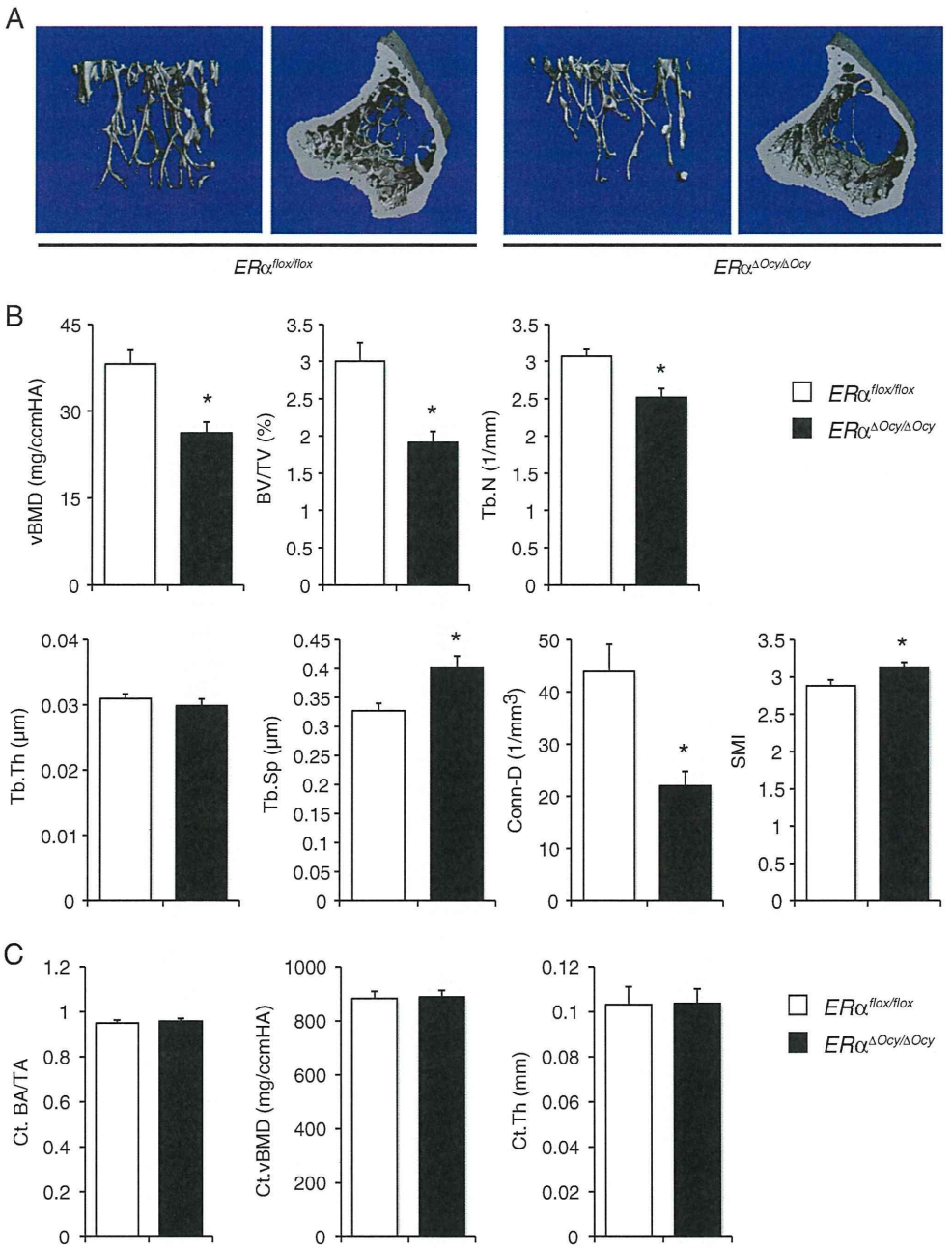

Fig. 2. μCT analyses of the mice lacking ERα in osteocytes. (A) Representative μCT views. (B) 3D measurements of proximal tibiae from $ERα^{flox/flox}$ and $ERα^{ΔOcy/ΔOcy}$ mice. Data are represented as mean ± SEM (n = 10). * indicates $p < 0.05$.

