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Conditional expression of constitutively active estrogen receptor α in osteoblasts increases bone mineral density in mice

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ABSTRACT

Estrogen plays an important role in maintaining bone density in women. Estrogen receptor (ER) is expressed in osteoblasts and osteoclasts; however, the precise mechanism of ER in bone is not fully understood. In the present study, we generated a conditional transgenic mouse caERα^{Coll} that expresses the constitutively active ERα in osteoblasts using collagen type I promoter-driven Cre transgenic mice. The caERα^{Coll} mice showed increased bone mineral density (BMD). Osteoblasts prepared from caERα^{Coll} mice expressed high levels of osteoprotegerin and decreased levels of IL-6, both of which are known to regulate osteoclast differentiation. These results suggest that ERα regulates osteoprotegerin and IL-6 production in osteoblasts and modulates BMD. The conditional transgenic mouse model is useful for understanding the in vivo function of ERα.

1. Introduction

Estrogen, a sex steroid hormone regulates bone metabolism that is controlled by a balance between bone resorption by osteoclasts and bone formation by osteoblasts [1]. Estrogen is beneficial for bone health. The physiological function of estrogen is mediated by the estrogen receptors (ER)α and ERβ [2]. ERs are transcription factors that regulate the expression of estrogen-targeted genes in response to hormone binding. ERs are expressed on both osteoblasts [3,4] and osteoclasts [5].

The mechanisms by which estrogen increases bone mass and its deficiency leads to bone loss are not fully understood. Estrogen deficiency increases the expression of receptor activator of nuclear factor κ B ligand (RANKL) [6]. RANKL is expressed on the surface of osteoblasts. Binding of RANKL to its receptor RANK on pre-osteoclasts is necessary for the differentiation and proliferation of pre-osteoclasts into mature osteoclasts [7]. RANKL is neutralized by its soluble decoy receptor osteoprotegerin (OPG), which is produced and secreted by osteoblasts [8]. It has been suggested that estrogen suppresses RANKL production [9] and increases OPG production [10] by osteoblastic cells. Therefore, estrogen deficiency induces an increase in the RANKL/OPG ratio and leads to an imbalance in bone resorption over bone formation [11,12]. Estrogen treatment has been shown to inhibit osteoclast formation from mononuclear hematopoietic stem cells [13,14]. In addition, several lines of evidence indicate that estrogen directly stimulates osteogenic activity and protects against osteoblastic apoptosis [15,16]. These results imply that ER has a crucial role in osteoblasts; however, its in vivo function is poorly understood.

To examine the role of ERα in a mouse model, we utilized a constitutively active ERα (caERα) mutant that had a substitution of tyrosine to serine at 537 (ERα Y537S) [17]. It is known that even in the absence of ligand stimulation, the ERα Y537S exhibits high transcription activity. Thus, we assumed that the ERα Y537S is useful for analyzing the gain-of-function of ERα in vivo. We generated conditional transgenic mice that express the caERα in osteoblasts by using Coll-Cre mice [18]. These mice displayed increased bone mineral density (BMD). In addition, primary osteoblasts prepared from caERα^{Coll} mice expressed higher levels of OPG and lower levels of IL-6 compared to those from wild-type (WT) mice. This mouse model shows a crucial role of ERα in the bone and aids in our understanding of in vivo function of ERα.

Abbreviations: BV/TV, bone volume per tissue volume; MAR, mineral apposition rate; ES/BS, eroded surface per bone surface; Oc.S/BS, osteoclast surface per bone surface; N.Oc/BS, osteoclast number per bone surface

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2. Materials and methods

2.1. Construction of constitutive active ERα and luciferase assay

The Flag-tagged pcDNA3 (Invitrogen, San Diego, CA) construct pcDNA3-Flag-ERα was prepared by insertion of open reading frame of human ERα into pcDNA3 containing Flag tag. Constitutively active ERα (caERα) containing Y537S substitution was generated by site-directed mutagenesis using pcDNA3-Flag-ERα according to the previous report [17]. HeLa cells at a density of 1×10^4 cells/well on 24-well plates were transfected with a firefly luciferase reporter gene ERE-tk-Luc (0.8 μg), a control Renilla luciferase reporter gene pRL-CMV (0.7 μg) (Promega, Madison, WI), 5 ng of expression vectors for caERα or ERα in phenol red-free DMEM containing 10% dextran-coated charcoal-treated FCS (dccFCS) using Lipofectamine 2000 transfection reagent (Invitrogen). Twelve hours after transfection, cells were treated with or without 10 nM 17β-estradiol (E2) or 1 μM ICI182,780 (ICI) for 24 h and luciferase assay was performed. Data were represented as the mean ± S.D. of three independent experiments.

