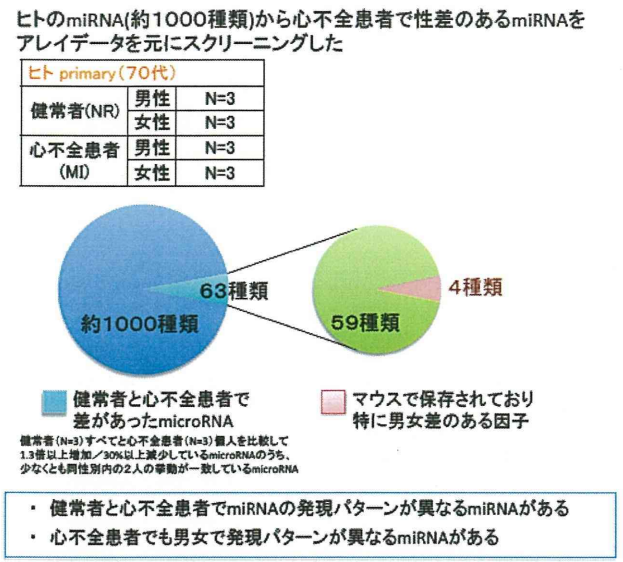
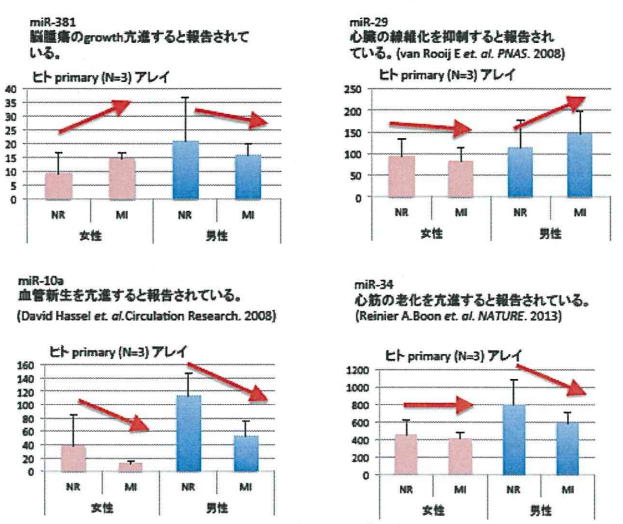
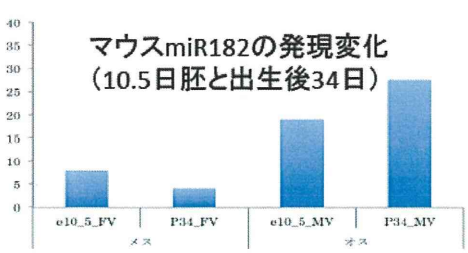
ングの結果から miR182 を含む複数の microRNA が単離された（図３０）。miR182 は心臓発生が進むにつれて発現量が増し、Baf60c の発現と相補的であることから、Baf60c 抑制因子として非常に期待される。



（図１６）ヒト心不全 miRNA アレイで単離された 1000 種類の候補 miRNA から、ヒト・マウス・ラット間で保存され、かつ発現量に性差のある miRNA。



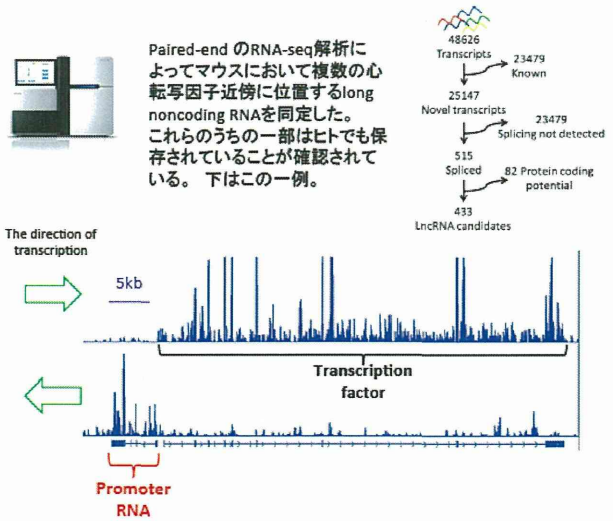
（図１７）性別によって発現変化応答が異なる miRNA 因子。ヒト・マウス間のみでも再探索する必要があるとも考えられる。