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

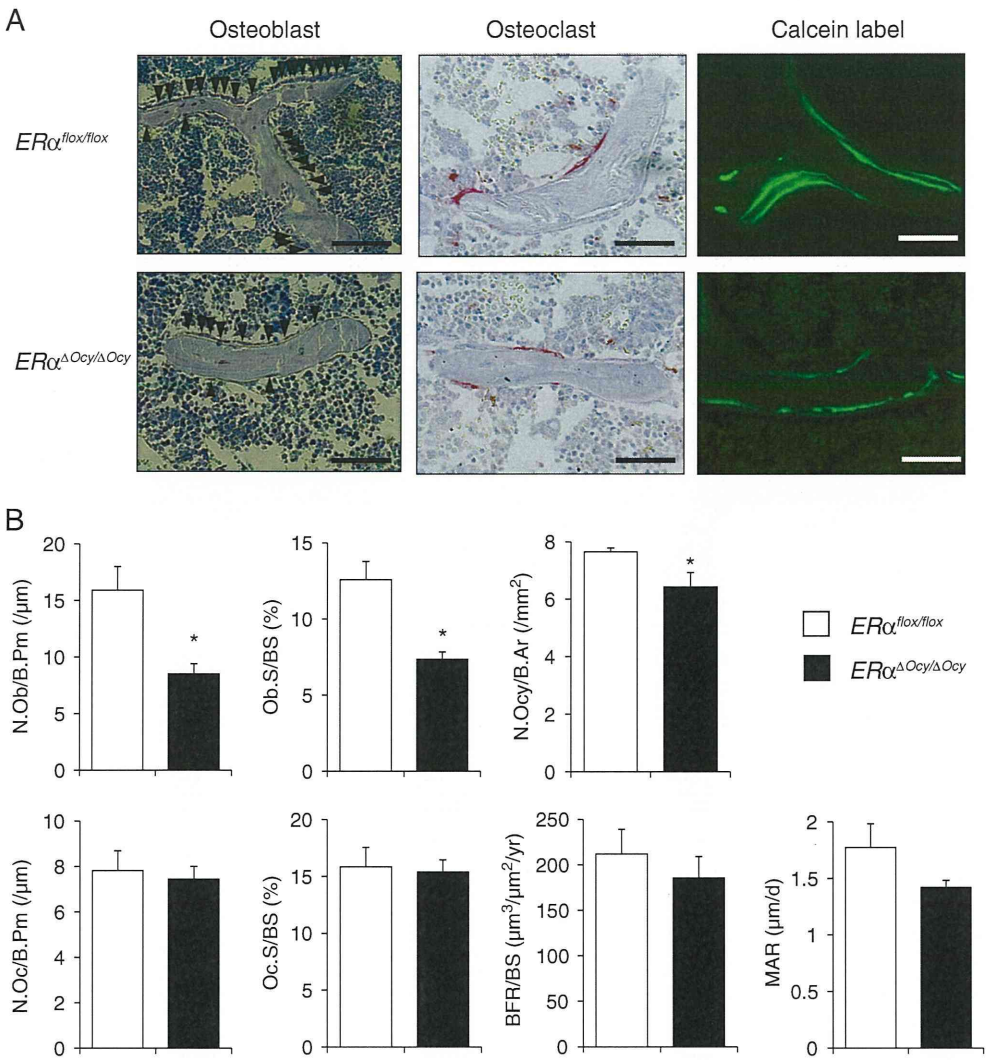

Fig. 3. *ERα*$^{\Delta Ocy/\Delta Ocy}$ mice exhibit decreased bone formation. (A) Representative views of Toluidine blue staining for mononuclear cuboidal osteoblasts (arrowhead), TRAP staining for multinuclear TRAP-positive osteoclasts and calcein labeling for dynamic parameters are shown. Bars indicate 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 ERα$^{\Delta Ocy/\Delta Ocy}$ *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 *ERα*$^{\Delta Ocy/\Delta Ocy}$ and *ERα*$^{flox/flox}$ 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 *ERα*$^{\Delta Ocy/\Delta Ocy}$ and *ERα*$^{flox/flox}$ 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 (*ERα*$^{\Delta Ocy/\Delta Ocy}$ and *ERα*$^{flox/flox}$) were measured using μCT. vBMD in the femoral diaphysis of tail suspended female *ERα*$^{\Delta Ocy/\Delta Ocy}$ was significantly higher than that of *ERα*$^{flox/flox}$ (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 *ERα*$^{\Delta Ocy/\Delta Ocy}$ was greater than that in *ERα*$^{flox/flox}$ (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

