Ⅱ．研究成果の刊行に関する一覧表

書籍

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竹内純	エピジェネティクスで組織可塑性を理解する		実験医学	羊土社	東京	2012	2896-2901
中村遼、塚原由布子、竹内純	心臓発生と心疾患のエピジェネティクス　－クロマチンモデリング因子・ヒストン修飾因子が織りなす複雑な臓器発生機構のモデルとして－		実験医学	羊土社	東京	2012	2923-2931
森田唯加、小柴和子、竹内純	心臓発生とその分子メカニズム		血管医学	メディカルレビュー社	東京	2012	97-113
塚原由布子、小柴和子、竹内純	心臓発生にかかわるクロマチン・ヒストン制御の役割		Heart View	メジカルビュー社	東京	2011	55-63

雑誌

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Koshiba-Takeuchi K, Morita Y, Takeuchi JK.	Heart cells survival by defined factors.	Etiology and Morphogenesis of Congenital Heart Disease (book) by Springer (in press)			2014
Kondoh S, Inoue K, Igarashi K, Sugizaki H, Shirode-Fukuda Y, Inoue E, Yu T, Takeuchi JK, Kanno J, Bonewald LF, Imai Y.	Estrogen receptor α in osteocytes regulates trabecular bone formation in female mice.	Bone	60	68-77	2014
Xu B, Hrycaj SM, McIntyre DC, Baker NC, Takeuchi JK, Jeannotte L, Gaber ZB, Novitch BG, Wellik DM	Hox5 interacts with Plzf to restrict Shh expression in the developing forelimb.	Proc. Natl. Acad. Sci. USA.	110	19438 -19443.	2013
van Weerd JH, Koshiba-Takeuchi K, Kwon C, Takeuchi JK.	Epigenetic factors and cardiac development.	Cardiovascular Res.	91	203-211	2011
Takeuchi JK, Lou X, Alexander JM, Sugizaki H, Delgado-Olguín P, Hollowray AK, Mori A, Wylie JN, Munson C, Zhu Y, Yu-Qing Zhou, Ru-Fang Yeh, Henkelman M, Harvey RP, Metzger D, Chambon P, Stainier DYR, Pollard KS, Scott IC, & Brunea u BG	Chromatin Remodeling Complex Dosage Modulates Transcription Factor Function in Heart Development.	Nature Communications	1187	1-11	2011

Ⅲ. 研究成果の刊行物・別刷

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Original Full Length Article

Estrogen receptor α in osteocytes regulates trabecular bone formation in female mice

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ABSTRACT

Estrogens are well known steroid hormones necessary to maintain bone health. In addition, mechanical loading, in which estrogen signaling may intersect with the Wnt/β-catenin pathway, is essential for bone maintenance. As osteocytes are known as the major mechanosensory cells embedded in mineralized bone matrix, osteocyte ERα deletion mice ($ER\alpha^{\Delta Ocy/\Delta Ocy}$) were generated by mating ERα floxed mice with Dmp1-Cre mice to determine the role of ERα in osteocytes. Trabecular bone mineral density of female, but not male $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice was significantly decreased. Bone formation parameters in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ were significantly decreased while osteoclast parameters were unchanged. This suggests that ERα in osteocytes exerts osteoprotective function by positively controlling bone formation. To identify potential targets of ERα, gene array analysis of Dmp1-GFP osteocytes sorted by FACS from $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and control mice was performed. Gene expression microarray followed by gene ontology analyses revealed that osteocytes from $ER\alpha^{\Delta Ocy/\Delta Ocy}$ highly expressed genes categorized in 'Secreted' when compared to control osteocytes. Among them, expression of Mdk and Sostdc1, both of which are Wnt inhibitors, was significantly increased without alteration of expression of the mature osteocyte markers such as Sost and β-catenin. Moreover, hindlimb suspension experiments showed that trabecular bone loss due to unloading was greater in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice without cortical bone loss. These data suggest that ERα in osteocytes has osteoprotective functions in trabecular bone formation through regulating expression of Wnt antagonists, but conversely plays a negative role in cortical bone loss due to unloading.

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Introduction

Estrogens clearly maintain physiological homeostasis through the development of reproductive organs and the mammary gland, potentiation of muscles, and through osteoprotection. The osteoprotective actions of estrogens are clearly demonstrated by post-menopausal osteoporosis [1]. The effects of sex steroid hormones on bone tissue can be considered as the combination or sum of the direct effects on bone cells and the indirect effects on other tissues [2]. The indirect effects of estrogen on bone through other tissues have been well described, such as modulation of cytokine production by immune cells and the increased induction of pituitary gland hormones [3,4]. However, the direct effect of estrogens on bone tissue is not fully understood.

Estrogens exert their effects by binding to their own nuclear receptors, such as Estrogen Receptor (ER) α and β, which also function as transcription factors. The conventional ERα null mouse model could not be used to address the direct functions of the receptor in bone due to hormonal imbalance and endocrine disturbances [5–7]. Therefore, the generation and analyses of bone cell type specific deletion is required to clarify the functions of ERα in bone.

Osteoclastic ERα null mice were generated showing that osteoclastic ERα shortens the life span of osteoclasts by promoting apoptosis [8,9]. Ovariectomy can induce osteocyte apoptosis [10] and conventional ERα null mice do not increase bone mass in response to anabolic mechanical loading [11]. Moreover, various groups reported murine skeletal phenotype due to ERα deletion in cells of the osteoblast lineage, suggesting ERα in osteoblastic lineage cells could play important roles in the maintenance of bone metabolism [12–15]. Recently, Windahl

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et al. [13] reported that ERα in osteocytes regulates trabecular bone formation and thus trabecular bone volume in male mice. These results are in contrast to our own findings showing that the precise molecular functions and target genes of ERα in osteocytes still remain elusive.