2.2. Construction of the transgene

A CAG-GFP^{floxed}-caERα transgene plasmid was constructed using a pCALwL vector which contains a CAG promoter, being a composite promoter consisting of a cytomegalovirus IE enhancer, a chicken β-actin promoter, two functional loxP sites, and a rabbit β-globin polyadenylation signal [19]. cDNAs for GFP fused to Histone H2B [20] and caERα were subcloned into sites between the two loxP sites and upstream of the polyadenylation signal, respectively.

2.3. Generation of transgenic mice

The plasmid CAG-GFP^{floxed}-caERα was linearlized with restriction enzyme digestion. Transgenic mice were produced by microinjecting the linearlized plasmid into the pronuclei of fertilized eggs from C57BL/6 mice as described previously [21]. Transgenic mice were identified by PCR assays of genomic DNA extracted from the tail, and transgene expression was monitored by GFP fluorescence using a stereomicroscope. Primers derived from the 5′- and 3′-flanking sequences of GFP (5′-ACGTGCTGGTTGTTGTGCTGTCTCTCATCA-3′ and 5′-TGATTTGATCCCCGGGTACCGAGCGAC-3′) were used to amplify a 1.9 kb product. ColI-Cre mice were kindly provided by Dr. G. Karsenty [18], and ROSA26LacZ reporter mice were obtained from The Jackson Laboratory (Bar Harbor, ME). For Cre transgenic mice, primers derived from Cre (5′-CCTGGAAAATGCTTCTGTCCGTTTGCC-3′ and 5′-GAGTTGATAGCTGGCTGGTGGCAGATG-3′) were used to amplify a 653-bp product. For ROSA26LacZ transgenic mice, primers; 5′-GCGAAGAGTTTGTCCTCAACC-3′, 5′-AAAGTCGCTCTGA GTTGTTAT-3′ and 5′-GGAGCGGGAGAAATGGATATG-3′ were used to amplify a 340-bp product for the transgene and a 650-bp product for wild-type allele (The Jackson Laboratory). CAG-GFP^{floxed}-caERα transgenic mice were mated with ColI-Cre transgenic mice to obtain the conditional transgenic mice caERα^{ColI}. ColI-Cre/ROSA26LacZ reporter mice were generated by a cross-breeding of ROSA26LacZ and ColI-Cre mice and used to track the activity of the ColI promoter throughout the ontogeny of the mouse. All animal experiments were approved by the Institutional Animal Care and Use Committee.

2.4. LacZ staining

Assessment of β-galactosidase (β-gal) activity was performed according to the method of Nagy et al. with a slight modification [21]. Embryos (10 dpc) and frozen sections (10 μm thick) of spine from neonatal ColI-Cre/ROSA26LacZ mice (P1) were fixed with a 0.25% glutaraldehyde solution for 10 min. The sections were then washed three times and incubated with a staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 0.1% phosphate buffer, pH 7.5, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.02% NP-40, 0.01% sodium deoxycholate, and 2 mM $MgCl_2$) for 4–5 h to visualize β-gal activity.

2.5. Bone histomorphometry

To assess the parameters for bone histomorphometry, 15-week-old female mice of caERα ($n = 7$) and WT ($n = 9$) were double labeled with subcutaneous injections of 30 mg/kg of tetracycline hydrochloride (Sigma, St. Louis, MO) at 4 days before sacrifice, and 16 mg/kg of calcein (Dojindo, Kumamoto, Japan) at 2 days before sacrifice. Tibiae were removed from each mouse, and fixed with 70% ethanol. They were trimmed to remove the muscle, stained with Villanueva bone stain for 7 days, dehydrated in graded concentrations of ethanol, and embedded in methyl-methacrylate (Wako Chemicals, Kanagawa, Japan) without decalcification. Frontal plane sections (5-μm-thick) of the proximal tibia were cut using a Microtome (LIECA, Germany). The cancellous bone was measured in the secondary spongiosa located 500 μm from the epiphyseal growth plate and 160 μm from the endocortical surface. Bone histomorphometric measurements of the tibia were made using a semiautomatic image analyzing system (System Supply, Nagano, Japan) and a fluorescent microscope (Optiphot; Nikon, Tokyo, Japan) set at a magnification of 400×. Standard bone histomorphometrical nomenclatures, symbols, and units were used as described in the report of the ASBMR Histomorphometry Nomenclature Committee [22]. Bone mineral density (BMD) of the femora was measured using a PIXImus instrument (Lunar Corp., Madison, WI). Statistical analysis was done using Student's *t*-test.