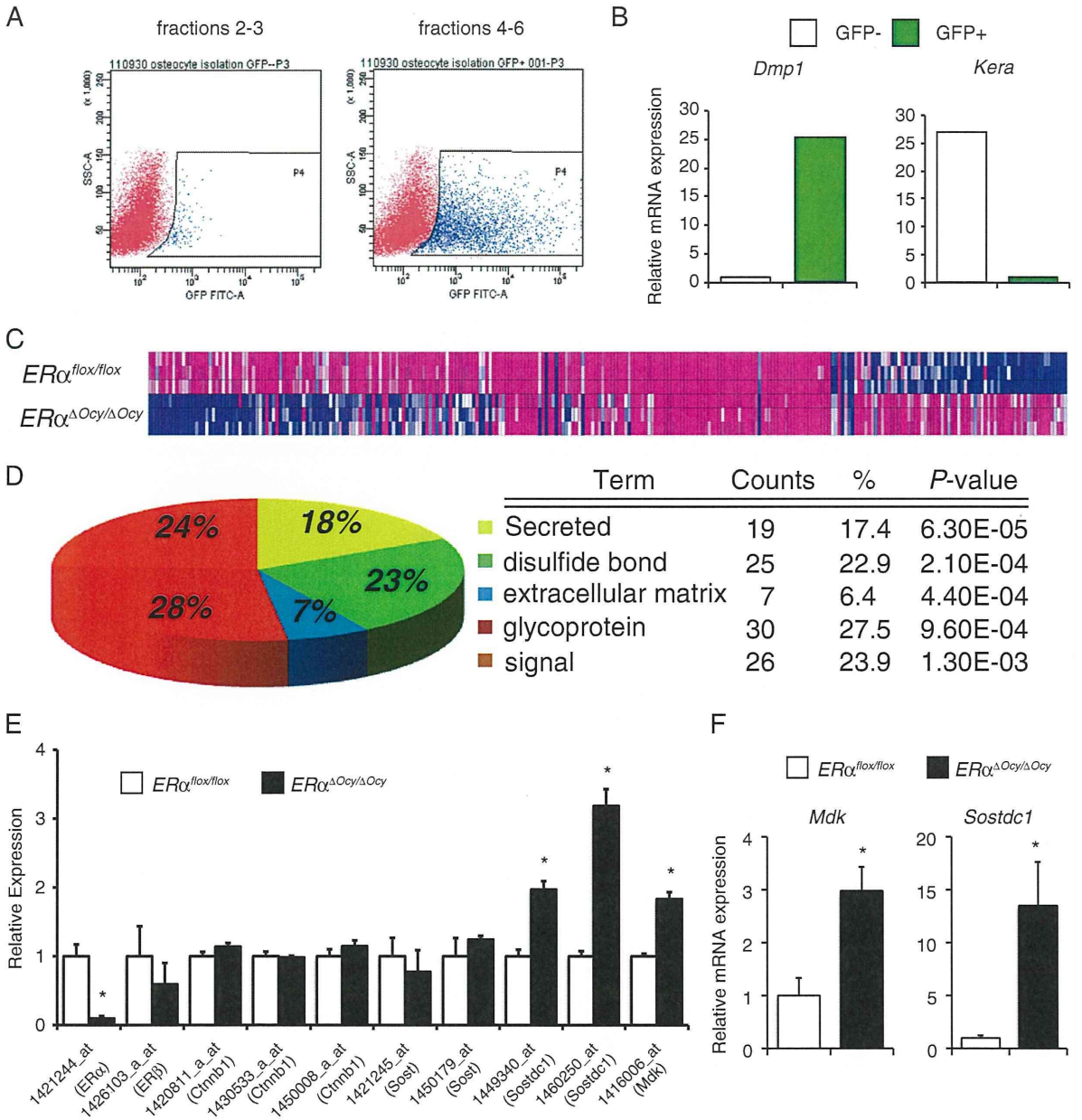

Fig. 4. Osteocytes lacking ERα show increased Mdk and Sostdc1 expression. (A) Two-dimensional dot plot of cells obtained from sequential enzymatic digestion of calvariae of mice expressing Dmp1-GFP. Left: fractions 2–3, right: fractions 4–6. (B) Expression of osteocyte (Dmp1) and osteoblast (Kera) marker 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