Osteocytes are embedded in the extracellular matrix of bone and represents more than 90% of the cells existing in bone. Osteocytes possess dendrites that extend throughout the bone and are used to communicate with each other and also with osteoblasts and osteoclasts on the surface of the bone. The function of osteocytes as mechanosensory cells is inferred from their shape and location [16]. In fact, mechanical loading and unloading change osteocyte gene expression *in vivo*, indicating that osteocyte function is affected by loading conditions [17–20]. In addition, they are known to be involved in mineral metabolism through expression of proteins such as FGF23, Phex, Mepe, and Dmp1 [21–24] (for review, see [25]). Recently, it has been postulated that osteocytes can orchestrate skeletal homeostasis through mineral metabolism as well as the regulation of osteoblastic bone formation and osteoclastic bone resorption by secretory proteins such as sclerostin and FGF23. Osteocytes are also reported to regulate osteoblastic bone formation through IGF-1, TGFβ, NO, PGE_2 and sclerostin and to regulate osteoclastic bone resorption through TGFβ, NO, and PGE_2, and RANKL/OPG [26].

Bone mass can be maintained by mechanical loading while unloading or immobilization decreases bone mass. *In vivo* unloading rodent models such as tail suspension can induce bone loss in hind limbs [27] and mechanical loading can increase bone mass in forelimbs [28]. The regulation of bone mass by mechanical loading is mediated, at least in part, through β-catenin signaling [29–31], and estrogen/ER signaling might also be involved in this mechanism [32].

In this study, we examined the functions of ERα in osteocytes by generating mice lacking ERα in osteocytes and analyzing osteocyte gene expression profiles and subjecting them to hindlimb unloading.

Materials and methods

Animals

The ERα floxed mutant ($ER\alpha^{L2/L2}$) mice kindly provided by Dr. Chambon and null alleles with a C57BL/6 J background have been previously described [5]. $ER\alpha^{L2/L2}$ mice were crossed with $Dmp1^{Cre}$ mice [33] to generate $Dmp1^{Cre}$; $ER\alpha^{L2/+}$ mice, and $Dmp1^{Cre}$; $ER\alpha^{L2/L2}$ ($ER\alpha^{\Delta Ocy/\Delta Ocy}$) and $ER\alpha^{L2/L2}$ ($ER\alpha^{flox/flox}$) were obtained by crossing $Dmp1^{Cre}$; $ER\alpha^{L2/+}$ and $ER\alpha^{L2/L2}$. *Dmp1-GFP* mice were kindly provided by Dr. Ivo Kalajzic [34]. All mice were housed in a specific-pathogen-free facility under climate-controlled conditions with a 12-hour light/dark cycle and were provided with water and standard diet (CE-2, CLEA, Japan) *ad libitum*. All animals were maintained and examined according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

Genome DNA extraction and cell culture

Various tissues (0.5 g) from $ER\alpha^{\Delta Ocy/\Delta Ocy}$ were harvested, washed with PBS and lysed in 2 ml of lysis buffer with proteinase K (150 μg/ml) overnight. Also, DNA of osteocytes was isolated from the calvariae of $ER\alpha^{\Delta Ocy/\Delta Ocy}$ in which cells on the surface of the bone such as osteoclasts and osteoblasts were removed by sequential enzymatic treatment. Primary osteoblasts obtained from the neonatal calvariae were cultured in αMEM (Life Technologies) containing 10% FBS (Cell Culture Bioscience), 50 μg/ml ascorbic acid (Sigma-Aldrich) and 10 nM β-glycerophosphate (Sigma-Aldrich) for 21 days. Cells were cultured with phenol red free media 24 h before cells were treated with 17β-estradiol. Primary osteoclasts were differentiated from the bone marrow obtained from 6-week-old $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice using 10 ng/ml of M-CSF (R&D Systems) and 234 ng/ml of GST-RANKL (Oriental Yeast) for 5 days. The genomic DNA was extracted using phenol/chloroform and isopropanol precipitation.

ELISAs

Enzyme-linked Immunoassays, ELISAs, were was performed following the protocols of the Estradiol EIA Kit (Cayman Chemical Company) for estradiol, Testosterone EIA Kit (Cayman Chemical Company) for testosterone, and Rodent Luteinizing Hormone (LH) ELISA TEST (Endocrine Technologies) for LH.

Bone analyses

The BMD of femurs and tibiae obtained from 12-week-old littermates were measured by DXA using a bone mineral analyzer (DCS-600EX: ALOKA). Micro Computed Tomography scanning of the tibiae and femurs was performed using a Scanco Medical μCT35 System (SCANCO Medical) with an isotropic voxel size of 6 μm for trabecular analyses and 12 μm for cortical analyses according to the manufacturer's instructions and the recent guidelines of the American Society for Bone and Mineral Research (ASBMR) [35]. For bone histomorphometry, the mice were double-labeled with intra-peritoneal injections of 16 mg/kg of calcein (Sigma) at 5 and 2 days before sacrifice. Lumbar vertebral bodies were removed from each mouse and fixed with 4% PFA in PBS overnight. Lumbar vertebrae were embedded with MMA after dehydration and the plastic sections were cut by a standard microtome (LEICA) into 7 μm for von Kossa staining and 4 μm for TRAP and Toluidine-blue staining. The region of interest was the secondary spongiosa of L3 and L4. Sections were used for analyses when the bases of the bilateral transverse processes were opened. The region of interest (ROI) in the lumbar vertebral body is the secondary spongiosa, which is separated from the primary spongiosa, cranial and caudal growth plate, according to the same protocol as previously performed [8,36]. Histomorphometric analyses were performed using OsteoMeasure (OsteoMetrics, Inc., GA, USA) according to the ASBMR guideline [37].