2.6. Quantitative PCR and measurement of OPG protein level

Primary mouse osteoblastic cells were isolated from calvariae of 5-day-old neonates of caERα^{ColI} mice and wild type littermates as described previously [23]. The cells were maintained in α-MEM containing 10% FCS and antibiotics. Total RNA was extracted from the cells at the third passage using the ISOGEN reagent (Nippon Gene, Tokyo, Japan). To examine estrogen-regulated gene expression, primary osteoblastic cells prepared from wild type mice were seeded in phenol-red free α-MEM containing 10% dextran-coated charcoal-stripped FCS and infected with recombinant adenovirus expressing caERα (Ad-caERα) or fluorescent protein DsRed (Ad-DsRed) at m.o.i. 10 for 24 h. The adenoviral vectors, Ad-caERα and Ad-DsRed, were constructed using the Adenovirus Expression Vector Kit (Takara, Tokyo, Japan) following the manufacturer's protocol. The cells were treated with 10 nM 17β-estradiol (E2), 1 μM ICI182,780 (ICI) or vehicle for 24 h and then total RNA was isolated. Quantitative real-time RT-PCR (qPCR) analysis was performed as described previously [24]. The sequences of PCR primers were as follows: OPG, 5′-GCCTGGGACCAAAGTGAATG and 5′-TCTTGTGA GCTGTGTCTCCGTTT; RANKL, 5′-TGTCGTTAAAACCAGCATCAAAAT and 5′-TTTCGTGCTCCCTCCTTTCA; IL-6, 5′-TCCTACCCCAATTTC-CAATGC and 5′-GTCCTTAGCCACTCCTTCTGTGA; and ERα, 5′-ACA-GACACTTTGATCCACCTGATG and 5′-AGATGCTCCATGCCTTTGTTA CTA. The primers for ERα were designed in common sequences of human and mouse ERα. To assess the caERα transgene expression, primers; 5′-ATGGACTACAAGGACGATGATGAC-3′ and 5′-GCAGTAGGGCCATCCCAGAT-3′ corresponding to Flag and human ERα sequences, respectively, were used. The comparison of PCR product amounts was carried out by the comparative cycle threshold (CT) method, using Gapdh as a control. The amount of OPG released into the culture medium was determined using a mouse OPG/TNFRSF11B Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Values were

normalized with the amount of total protein that was purified from a protein-containing fraction of the RNA isolation procedure. The experiments were independently repeated at least three times, each performed in triplicate. Results were shown as mean ± S.D. Statistical analysis was performed using Student's *t*-test.

2.7. Osteoclast formation assay

Wild type mouse bone marrow cells from long bones were cultured in α-MEM containing 10% FCS and 5 ng/ml M-CSF (PeproTech, Rocky Hill, NJ) for 16 h. Non-adherent cells were harvested and cultured for three more days in the presence of 30 ng/ml M-CSF. Floating cells were removed and adherent cells were used as osteoclast precursors. The cells were further cultured in medium supplemented with 30 ng/ml M-CSF and 50 ng/ml RANKL (PeproTech) for 7 days. Equal volume of conditioned medium prepared by caERα^{Coll} primary osteoblasts were added to the medium. The culture plate was stained for TRAP-positive multinuclear cells (TRAP + MNCs) using the leukocyte acid phosphatase (TRAP) kit (Sigma–Aldrich). TRAP + MNCs containing more than three nuclei were counted.

3. Results

3.1. Generation of caERα

To assess the function of ERα in vivo, we generated a constitutively active mutant of human ERα with a Y537S substitution (Fig. 1A). Studies have demonstrated that this tyrosine 537 is an important phosphorylation site with potential roles in regulating ERα ligand binding, homodimerization, and transactivation [25]. A luciferase assay using estrogen-responsive element (ERE)-driven reporter plasmids showed that caERα enhances ERE-driven transcription regardless of estrogen (Fig. 1B). The caERα transcription activity could be suppressed by the anti-estrogen ICI182,780 (Fig. 1B). The transcriptional activity of caERα was the same level to wild type ERα (Fig. 1C). We utilized this caERα mutant to examine the in vivo gain-of-function effects of ERα.

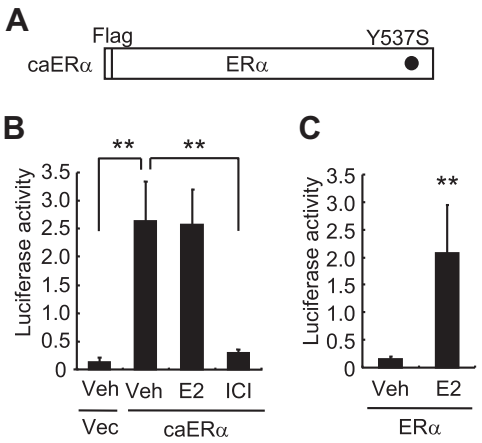

Fig. 1. Construction of constitutively active ERα (caERα). (A) Schematic representation of caERα that has a substitution of tyrosine to serine at position 537. The caERα-expressing plasmid was generated by mutagenesis using Flag-tagged ERα cDNA. (B) Transcription activity of caERα. HeLa cells were transfected with the estrogen responsive element-driven luciferase reporter (ERE-tk-Luc), the luciferase reporter for internal control (pRL-CMV), and the expression plasmid for caERα in phenol red-free DMEM containing 10% dccFCS. After transfection, the cells were treated with or without 10 nM 17β-estradiol (E2) or 1 μM ICI182,780 (ICI) for 24 h, and then luciferase assays were performed. (C) Transcription activity of ERα. Luciferase assay was performed using an expression vector for wild type ERα instead of caERα. Data were represented as the mean ± S.D. of three independent experiments. Statistical analysis was performed using Student's *t*-test, $^{**}P < 0.01$.

