BMMs cultured under non-adherent conditions retained mature macrophage phenotypes and failed to express *Rank* mRNA. Similar to their report, we found that BMMs cultured on Lm-332-coated plates failed to induce *Rank* mRNA even under RANKL-stimulation, and the BMMs retained macrophage phenotypes evidenced by NSE activity and expression of *lyz* and *Emr1*. Our results show that Lm-332 maintains macrophage phenotypes of BMMs even in the presence of RANKL. Under the non-coated culture condition, BMMs exhibited significant induction of *Rank* mRNA (~8-fold) in response to RANKL-stimulation, whereas in RAW-D cells the induction was moderate (~2-fold). This might be due to differences in the cell population between BMMs and RAW-D. BMMs are M-CSF responsive cells prepared from bone marrow cells, in which the number of RANK-positive osteoclast precursors is low before exposure to RANKL. When these cells were treated with RANKL in the presence of

M-CSF stimulation, *Rank*-expressing early osteoclast precursors proliferated to form large numbers of RANK-positive late precursors. In contrast, RAW-D is an osteoclast precursor cell line that was established from one cell that was highly responsive to RANKL-stimuli. As RAW-D cells at baseline express high levels of *Rank* mRNA,²⁸ it is reasonable that the *Rank* mRNA level upregulated only twofold in response to RANKL. It will be important to determine whether upregulation of *Rank* mRNA expression by RANKL is suppressed by Lm-332 by use of the reporter assay system using *Rank* promoter.

It has been proposed that there are two types of bone marrow niches for HSC maintenance: the endosteal niche, populated by osteoblasts, and the vascular niche, which includes sinusoids and endothelial cells.¹ Osteoclasts are derived from HSCs, and their differentiation is supported by bone marrow stromal cells or osteoblasts through cell–cell interactions.¹⁸ In the current study, immunohistochemical analysis for laminin $\beta 3$ indicated that the fluorescence signal was detected in cells lining limited areas of the bone surface, indicating that Lm-332 is possibly expressed in a subset of cells in the osteoblast lineage. We have confirmed the expression of all chains of Lm-332 in primary osteoblasts by use of RT-PCR and immunofluorescence staining.