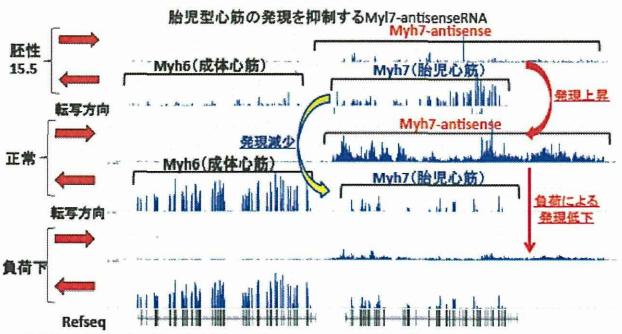
（図１８）マウス胎児期と出生後における miR182 の発現変化を追跡すると雌雄によって発現パターンが異なる。BAF の発現も雌雄によってことなる。

３：RNA シーケンスを用いた ncRNA の単離

組織からの RNA 抽出はナカライテスク社の Sepasol-RNA I Super G(1)を用い、クリーンアップに Macherey-Nagel 社の Nucleospin RNA XS を用いた。cDNA ライブラリ作成は rRNA などを total RNA から取り除く kit である Epicentre 社の Ribo-Zero Gold (Human/Mouse/Rat)と、Illumina 社の Truseq Stranded mRNA sample prep kit を用いた。これにより、どちらの鎖から RNA が転写されているかを知ることが可能である。Illumina Hiseq 2000 を用い、シーケンス長 101 base、Paired-end read でシーケンシングを行った。Paired-end とは cDNA ライブラリの各フラグメントに対し、両端からシーケンスを行うことであり、Single-end の場合に比べてはるかに詳細で正確な転写産物の再構成が可能である。遺伝子発現変動解析ソフト Cuffdiff を用い、gene model を再構築するソフトウェア Cufflinks による転写産物の再構成の結果、全サンプル合わせて 48,626 個の転写産物単位を得た。この内、Refseq 中の既知遺伝子と一致するものが 23,479 個であり、残りの 25,147 個は未知転写産物と考えられる。更にこの未知転写産物の内で、Splicing が検出され、タンパク質に翻訳されないと推定されるものが 515 個存在した。Splicing を機能的な lncRNA の基準の一つとしたのは、既知の機能的な lncRNA のほとんどが splicing を受けていること、splicing を検出できない程度の発現量/read 数の遺伝子は実際に機能解析を行うことが難しいであろうと考えた。また、翻訳能に関しては *ensembl* の基準に従い、ORF が全長の 35%以上のものを翻訳され得る (http://asia.ensembl.org/info/genome/genebuild/ncrna.html)。82 個がこれに該当し、残る 433 個が心臓において機能する lncRNA の最終的な候補となった。発現量の比較により lncRNA 候補の発現を発現量変化が 4 倍以上であるものを 83 個同定した。この内、Sham との比較でも変動していると判定されたものを 6 個同定した（図１８参照）。その中で、心負荷により発現調節を受ける ncRNA を同定した（図１９）。



（図１９）バイオインフォマティクス解析を用いた ncRNA スクリーニング

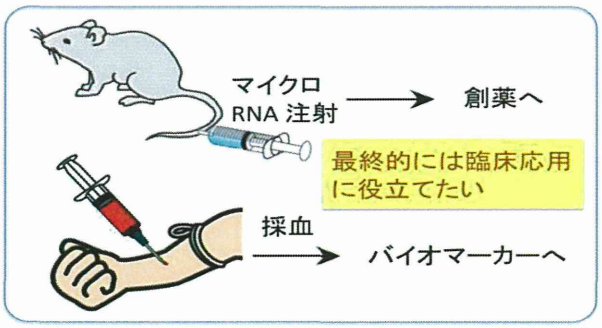


（図２０）心筋成熟に伴って発現亢進するMyl7ncRNA

【D．考察】

本研究により、心不全患者から作製された遺伝子発現プロファイルを元に複数のエピジェネティック因子が単離され、それぞれの機能が明確化されてきた。これら因子が心臓発生に重要な機能を担っていることを報告し、モデルマウスをプロデュース出来た。しかしながら、単離されたすべての候補因子について研究論文としてまとめることまでには至らなかった。今年度に①Brg1 KO（平成 24 年度報告）、②Baf60c 恒常発現解析、③Dnmt1 KO（平成 24 年度報告）、④miRNAs に関する研究を発表することを目指す。さらに、どの程度特定のゲノム構造に変化をもたらすのか、ChIP シーケンスを用いた解析系を立ち上げる必要性があり、今後の中心的な課題である。