3.2. Generation of conditional transgenic mice that express caERa in osteoblasts

To investigate the in vivo effects of ERα overexpression, we employed a conditional transgenic mouse system to express the caERα transgene (Fig. 2A). We first established a floxed GFP-caERα transgenic mouse line bearing the CAG-GFP^{floxed}-caERα transgene. The CAG promoter has the potential for ubiquitous expression in various tissues. We expected that mice bearing this construct would express GFP but not caERα due to the poly(A) signal sequence that is located immediately after the GFP sequence. In the presence of Cre recombinase, the GFP sequence would be deleted and caERα would be expressed instead of GFP under CAG promoter control. The CAG-GFP^{floxed}-caERα construct was introduced into pronuclear stage eggs of C57BL/6 by microinjection, and we obtained CAG-GFP^{floxed}-caERα transgenic mice. Genomic polymerase chain reaction (PCR) using GFP-specific primers was used to verify the transgene integration (Fig. 2B). Fluorescent stereomicroscopic analysis of the tail (Fig. 2C) from the transgenic mice confirmed expression of the GFP protein.

We utilized Coll-Cre mice [18] to achieve conditional caERα expression in osteoblasts. In order to inspect Cre expression in osteoblasts, we generated mice heterozygous for the Coll-Cre and ROSA26LacZ transgenes by breeding a homozygous ROSA26LacZ mice with homozygous Coll-Cre mice. Embryos and spines were prepared from heterozygous neonates at 10 dpc and P1, respectively, and monitored by X-gal staining (Fig. 2D and E). As shown in the figure, LacZ-positive signals were detected in osteoblasts in these tissues. The osteoblast-specific expression of Cre recombinase to induce recombination in this Coll-Cre mouse has been previously demonstrated by Karsenty and co-workers [18]. Next, we generated the conditional transgenic mice caERα^{Coll} that express the caERα protein in osteoblasts.

3.3. Increased BMD of mice expressing caERα in osteoblasts

Dual energy X-ray absorptiometry was used to measure the femoral BMD of caERα^{Coll} female transgenic mice compared with WT controls. Fig. 3A shows an increase in femoral BMD observed in the caERα^{Coll} compared with WT controls. The effects of caERα on bone remodeling were further analyzed by histomorphometry at the proximal tibia. As shown in Fig. 3B, caERα^{Coll} mice displayed increased osteoid formation within the marrow cavity. Fig. 3C indicated that the marks produced by calcein and tetracycline appeared to be similar in width. The caERα^{Coll} mice significantly increases bone mass – bone volume per tissue volume (BV/TV) (Fig. 3D). As shown in Fig. 3H, a histological index of bone resorption, the osteoclast number per bone surface (N.Oc/BS) was significantly decreased in caERα^{Coll} mice compared with WT mice. Additional histological indices of bone resorption, both the bone surface covered by osteoclasts (Oc.S/BS) and the eroded surface (ES/BS), tended to decrease (Fig. 3F and G). Unexpectedly, the caERα^{Coll} mice revealed no difference in mineral apposition rate (MAR) (Fig. 3E). These data suggest that caERα overproduction in osteoblasts decelerates bone resorption and elevates bone mass.

3.4. OPG mRNA and protein are upregulated in caERα-expressing osteoblasts

To further examine the mechanism of increase in BMD in caERα-^{Coll} mice, expression levels of OPG, RANKL and IL-6 mRNA were quantified in primary osteoblasts prepared from calvaria. Transgene expression was confirmed in the primary osteoblasts prepared from caERα^{Coll} mice (Fig. 4A). Total ERα expression levels of mouse endogenous ERα and the transgene caERα were examined using primers that designed in common regions between mouse and human ERα