Isolation of Dmp1-GFP positive osteocytes by FACS

A highly purified population of osteocytes was isolated from neonatal calvariae by FACS using a modified version of the protocol of Paic F et al. [38]. Cells were isolated from 10-day-old fetal mice calvariae of $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ also expressing *Dmp1-GFP*. After removal of the sutures, pooled calvarial tissue was subjected to six sequential, 30-minute digestions in a mixture containing 0.05%/0.2 mM trypsin/EDTA and 1.5 U/ml collagenase-P (Roche) at 37 °C. Cell fractions 4 to 6 were collected, pooled, and re-suspended in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% FBS (Hyclone) and centrifuged. Cells were rinsed with PBS and re-suspended in PBS/2% FBS and filtered through a 70-μm filter. Cell sorting was performed using a BD FACS Aria cell sorter. The gate for collecting GFP + cells was set as GFP + population to represent 10% to 15% of the total cells in GFP + mice and 0.8% to 1.0% of total cells in GFP − mice (negative control). GFP + cells were collected in a tube with 500 μl of PBS/3% FBS.

Gene expression microarray

Gene expression microarray was generated using total RNA extracted from the isolated GFP + osteocytes of $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ as previously described [8] and RNA samples were evaluated using the Affymetrix Mouse Genome 430 2.0 Array following standard Affymetrix protocols (GEO: GSE41997). Gene ontology analyses were performed using DAVID Bioinformatics Resources 6.7 [39].

RNA extraction and RT-qPCR

Total RNA from the pulverized femurs or sorted cells was extracted using TRIZOL (Invitrogen) and RNeasy purification kit (QIAGEN). First-strand cDNA was synthesized from total RNA using PrimeScript RT Master Mix (TaKaRa) and subjected to RT-qPCR using SYBR Premix Ex Taq II

(TaKaRa) or KAPA SYBR Fast qPCR Kits (KAPA Biosystems) with Thermal Cycler Dice (TaKaRa) according to the manufacturer's instructions. Primers were purchased from Takara Bio Inc. (Otsu, Japan) or Operon Biotechnologies (Tokyo, Japan) [8]. Gene expression levels were normalized by *Gapdh* or *Rplp0*. Primer sequences were as follow; *Rplp0*: F 5′-TTCCAGGCTTTGGGCATCA-3′ and R 5′-ATGTTCAGCATGTTCAGCAGTGTG-3′, *Gapdh*: F 5′-AAATGGTGAAGGTCGGTGTG-3′ and R 5′-TGAAGGGGTCGTTGATGG-3′, *ERα*: F 5′-CATGGTCATGGTAAGTGGCA-3′ and R 5′-TCTCTGGGCGACATTCTTCT-3′, *Dmp1*: F 5′-TGAAGAGAGGACGGGTGATT-3′ and R 5′-TCCGTGTGGTCACTATTTGC-3′, *Kera*: F 5′-TGGGATGTCCACGACGACTT-3′ and R 5′-AAGGCAGTAGGGAAACTGGGA-3′, *Mdk*: F 5′-TGGAGCCGACTGCAAATACAA-3′ and R 5′-GGCTTAGTCACGCGGATGG-3′, *Sostdc1*: F 5′-AAATGTATTTGGTGGACCGC-3′ and R 5′-GAATCAAGCCAGGAATGGAG-3′.

Tail suspension

Tail suspension experiments were performed for female $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$ mice for 4 weeks starting at 8 weeks of age according to previous reports [40,41]. Briefly, a stainless steel harness was superglued to the sides of the tail. Female $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$ mice were then suspended from an eye bolt which was secured into the bars of the top of the rat cage. The animal could rotate 360° with the fish swivel and could also move backwards and forwards about 7.5 cm. Water was provided through a standard water bottle with an extra long angled sipper tube to allow the animals to reach the water. Control female $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$ mice were chained to the cage top during the same period of time, but were allowed to load their hindlimbs to minimize the difference in stress-related effects between the tail-suspended groups and the control groups ($n = 6$ per group).

Statistical analysis

Data were analyzed by a two-tailed student's *t*-test or one-way analysis of variance (ANOVA) to initially determine whether an overall statistically significant change existed before using Tukey's *post hoc* test. For all graphs, data are represented as mean ± SEM. A *p*-value less than 0.05 was considered statistically significant.

Results

Generation of osteocytic ERα deletion mice

To investigate the function of ERα in osteocytes, we generated mice lacking ERα in late-osteoblasts/osteocytes by crossing ERα floxed mice with Dmp1-Cre mice, which express Cre recombinase driven by the Dmp1 promoter. The mice harboring the genotypes of $Dmp1^{Cre}$; $ERα^{L2/L2}$ and $ERα^{L2/L2}$ were analyzed as $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$, respectively. First, to assess cell type specificity of the deletion of the ERα gene locus by Dmp1 promoter-driven Cre recombinase, genomic PCR was performed using DNA extracted from $ERα^{ΔOcy/ΔOcy}$. As a result, a relatively specific deletion of ERα in osteocytes, which were isolated by sequential enzymatic digestion, was detected as an L-band, which was seen only in osteocytes and not in primary cultured osteoblasts or osteoclasts (Fig. 1A). In addition, the ERα mRNA level was examined by qPCR using RNA extracted from femoral bones and GFP-mediated FACS sorted osteocytes of $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$ mice. As a result, there was an approximately 30% and 90% reduction of ERα expression in whole bone and osteocytes, respectively, in $ERα^{ΔOcy/ΔOcy}$ compared to $ERα^{flox/flox}$ mice (Fig. 1B). This significant but low percent deletion in whole bone might reflect ERα expression by other cell types, which are present in the intact femur even though the bone marrow was removed. Also, one group reported that clear deletion of the target gene was detected at the genome level but not the mRNA level when using the Dmp1-Cre mice [42]. Next, body weight was measured every other week from 3 to 12 weeks old. There was no significant difference in body weight between $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$, whereas it was previously reported that ERα total KO mice exhibited a significant increase in body weight [43] (Fig. 1C). Next, we asked if these mice could be a suitable model for analyzing ERα function without the systemic influence of hormones (endocrine disturbances) as described in the conventional ERα null mouse, by examining the concentration of sex steroid hormones. Serum estradiol, testosterone and luteinizing hormone concentrations were measured by ELISA, showing that there were no significant differences between the 12-week-old $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$, regardless of gender (Fig. 1D). Since $ERα^{ΔOcy/ΔOcy}$ mice exhibited a relatively specific deletion of ERα in osteocytes and normal serum sex steroid hormone levels, we concluded that $ERα^{ΔOcy/ΔOcy}$ could be used for analysis of ERα function in osteocytes without the complications of endocrine disturbances.