さらに今後の課題は、どのようにして心筋再生能を高めるか、また恒常因子を見いだすのか、であると考えている。本研究により Baf60c が心筋の増殖活性を亢進し、炎症作用を抑制するなど萌芽的な結果が見いだせた点は大きい。Baf60c がどのようなシグナルまたは因子によって誘導を受け、また発現抑制されるのか課題が残されたが、本研究によって単離された miRNA は一つの候補であると考えている。



（図２１）miRNA を用いた臨床応用概念図

本研究期間内に生じた疑問を発展させて、詳細にグループ分けをした心不全患者プロファイルの作成に成功したことにより、男女で発現の異なる miRNA が存在することを見出した。マウス・ラット間においても保存された miRNA が存在し、かつ、発生過程においても興味深い発現変化をしていることも明らかとなった。本研究結果により、miRNA の発現にのみならず、ゲノム修飾や構造変換においても性差の相違が存在すると考えられる。ChIP シーケンスを駆使することで、性差により発現の異なる遺伝子や ncRNA の存在理由という本疑問を紐解けるものと考え、今後の課題の一つとして取り組む。

本研究で作製されたモデルマウスは疾病研究に有効である。本年度内に全てプロデュースすることを目標にする。それとともにバイオインフォマティクス解析系を立ち上げられた点は、今後のゲノム解析において有意義な研究を期待できる。両結果は、本研究により構築できた点であり、ヒト心不全で発現変化する miRNA に関しては早急にプロファイルし国際論文に報告する。

性差医学に貢献することにより、男女別の創薬・処方を提案できる。現在、心不全を軽減する製剤として利尿薬やジギタリス製剤が一般的に使われているが、有効な治療薬ではない。また、男女の薬効の差を考慮したものでもない。しかし、本研究により、男女で選べる治療法を新たに提案することができる。本研究を成し遂げることにより、生物学的特性の理解や、診断・治療・病態把

握・予後予測といった臨床応用の現状と問題の解決に役立つと考えられる。

【E．結論】

今まで、再生研究は様々な視点から研究がなされてきたが、クロマチン環境変化に着目して再生メカニズムを理解する研究は行われてこなかった。本研究により、再生過程おいてクロマチン環境は刻々と変化していることが分かり、クロマチン環境変換を引き起こす分子機構解明から心筋再生能の向上を目指す本研究は、独創的であり、心臓再生現象の理解のみならず他器官における再生現象の理解に貢献できると考える。今後は、*in vivo* ChIP-seq法を立ち上げ、ゲノムワイドに標的領域を絞り込み、心筋再生向上因子の網羅的な転写調節領域の解析を行うことを目指していきたい。さらに本研究では、心臓再生に寄与する生体内機能因子の同定のみならず、再生環境の向上させる生理活性物質の単離を試みた。これらの心臓再生可能時と不可能時のクロマチン構造変換の網羅的な解析の成果は、先天的な心臓病における心筋再生能力向上および後天性心疾患を引き起こす制御領域の発見への応用につながる可能性を秘めており、さらに、心筋再生環境を整える因子の発掘は、心筋梗塞患者の予後を救い、心不全をプロテクトする手法になると考えられ、再生医科学に大きなインパクトを与えると期待している。

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【G．知的財産権の出願・登録状況】

1．特許取得

現在、東大TLOと協議中。

2．実用新案登録

該当事項無し。

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別紙４

Ⅱ．研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
堀優太郎、森下環、中村遼、小柴和子、竹内純	エピジェネティック因子と心臓発生・心疾患		エピジェネティクス キーワード事典(実験医学)	羊土社	東京	2013	192-200

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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

Ⅲ. 研究成果の刊行物・別刷

Bone 60 (2014) 68–77

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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′-ATGTTCAGCATGTTCAGCAG TGTG-3′, *Gapdh*: F 5′-AAATGGTGAAGGTCGGTGTG-3′ and R 5′-TGAA GGGGTCGTTGATGG-3′, *ERα*: F 5′-CATGGTCATGGTAAGTGGCA-3′ and R 5′-TCTCTGGGCGACATTCTTCT-3′, *Dmp1*: F 5′-TGAAGAGAGGACGGGT GATT-3′ and R 5′-TCCGTGTGGTCACTATTTGC-3′, *Kera*: F 5′-TGGGATGT CCACGACGACTT-3′ and R 5′-AAGGCAGTAGGGAAACTGGGA-3′, *Mdk*: F 5′-TGGAGCCGACTGCAAATACAA-3′ and R 5′-GGCTTAGTCACGCGGA TGG-3′, *Sostdc1*: F 5′-AAATGTATTTGGTGGACCGC-3′ and R 5′-GAATCA AGCCAGGAATGGAG-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