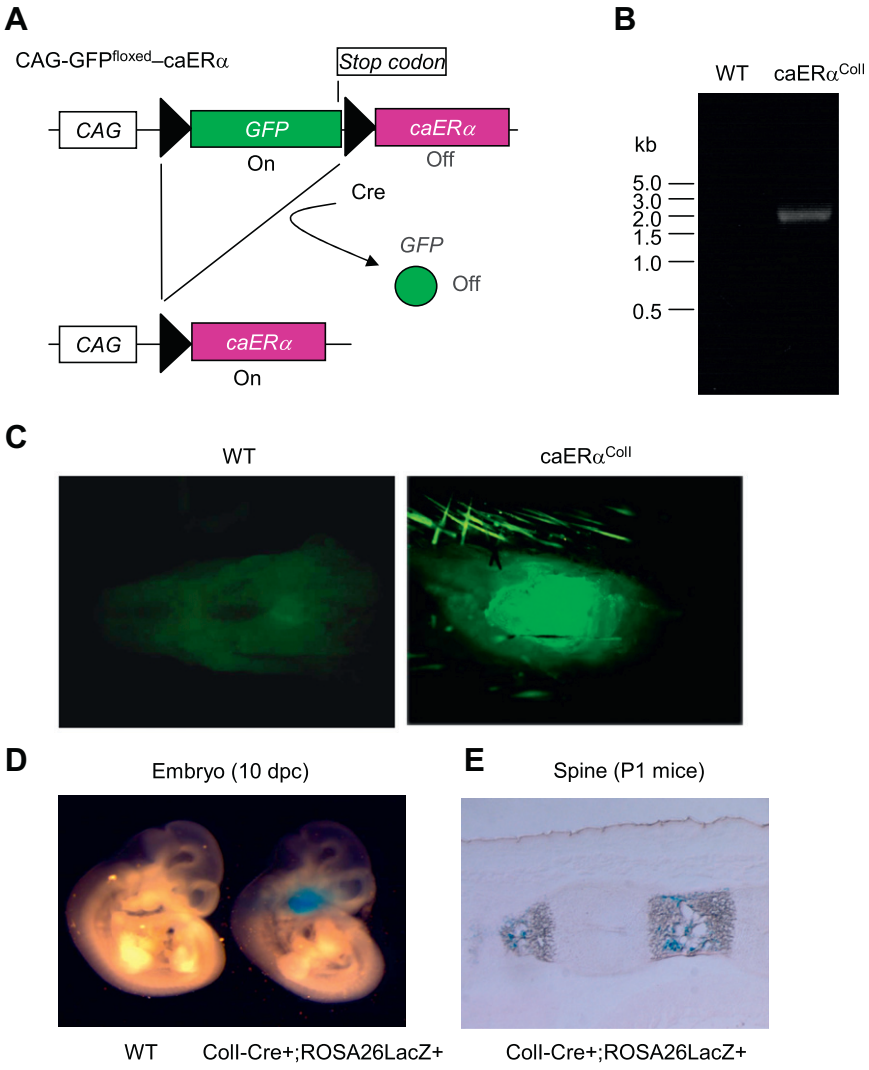

Fig. 2. Generation of conditional transgenic mice expressing caERα in osteoblasts. (A) Schematic representation of CAG-GFP^{Floxed}-caERα transgene. The construct expresses GFP but not caERα since the loxP-flanked GFP gene has a stop codon at the end of the gene. In the presence of Cre recombinase, the GFP sequence would be excised and caERα would be expressed instead of GFP under CAG promoter control. (B) Genotyping of caERα transgenic mice. Transgenic mice were identified by PCR assays of genomic DNA extracted from the tail using primers derived from the 5′- and 3′-flanking sequences of GFP. A positive signal was detected as a 1.9-kb product. (C) Stereomicroscopic image of tail from CAG-GFP^{Floxed}-caERα transgenic mouse. GFP fluorescence (green) was visualized by stereomicroscopy. (D and E) A Coll-Cre transgenic mouse was mated with a ROSA26LacZ tester mouse, and the resulting embryos (10 dpc) and spines from Coll-Cre+; ROSA26LacZ+ newborn mice (P1) were stained with X-gal.

mRNA (Fig. 4B). The figure indicated that caERα^{Coll} primary osteoblasts express ERα mRNA by more than 4-fold compared to wild type primary osteoblasts. As shown in Fig. 4B, OPG mRNA was significantly upregulated in primary osteoblasts from caERα^{Coll} mice. In addition, IL-6 mRNA was downregulated in caERα^{Coll} primary osteoblasts, but RANKL mRNA was not (Fig. 4D and E). OPG protein levels were obviously higher in culture media from primary osteoblasts derived from caERα^{Coll} mice compared to those from WT mice (Fig. 4F). These results suggest that estrogen receptors stimulate OPG expression in osteoblasts, whereas they inhibit IL-6 expression.

To examine the effect of estrogen on these osteoclast-associated gene expressions, primary osteoblasts derived from wild type mice were infected with or without recombinant adenovirus expressing the caERα or DsRed for control, and treated with E2 or ICI. The overexpression of caERα was confirmed in the Ad-caERα infected primary osteoblasts (Fig. 4G). As shown in Fig. 4H, qPCR assay showed that OPG mRNA was upregulated by either E2 treatment or Ad-caERα infection. The expression levels of RANKL mRNA were not changed by these treatments; however, IL-6 mRNA was downregulated by either E2 treatment or Ad-caERα infection (Fig. 4I and J). Moreover, ICI inhibited the estrogen-mediated increase of OPG mRNA and reversed E2-dependent repression of IL-6 mRNA. These data indicated that the caERα signaling mimics the endogenous ERα signaling in wild-type osteoblasts, and the differential gene expression of OPG and IL-6 in caERα-expressing osteoblasts will contribute to the alteration of bone tissue with reduced bone resorption and high BMD in caERα transgenic mice.