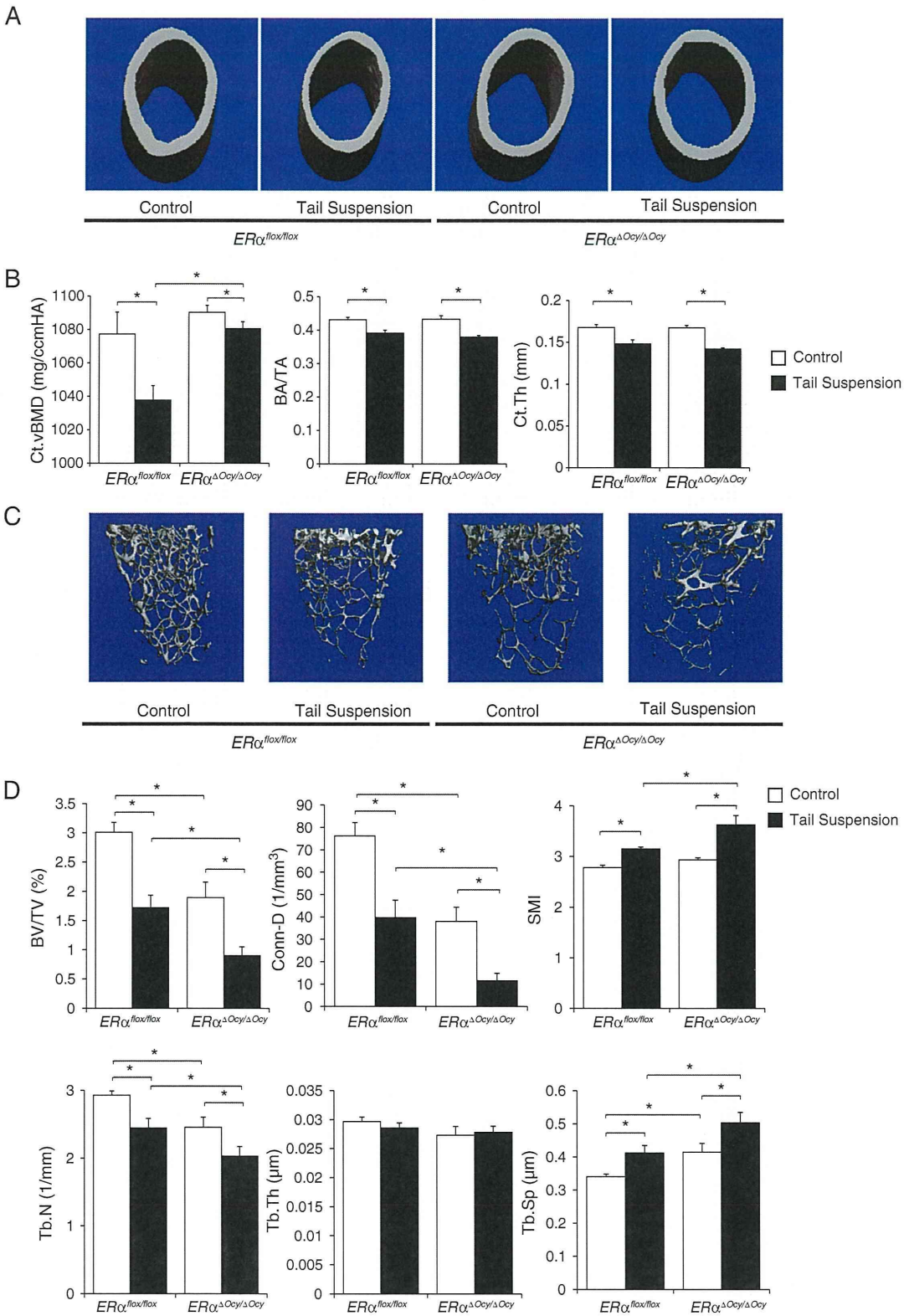

Fig. 5. Effects of unloading on trabecular and cortical bone in mice with targeted deletion of ERα in osteocytes. (A and C) Representative μCT views. (B) 3D measurements of femoral distal trabecular area and (D) 3D measurements of femoral diaphyses from $ERα^{flox/flox}$ and $ERα^{ΔOcy/ΔOcy}$ mice subjected or not subjected to tail suspension. Data are represented as mean ± SEM (n = 6). * indicates $p < 0.05$.

knockout mice by Maatta et al. and Almeida et al., and in previous reports regarding osteoclast-specific ERα knockout mice [8,9]. As would be predicted, androgen receptor knockout mice (ARKO), including both systemic ARKO [51] and osteocyte conditional ARKO [52], exhibited bone loss in male mice. These gender-specific phenotypes are probably caused by differences in concentration of circulating sex steroids, estrogens and androgens. In contrast to these studies and our present study, a recent report showed that mice lacking ERα using the same Dmp1-Cre mouse exhibited trabecular bone loss only in male mice, but not in female mice [13]. In this report, Windahl et al. proposed that the physiological trabecular bone-sparing effect of estrogen is mediated *via* ERα in osteocytes in males, but also *via* ERα in osteoclasts in females [13]. At present, it is difficult to provide a convincing explanation to describe the discrepancies between our current study and this report [13]. However, one possible reason may be differences in the genetic background of the mouse strain of the ERα-floxed mice since the Dmp1-Cre mice were identical. The ERα-floxed mice used in our study have been registered as *Esr1*^{tm1Mma} and originated from 129S2/SvPas mixed background, and published in 2000 [5], then backcrossed with C57BL6 line for more than 10 times. On the other hand, the ERα-floxed mice used in the study by Windahl et al. have been registered as *Esr1*^{tm1Gust} and originated from 129X1/SvJ mixed background, and published in 2012 [53]. These differences might be responsible for the discrepancies between the two studies. Regardless, the results of these two studies suggest that osteocytic ERα may have a role in maintenance of trabecular bone homeostasis regardless of gender.