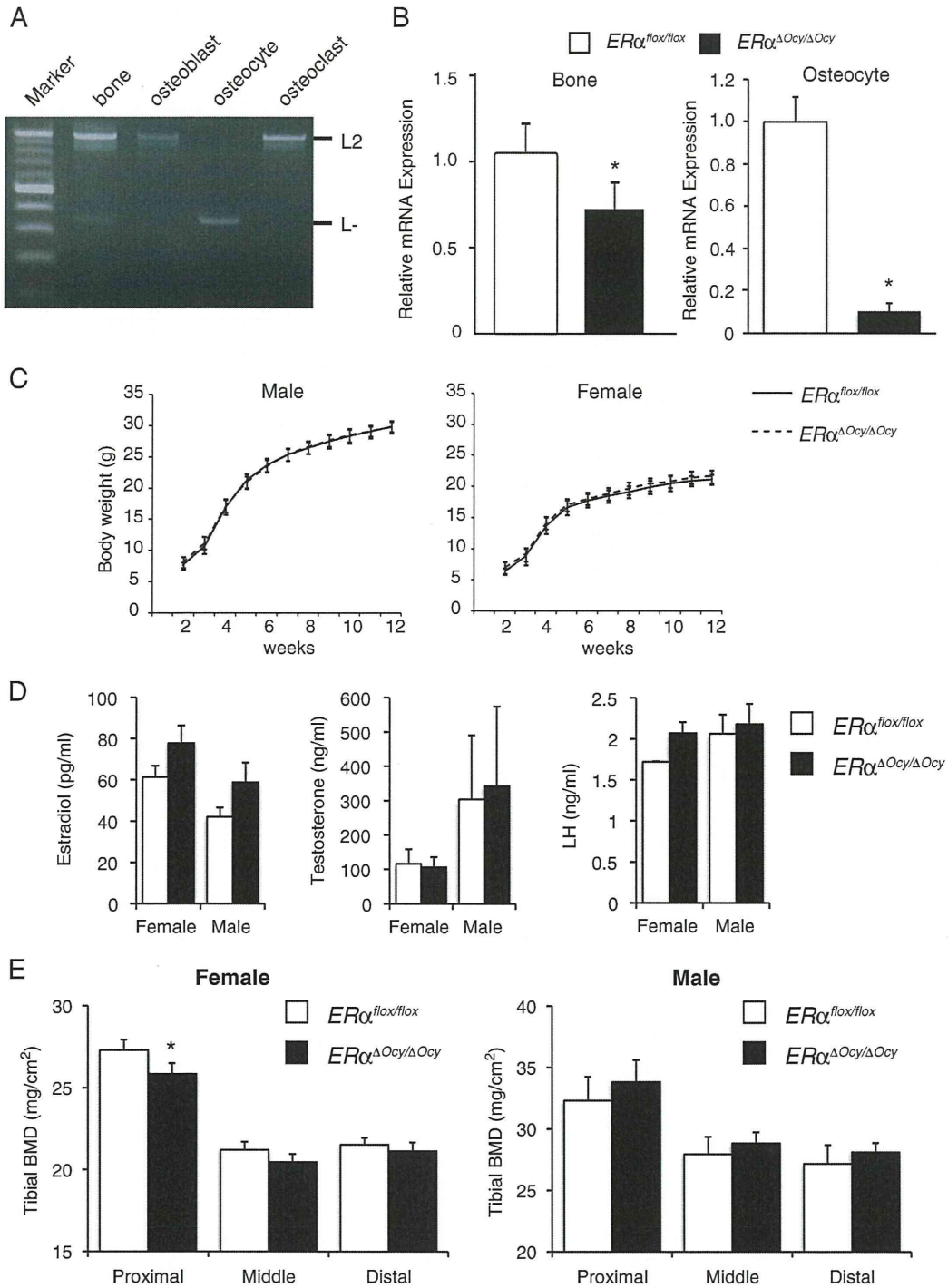

Fig. 1. Generation of mice with targeted deletion of ERα in osteocytes. (A) Deletion of ERα gene locus in osteocyte was detected by genome PCR in $ER\alpha^{\Delta Ocy/\Delta Ocy}$. (B) mRNA levels of ERα from whole femurs (left panel) and isolated osteocytes (right panel) of $ER\alpha^{flox/flox}$ and $ER\alpha^{\Delta Ocy/\Delta Ocy}$ 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