Osteocytic ERα deletion female mice exhibit an osteopenic phenotype

The BMD of 12-week-old $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$ were measured by DXA, showing that the BMD of female $ERα^{ΔOcy/ΔOcy}$ was significantly decreased in the proximal, not in middle and distal, tibiae compared to that of female $ERα^{flox/flox}$ (Fig. 1E). However, the BMD of tibiae from male $ERα^{ΔOcy/ΔOcy}$ were not significantly different from that of male $ERα^{flox/flox}$ (Fig. 1E). Next, to assess changes in bone structure between female $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$ mice, μCT analysis was performed. Decreased trabecular bone mass in $ERα^{ΔOcy/ΔOcy}$ mice was observed by μCT analysis (Fig. 2A). Trabecular bone of female $ERα^{ΔOcy/ΔOcy}$ exhibited a significant decrease in BV/TV, vBMD, Tb.N and Conn-D, and an increase in Tb.Sp and SMI compared to those of female $ERα^{flox/flox}$ (Fig. 2B). The parameters in metaphyseal cortical bone of female $ERα^{ΔOcy/ΔOcy}$ were not significantly different from that of female $ERα^{flox/flox}$ (Fig. 2C).

Osteocytic ERα regulates bone formation through control of osteoblasts

To examine whether the reduced bone phenotype of $ERα^{ΔOcy/ΔOcy}$ could be caused by alterations in the potential interaction between osteocytes and either osteoblasts or osteoclasts, bone histomorphometry was performed. The number and/or activity of osteoblasts/osteoclasts were examined in $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$, using lumbar vertebrae of 12-week-old female $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$. Parameters related to osteoblastic bone formation, such as N.Ob/B.Pm and Ob.S/BS, were significantly decreased in $ERα^{ΔOcy/ΔOcy}$ compared to $ERα^{flox/flox}$ (Fig. 3). In addition, N.Ocy/B.Ar was also decreased in $ERα^{ΔOcy/ΔOcy}$, which might be due to a decreased number of osteoblasts, which are precursors of osteocytes. Also, the reduction of BFR/BS and MAR in $ERα^{ΔOcy/ΔOcy}$ tended to be significant ($p = 0.07$), due to the reduction of osteoblastic parameters. On the other hand, parameters related to osteoclastic bone resorption, such as N.Oc/B.Pm and Oc.S/BS, were not altered in $ERα^{ΔOcy/ΔOcy}$ when compared to $ERα^{flox/flox}$ (Fig. 3). These results suggested that deficiency of ERα in osteocytes could decrease the number of osteoblasts and consequently their bone forming activity, indicating that bone mass reduction in $ERα^{ΔOcy/ΔOcy}$ could be caused by a reduction of osteoblastic bone formation, not a promotion of osteoclastic bone resorption. In addition, this result implies that osteocytic ERα might positively regulate osteoblastic bone formation by signaling from osteocytes, such as in a paracrine manner or by cell–cell contact.

Gene expression profiles of osteocytes lacking ERα

To determine what secretory proteins or signaling pathways *ERα* may utilize in osteocytes, a gene array analysis of Dmp1-GFP-positive cells from controls and mice with a targeted deletion of *ERα* in osteocytes was performed. Dmp1-GFP mice were crossed with $Dmp1^{Tg/0}$; $ERα^{L2/L2}$ mice to generate *Dmp1-GFP+*; $Dmp1^{Tg/0}$; $ERα^{L2/+}$ mice, and then *Dmp1-GFP+*; $Dmp1^{Tg/0}$; $ERα^{L2/L2}$ (*Dmp1-GFP+*; $ERα^{ΔOcy/ΔOcy}$) and