To investigate the possible molecular basis underlying ERα function in osteocytes, we performed an osteocyte isolation technique using FACS analysis of Dmp1-GFP positive cells from conditional null mice and their controls. The results obtained from the Functional Annotation Clustering of differentially expressed genes suggested that osteocytic ERα might regulate transcription of the genes related to secretory proteins, which may regulate osteoblastic bone formation and contribute to maintenance of bone homeostasis. In fact, Sostdc1, an antagonist of the Wnt signaling [45,54], was elevated as a downstream gene of osteocytic ERα. Sostdc1 is a gene also called Wise or Ectodin whose domain is similar to Sost (Sclerostin). Sost and Sostdc1 bind to Wnt co-receptors called Lrps and regulate the Wnt/β-catenin pathway negatively [55]. Wnt signal proteins are reported to modulate bone mass *in vivo* by acting directly on mesenchymal stem cells [56–59]. Genes involved in the Wnt signaling are known to regulate the cell proliferation, differentiation, and apoptosis of osteoblasts [60]. Interaction between β-catenin and ERα has been previously reported [61] and the expressions of some Wnt family genes are important for responding to mechanical stress and are reportedly regulated by ERα [32]. Conventional Sostdc1 KO mice are reported to exhibit abnormal tooth development, which has similar characteristics as bone [45,54]. Also, it has been reported that estradiol regulates mRNA levels of Sostdc1 in U2OS cells [62]. In addition, a meta-analysis of BMD in a female Chinese population revealed that a mutation in the Sostdc1 cording region was correlated with BMD, suggesting that Sostdc1 might play a role in homeostasis of bone metabolism [46].