Next, we examined whether osteoclast differentiation is impaired in the presence of medium conditioned by caERα^{Coll} primary osteoblasts. In support of our findings, the number of TRAP-positive multinuclear cells were decreased in the medium conditioned by caERα^{Coll} primary osteoblasts compared to that of wild-type primary osteoblasts (Fig. 4K and L).

4. Discussion

In the present study, we generated conditional transgenic mice using a Cre/loxP system to explore the in vivo function of human ERα in osteoblasts by using the caERα mutant. CAG-GFP^{Floxed}-caERα transgenic mice expressing GFP but not caERα were gener-

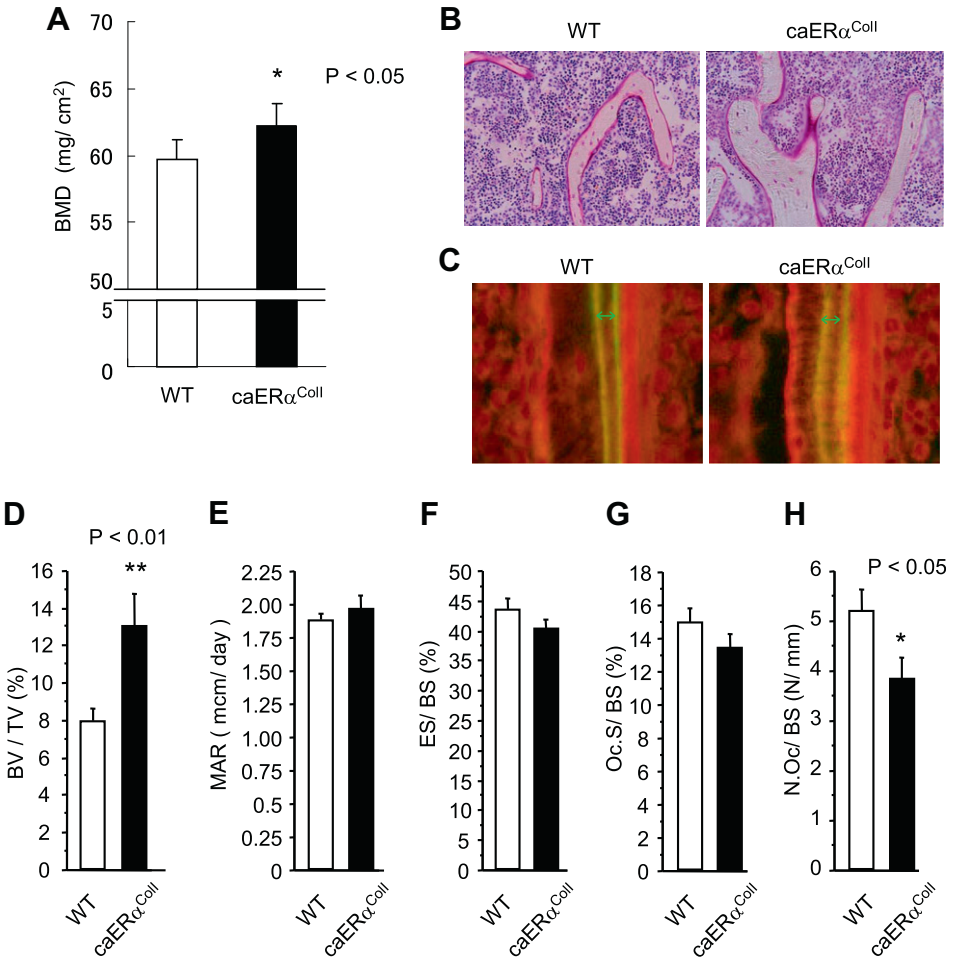

Fig. 3. Increased bone mineral density (BMD) of caERα^{Coll} mice. (A) BMD of 15-week-old female mice from caERα^{Coll} (n = 7) and WT (n = 9) were measured using dual energy X-ray absorptiometry (DEXA). Values are represented as mean ± S.D. *Significantly different versus control at $P < 0.05$. (B) Bone histomorphometric analysis was performed as described in Section 2. Panels are representative sections stained with Villanueva bone stain. (C) Fluorescence microscopy of bone sections derived from the caERα^{Coll} and WT mice. The widths between the labeling lines of calcein and tetracycline are indicated. (D–H) Bone turnover parameters as measured by dynamic bone histomorphometry after calcein and tetracycline labeling. Parameters are measured in the proximal tibia of 15-week-old caERα^{Coll} (n = 7) and WT (n = 9) female mice. BV/TV: bone volume per tissue volume; MAR: mineral apposition rate; ES/BS: eroded surface per bone surface; Oc.S/BS: osteoclast surface per bone surface; N.Oc/BS: osteoclast number per bone surface. Values are represented as mean ± S.D. Statistical analysis was performed using Student's t-test, $^{*}P < 0.05$; $^{**}P < 0.01$.