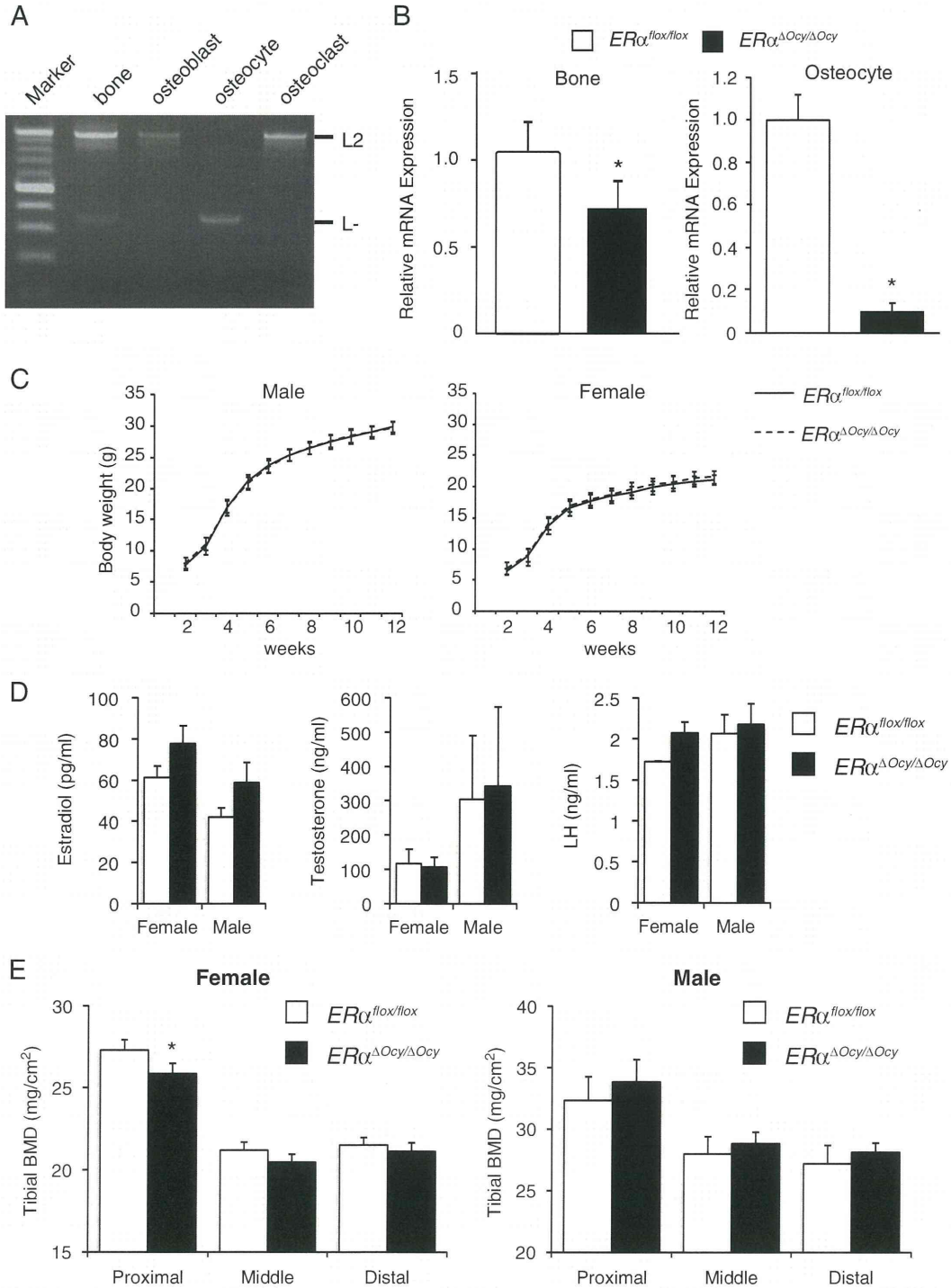

Fig. 1. Generation of mice with targeted deletion of ERα in osteocytes. (A) Deletion of ERα gene locus in osteocyte was detected by genome PCR in $ER\alpha^{\Delta Ocy/\Delta Ocy}$. (B) mRNA levels of ERα from whole femurs (left panel) and isolated osteocytes (right panel) of $ER\alpha^{flox/flox}$ and $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice was evaluated by RT-qPCR. Data are represented as mean ± SEM (n = 3). (C) The growth curves of $ER\alpha^{flox/flox}$ and $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice. Data are represented as mean ± SEM (n = 7–10). (D) Serum hormone levels of 12-week-old $ER\alpha^{flox/flox}$ and $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice. Data are represented as mean ± SEM (n = 4–7). (E) BMD of 1/3 portion of longitudinal divisions of tibiae from 12-week-old $ER\alpha^{flox/flox}$ and $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice. Data are represented as mean ± SEM (Female n = 8, Male n = 7). * indicates $p < 0.05$.

Dmp1-GFP+; $ER\alpha^{L2/L2}$ (*Dmp1-GFP*+; $ER\alpha^{flox/flox}$) were generated by crossing *Dmp1-GFP*+; $Dmp1^{Tg/0}$; $ER\alpha^{L2/+}$ and $ER\alpha^{L2/L2}$. Calvariae obtained from approximately 10-day-old female *Dmp1-GFP*+; $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and *Dmp1-GFP*+; $ER\alpha^{flox/flox}$ were treated with sequential enzymatic digestion and subjected to FACS. The percentage of GFP+ cells in fractions 4 to 6 was increased compared to that in fractions 2 to 4 (23.3% and 8.2%, respectively) (Fig. 4A). To determine if osteocytes were highly purified in this system, gene expression of cell-type specific marker genes in GFP+ cells (osteocytes) and GFP− cells (osteoblasts) was confirmed by RT-qPCR. As a result, the expression of Dmp1 (osteocyte marker