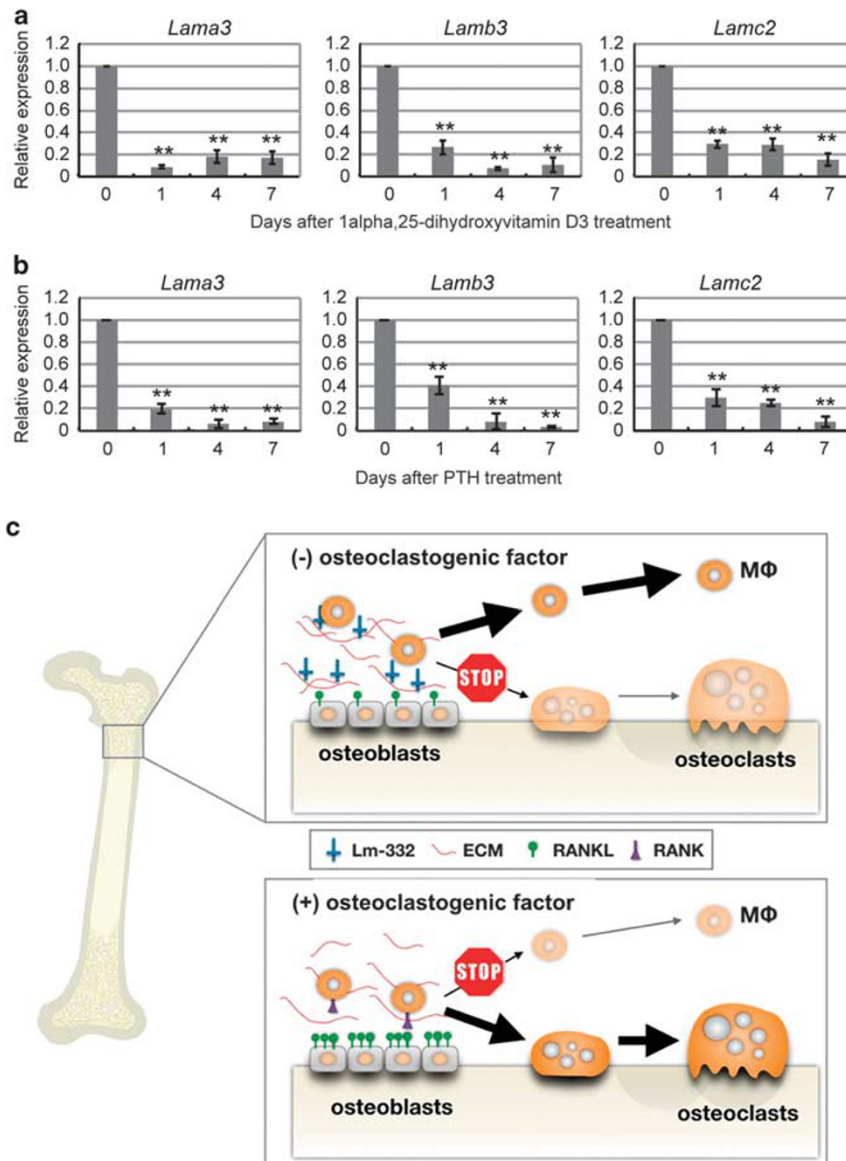


Figure 6 Suppression of Lm-332 expression by treatment with osteoclastogenic factors. Osteoblasts prepared from calvaria of newborn mice were treated with 10 nM of 1α,25-dihydroxyvitamin D₃ (a) or 100 ng/ml of PTH (b) for 7 days. mRNA expression of laminin-α3 (*Lama3*), -β3 (*Lamb3*), and -γ2 (*Lamc2*) was analyzed by quantitative real-time PCR. For each target gene, the expression was normalized to *Actb*, and the expression level is shown relative to the control of day 0 culture (designated as 1) for comparison; bars, ± s.d. ***P* < 0.01 by paired *t*-test. (c) Hypothetical model of the role of Lm-332 in modulation of osteoclastogenesis. In the absence of osteoclastogenic factors, Lm-332 secreted by osteoblasts suppresses osteoclastogenesis and supports the maintenance of the macrophage population. In the presence of osteoclastogenic factors such as 1α,25-dihydroxyvitamin D₃ or PTH, Lm-332 production in osteoblasts is markedly suppressed, which allows promotion of osteoclastogenesis through RANK/RANKL signaling. MΦ, macrophage.

Osteoblasts secrete proteins such as OPG and Semaphorin 3A, both of which exert osteoprotective effects by suppressing osteoclast differentiation and function.³⁹ Osteoclast differentiation is induced by RANKL, which is expressed by osteoblasts and bone marrow stromal cells in response to osteotropic factors such as 1,25(OH)₂D₃, prostaglandin E2 or PTH. All three components of Lm-332 were detected in primary osteoblasts, but their expression was markedly suppressed by treatment with osteoclastogenic factors that

induce RANKL, such as 1,25(OH)₂D₃ and PTH. As illustrated in our model shown in Figure 6c, the interplay of these factors determines the formation or inhibition of osteoclasts (Figure 6c). Our findings suggest that Lm-332 is a novel osteoprotective factor produced by osteoblasts.

The results presented here provide novel evidence indicating that Lm-332 exerts inhibitory effects on RANKL-induced osteoclastogenesis. This effect was accompanied by inhibition of induction of *Rank* mRNA expression and retention of

macrophage phenotypes by BMMs. In addition, Lm-332 was expressed by primary osteoblasts in culture, and its expression was negatively regulated by osteoclastogenic factors, suggesting that Lm-332 is a novel negative regulator that may define osteoclastogenesis spatiotemporally in bone tissues. It will be important to elucidate the molecular mechanisms by which adhesion signaling via Lm-332 regulates osteoclastogenesis. Insights from our findings may contribute to the development of strategies for the fine regulation of bone metabolism.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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