ated and mated with ColI-Cre mice to allow for caERα expression in osteoblasts. The caERα^{Coll} mice had higher BMD compared with the WT mice. Histomorphometric analysis revealed that the number of osteoclasts was significantly decreased in caERα^{Coll} mice. Furthermore, primary osteoblasts from caERα^{Coll} mice expressed higher levels of OPG mRNA and protein and lower levels of IL-6 mRNA. Therefore, these results suggested that ERα plays a critical role in gene expression associated with osteoclast differentiation in osteoblasts and affects bone resorption by osteoclasts.

OPG is a naturally occurring soluble member of the TNF receptor superfamily that binds to RANKL [26]. OPG acts as a secreted decoy receptor to neutralize RANKL and negatively regulate osteoclast differentiation, activity, and survival [8]. The ratio of RANKL to OPG is assumed to be a critical determinant for osteoclast development and bone resorption. OPG prevents bone loss when administered to ovariectomized rats [8], induces osteoporosis when ablated in knockout (KO) mice [27,28], and induces osteopetrosis when overexpressed in transgenic mice [8]. 17β-Estradiol also dose-dependently increases OPG mRNA and protein levels in cultured osteoblastic cells [10]. Our data strongly support that osteoblastic ERα mediates OPG secretion.

Estrogen also modulates the production of bone-resorbing cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, macrophage colony-stimulating factor (M-CSF), prostaglandins, and transforming growth factor-β by osteoblasts or osteoclasts [29]. We demonstrated in this study that primary osteoblasts prepared from caERα^{Coll} had decreased levels of IL-6 mRNA that is known to be produced by osteoblasts and induces bone resorption. It is also possible that ERα in osteoblasts can affect cytokine production by osteoblasts, affecting on osteoclasts.

The caERα^{Coll} mice showed no change of MAR in bone histomorphometric analysis, therefore, it is speculated that differentiation of osteoblasts expressing caERα is not affected in vivo. However, some reports showed that ERα and estrogen promote osteoblast differentiation [30,31]. Our results do not rule out this possibility because differentiation of caERα^{Coll} osteoblasts is not investigated. In addition, we speculate that the female caERα^{Coll} mice would resist to bone loss induced by ovariectomy. The caERα^{Coll} mice will be also useful to study the ERα function in male. These points will be addressed by future studies.

Gene KO mice disrupting ERα and ERβ were utilized to analyze ER function in bone tissues [32,33]. However, bone loss and high bone turnover were not markedly observed in these KO mice [1,33]. This unexpected maintenance of bone mass is considered to be caused by increased circulating estradiol and testosterone levels because ER inactivation impairs the negative feedback sys-