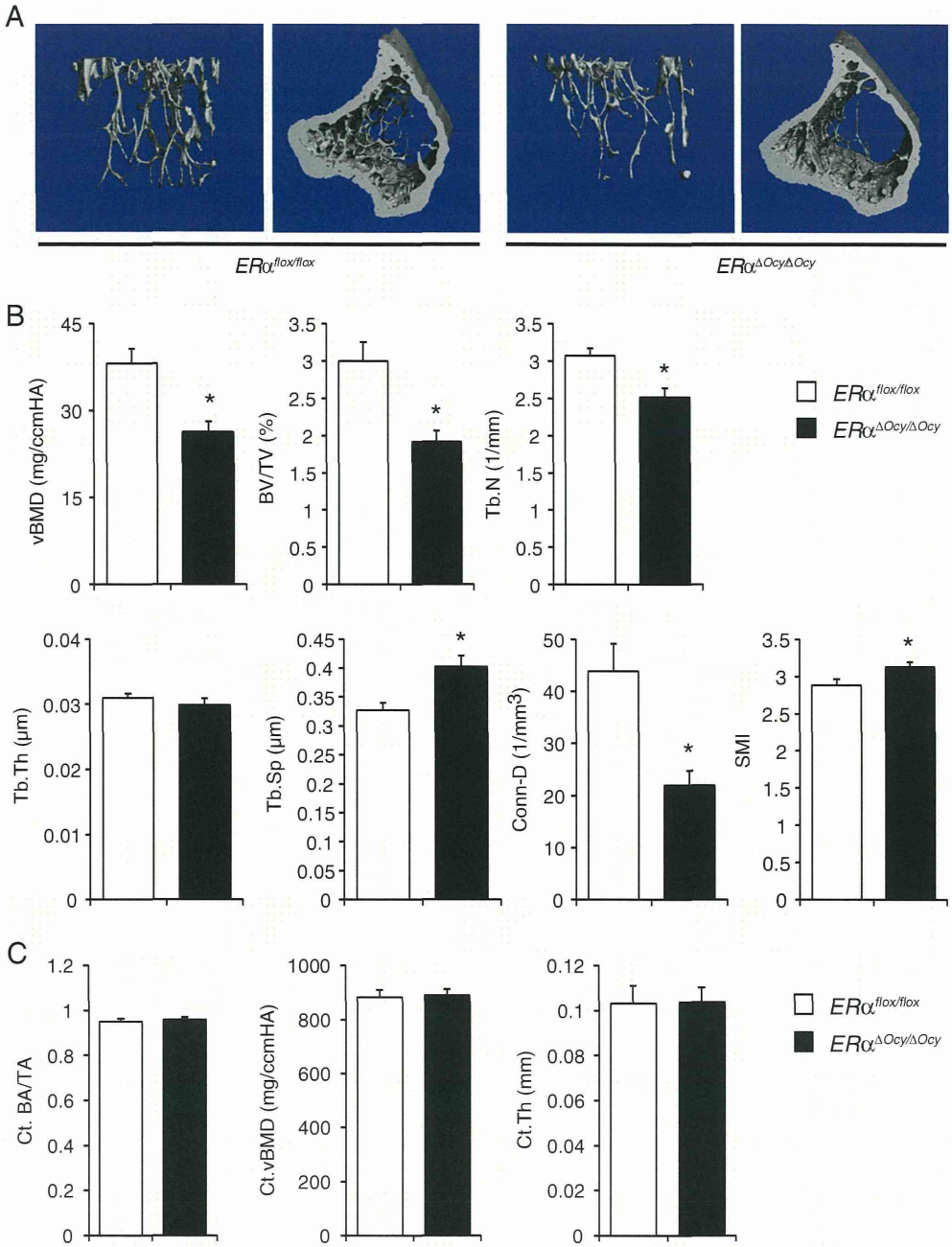

Fig. 2. μCT analyses of the mice lacking ERα in osteocytes. (A) Representative μCT views. (B) 3D measurements of proximal tibiae from $ERα^{flox/flox}$ and $ERα^{ΔOcy/ΔOcy}$ mice. Data are represented as mean ± SEM (n = 10). * indicates $p < 0.05$.

gene) in GFP + cells was about 25 times higher than in GFP − cells, while the expression of keratocan, Kera, (osteoblast marker gene) in GFP − cells was about 25 times higher than in GFP + cells (Fig. 4B). Extracted total RNA from *Dmp1-GFP+*; $ERα^{ΔOcy/ΔOcy}$ (n = 3) and *Dmp1-GFP+*; $ERα^{flox/flox}$ (n = 3) was subjected to a gene expression microarray analysis with GeneChip Mouse Genome 430 2.0 (Affymetrix). There were 276 genes found to be significantly differentially expressed between $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$ ($p < 0.01$). Among them, 76 genes were significantly down-regulated and 200 genes were up-regulated (Fig. 4C). Gene ontology analyses revealed that 'secreted' was listed top in the Keyword analysis when sorted by *p*-value (Fig. 4D). Among these genes, *Mdk* (Midkine) and *Sostdc1* (Sclerostin domain containing 1) were significantly up-regulated in $ERα^{ΔOcy/ΔOcy}$ although there were no significant differences in *Sost* or β-catenin (*Ctnnb1*) gene expression (Fig. 4E). Up-regulation of mRNA of *Mdk* and *Sostdc1* in $ERα^{ΔOcy/ΔOcy}$ was also validated when determined by RT-qPCR (Fig. 4F). From the results of functional annotation in differentially expressed genes between $ERα^{ΔOcy/ΔOcy}$ and $ERα^{flox/flox}$, osteocytic ERα could regulate the expression of secretory protein genes such as *Mdk* and *Sostdc1*, which have been shown to be inhibitors of Wnt signaling-related bone formation [44–46]. However, the expression levels of *Mdk* and *Sostdc1* were not significantly altered when late-stage primary cultured osteoblasts

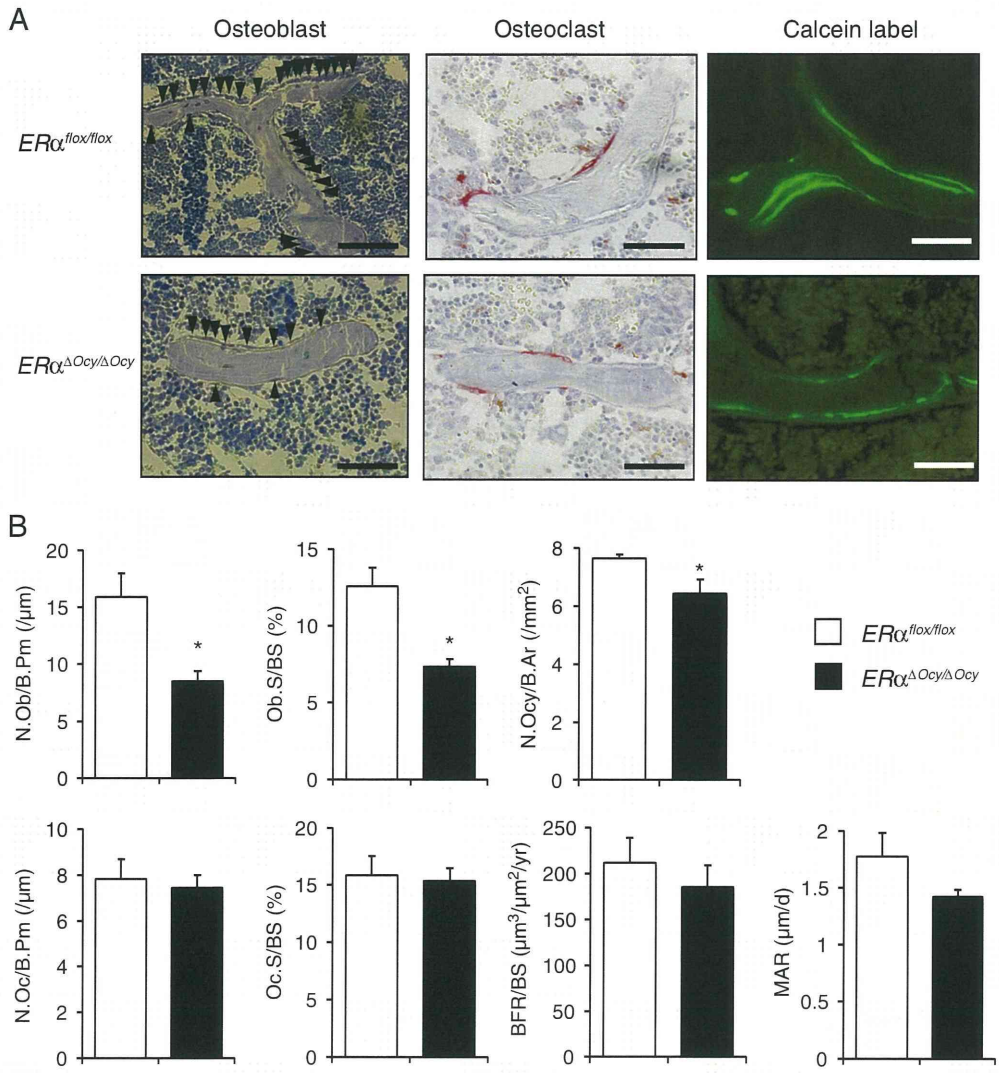

Fig. 3. $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice exhibit decreased bone formation. (A) Representative views of Toluidine blue staining for mononuclear cuboidal osteoblasts (arrowhead), TRAP staining for multinuclear TRAP-positive osteoclasts and calcein labeling for dynamic parameters are shown. Bars indicate 50 μm. (B) Data are represented as mean ± SEM (n = 6). * indicates $p < 0.05$.

were treated with 17β-estradiol for 2 or 6 h (Supplemental Fig. S1), indicating that *Mdk* and *Sostdc1* might not be early responsive genes, but be indirect target genes.

Trabecular bone loss is exacerbated in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ *in response to unloading while cortical bone is resistant to unloading-induced bone loss*

ERα has been reported to be involved in mechanosensing and increasing cortical bone formation under overloading conditions [11]. The hindlimb tail suspension model is a well-known model for unloading (or immobilization) and it is also reported that tail suspension-induced bone loss is significantly enhanced by ovariectomy [47]. To determine whether osteocytic ERα plays any roles in unloading-induced bone loss, a hindlimb suspension experiment was performed for female $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ for 4 weeks starting at 8 weeks of age. Control mice were chained to the cage top during the same period but allowed to load their hindlimbs to control for stress related effects.

During the 4-week experimental period, the average body weight of the experimental group increased 1 g, whereas the control group increased 2 g (Supplemental Fig. S2). Although there was a significant difference in body weight increase over the four weeks between the experimental and control groups, there was no significant difference in body weight between $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ within each group at the end of the experiment (Supplemental Fig. S2). Femoral diaphysis and distal metaphysis of the unloaded and loaded groups of both genotypes ($ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$) were measured using μCT. vBMD in the femoral diaphysis of tail suspended female $ER\alpha^{\Delta Ocy/\Delta Ocy}$ was significantly higher than that of $ER\alpha^{flox/flox}$ (Figs. 5A and B), although there were no significant differences in bone area or cortical thickness between genotypes. Upon further analysis, it was found that the trabecular bone mass was decreased in unloaded mice regardless of genotypes, and tail suspension induced trabecular bone loss in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ was greater than that in $ER\alpha^{flox/flox}$ (Figs. 5C and D). These data indicate that osteocytic ERα is protective against trabecular bone loss due to unloading.

Discussion

Based on reports on the functions of ERα in bone, estrogens are osteoprotective by regulating the life span of osteoclasts through osteoclastic and osteoblastic ERα and also by inhibiting apoptosis of osteoblasts and osteocytes [8,9,48–50]. Recently, it was reported that osteoblastic ERα has an osteoprotective function [12,14,15], however, little is known about the role of osteocytes in the osteoprotective actions