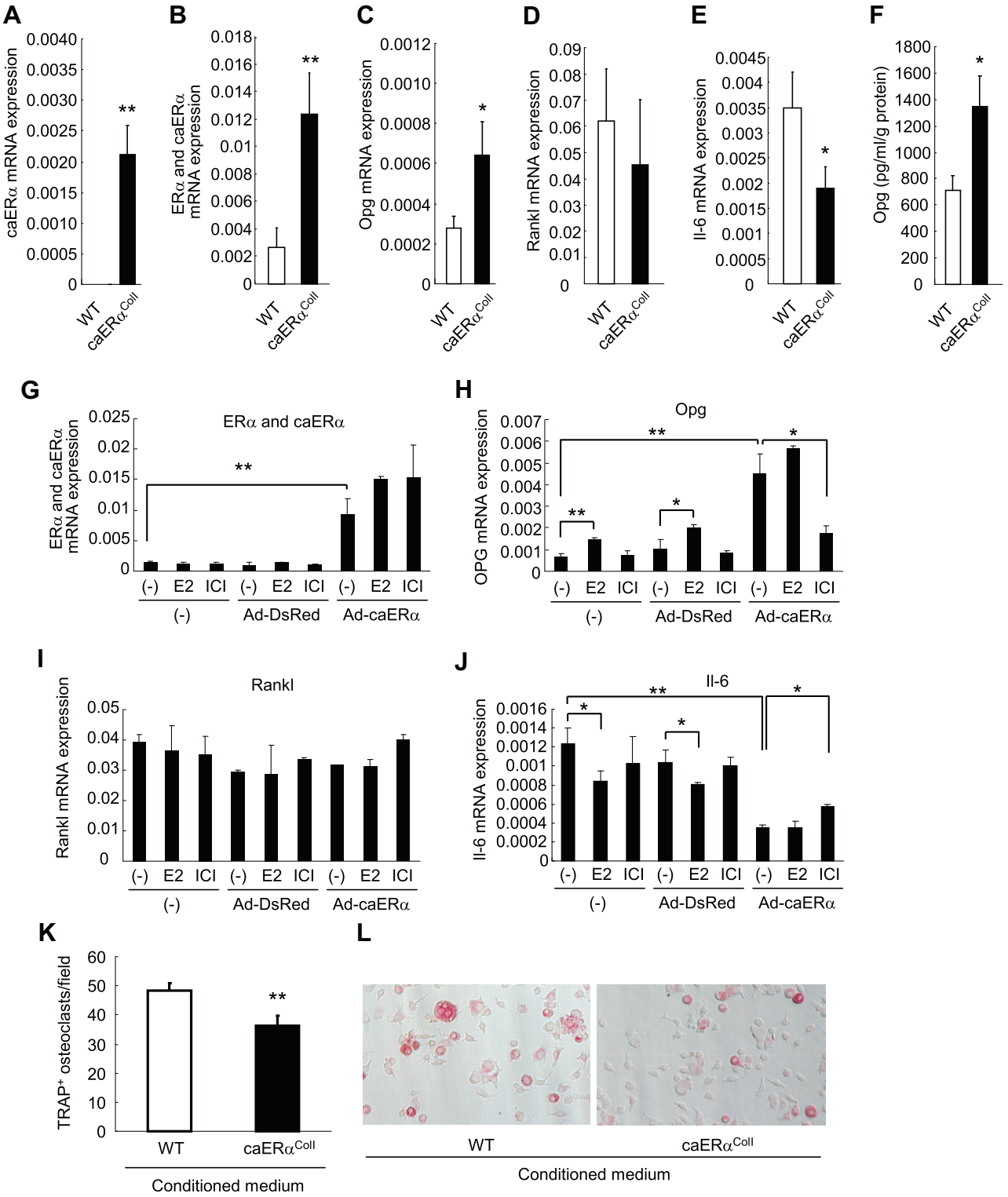

Fig. 4. Increased osteoprotegerin (OPG) expression in primary osteoblasts derived from caERα^{Coll} mice. Primary mouse osteoblasts were isolated from calvariae of 5-day-old neonates of caERα^{Coll} mice and their wild-type littermates. Total RNA was extracted from the cells and subjected to quantitative real-time RT-PCR (qPCR) analysis with the primers for amplifying caERα transgene (A), both mouse endogenous ERα and caERα transgene (B), OPG (C), RANKL (D) and IL-6 (E). Comparison of the PCR product amounts was carried out using the comparative cycle threshold (CT) method using GAPDH as a control. (F) The amount of OPG in the culture media of primary osteoblasts was determined using a mouse OPG immunoassay kit. Values were normalized with the amount of total protein. (G–J) Estrogen regulation of OPG, RANKL and IL-6 mRNA in primary osteoblasts. Primary osteoblasts isolated from wild-type mouse were infected with or without recombinant adenovirus expressing the caERα or DsRed (control). After 24 h, the cells were treated with 10 nM 17β-estradiol (E2), 1 μM ICI182,780 (ICI) or vehicle for 24 h, and then qPCR was performed using the primers for amplifying both mouse endogenous ERα and caERα transgene (G), OPG mRNA (H), RANKL mRNA (I) and IL-6 mRNA (J). (K and L) Conditioned medium prepared from caERα primary osteoblasts inhibits osteoclast differentiation. Bone marrow cells were cultured in the presence of conditioned medium prepared from caERα and wild-type primary osteoblasts for 7 days and then subjected to TRAP staining (original magnification, ×200) (L). TRAP-positive multinuclear cells containing more than three nuclei were counted (K). The experiments were independently repeated at least three times, each performed in triplicate. Results were shown as mean ± S.D. Statistical analysis was performed using Student's *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$.

tem of hormone production through the hypothalamus [1]. Nakamura et al. generated osteoclast-specific ERα KO mice and found that the mutant females exhibited trabecular bone loss, a finding similar to the osteoporotic bone phenotype in postmenopausal women [34]. The osteoclast-specific ERα KO mice did demonstrate that ERα is required for the induction of Fas ligand (FasL) expression and estrogen-mediated apoptosis in osteoclasts. These observations provide an explanation for the osteoprotective function of estrogen [34]. Transgenic mouse models were also generated to investigate the effects of ERα overexpression, although they were designated or utilized to investigate ERα function in reproductive organs of female mice [35,36]. In the present study, we generated a conditional transgenic mouse model that revealed an in vivo role of ERα in osteoblasts. This gene-engineered animal model will be a powerful tool for understanding cell-specific and tissue-specific ERα functions in vivo.

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