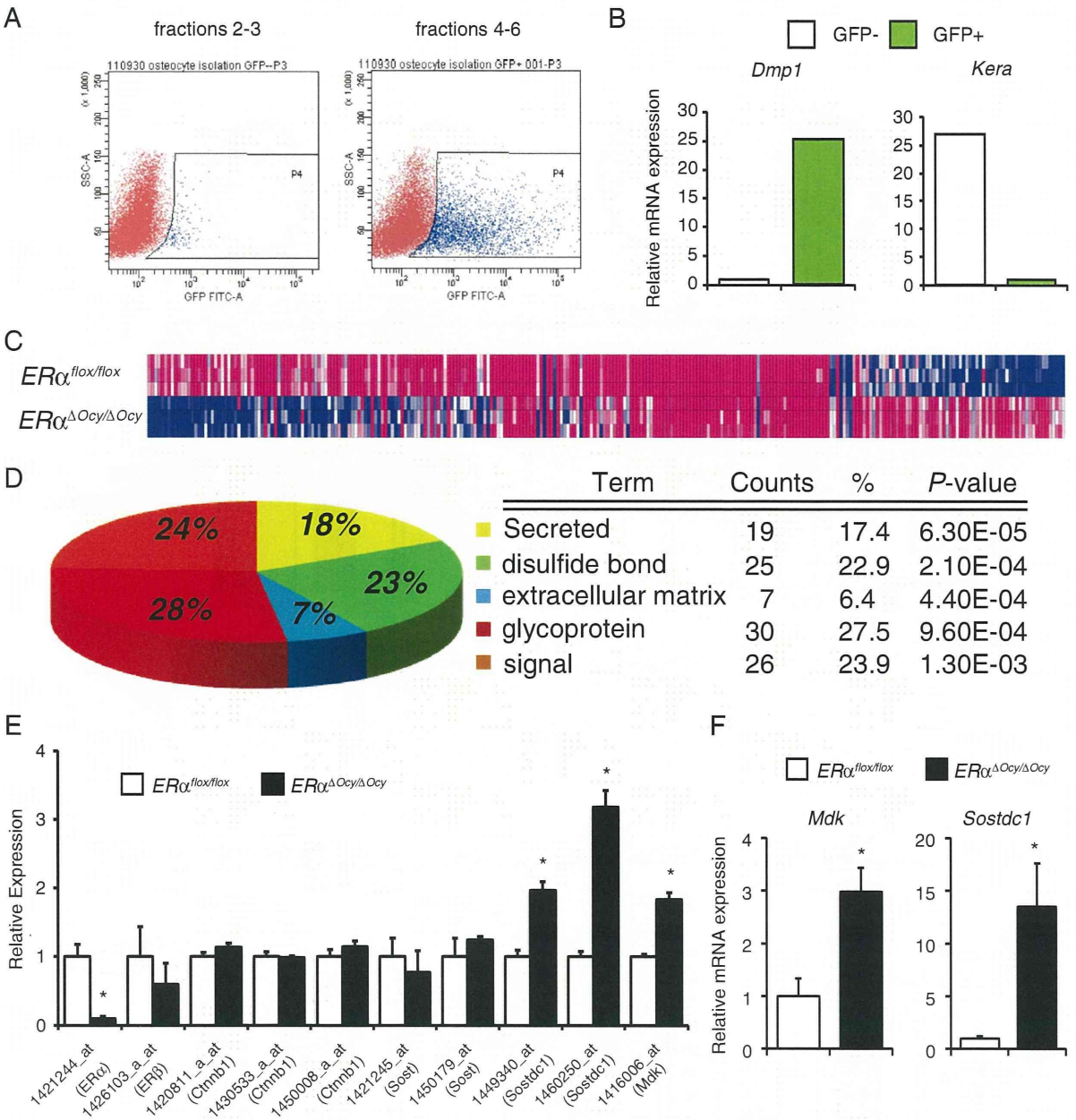

Fig. 4. Osteocytes lacking ERα show increased Mdk and Sostdc1 expression. (A) Two-dimensional dot plot of cells obtained from sequential enzymatic digestion of calvariae of mice expressing Dmp1-GFP. Left: fractions 2–3, right: fractions 4–6. (B) Expression of osteocyte (Dmp1) and osteoblast (Kera) marker genes in the GFP− and GFP+ population of isolated cells. (C) Heat map of significantly regulated genes in the gene expression microarray using total RNA from isolated GFP+ cells of $ERα^{flox/flox}$ and $ERα^{ΔOcy/ΔOcy}$ mice harboring Dmp1-GFP (n = 3). Red: high expression. Blue: low expression. (D) Functional annotation clustering of Keywords by DAVID Bioinformatic Resources. (E) Relative microarray intensity of each probe for ERα, ERβ, Ctnnb1 (β-catenin), Sost, Sostdc1 and Mdk. Data are represented as mean ± SEM (n = 3). (F) RT-qPCR for Mdk and Sostdc1 as same as panel E. * indicates $p < 0.05$.

of estrogens in skeletal homeostasis. To decipher the direct functions of ERα in osteocytes, the most abundant bone cell type in the adult skeleton, mice lacking ERα in osteocytes were genetically generated and their bone phenotype were analyzed in this study. ERα in osteocytes was found to play a significant role in maintaining bone mass by regulating osteoblastic bone formation only in females. It was further revealed that ERα in osteocytes is supportive for maintaining trabecular bone mass not only under normal loading conditions but also under tail suspension-induced unloading, which can be considered as experimental recapitulation of immobilization or space flight. However, the absence of this receptor protected against cortical bone loss. These results are consistent with a previous report in which bone mass adaptation induced by mechanical loading was impaired in ERα null mice [11]. Together, these results indicate that osteocyte mechanosensations at least in part *via* osteocytic ERα.

Maatta et al. and Melville et al. suggested that ERα in mature osteoblasts plays a role in maintaining trabecular bone mass in females based on analyses of mice lacking ERα in mature osteoblasts using Osteocalcin-Cre mice [12,15]. Almeida et al. suggested that ERα in osteoblast progenitors, but not in mature osteoblasts or osteocytes, is essential for regulation of female cortical bone [14]. As mentioned above, the functions of ERα in osteoblast lineage cells *in vivo* are still controversial and it is important to combine knowledge from various studies. All female mice exhibited an osteopenic phenotype in both the osteoblast-specific ERα