Also, Midkine, Mdk, was elevated as a downstream molecule of ERα in mice with this targeted deletion. Mdk is a member of a family of heparin-binding growth factors known primarily for their effects on neural cells [63]. Mdk expression is reported to increase during the course of primary osteoblast differentiation. Mdk has been shown to bind to a complex of protein tyrosine phosphatase zeta (Ptprx), low-density lipoprotein receptor-related protein-6 (Lrp6), and exert negative effects on Wnt signaling [64]. Conventional Mdk null mice exhibit increased bone formation, suggesting Mdk is a negative regulator of osteoblastic bone formation. Furthermore, Mdk KO mice are resistant to OVX-induced bone loss and sensitive to mechanical loading induced cortical bone increase [44]. In addition, the expression of ALP and the induction of canonical Wnt signaling in MC3T3E1, an osteoblastic cell line, were inhibited by Mdk treatments [64]. These reports and the results from our current study suggest that Sostdc1 and Mdk might be responsible for a component of estrogen's osteoprotective actions.

However, questions remain regarding how ERα negatively regulates the transcription of these genes because there are no reports of a negative transcriptional regulation of the estrogen receptor response element (negative ERE), although details of a negative glucocorticoid receptor response element (nGRE) have been reported [65]. Alternatively, it is possible that the expression of these factors might be regulated by an ERα-dependent miRNA. The precise molecular basis of transcriptional regulation or mRNA stabilization of these genes must be clarified in future studies. Neutralizing or deletion studies of these two proteins in this mouse model could provide possible answers for these questions.

In conclusion, osteocytic ERα might play a role in estrogen's osteoprotective action by controlling the expression of Wnt antagonists, which regulate osteoblastic bone formation in trabecular bone.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2013.12.005.

Conflict of interest

All authors state that they have no conflicts of interest.

Acknowledgments

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4 心血管疾患

cardiovascular disease

堀 優太郎，森下 環，中村 遼，小柴和子，竹内 純

Keyword 1先天性心疾患 2心筋症 3高血圧症 4心不全

概論

エピジェネティック因子と心臓発生・心疾患

1. 心血管疾患とは

心臓は発生学上，最初期に機能しはじめる臓器であり，心臓の停止が死の象徴として扱われるようにその拍動は個体の死まで続く．そのため自然と，心血管疾患は重篤な結果となるものが少なくない．心血管疾患は，悪性新生物に次いで日本人の死因の第2位で実に約16％を占めている．

心血管疾患とは心臓や血管系における疾患全般を指し，代表的なものとして虚血性心疾患（ischemic heart disease），**心筋症（cardiomyopathy）**（→Keyword 2），**高血圧症（hypertension）**（→Keyword 3），動脈硬化症（atherosclerosis），不整脈（cardiac dysrhythmia）などがあげられる．また，心血管系では心室中隔欠損症（ventricular septal defect）など**先天性心疾患（congenital heart disease）**（→Keyword 1）が新生児の約100人に1人と少なくない割合で発生することが知られている．

心臓は虚血，過剰な圧，遺伝的・免疫的負荷など外的，内的なストレスに対して心収縮を増加させるのみならず，心肥大や心拡大といった方法でその形までも変化させ，これに対応しようとする．この過程は遺伝的，機能的に非常に迅速に起こり，病的なものというよりはあくまでも生理的な過程として捉えられる．しかしながら，このような状態が長時間続くことで心血管系の恒常性は崩壊へと向かい，種々の疾患を生じ，最終的には心不全（heart failure）（→Keyword 4）などの重篤な病態へと至ることとなる．これが多くの成人心血管疾患の発症までの過程である．

2. 心血管疾患の発症原因とエピジェネティクス

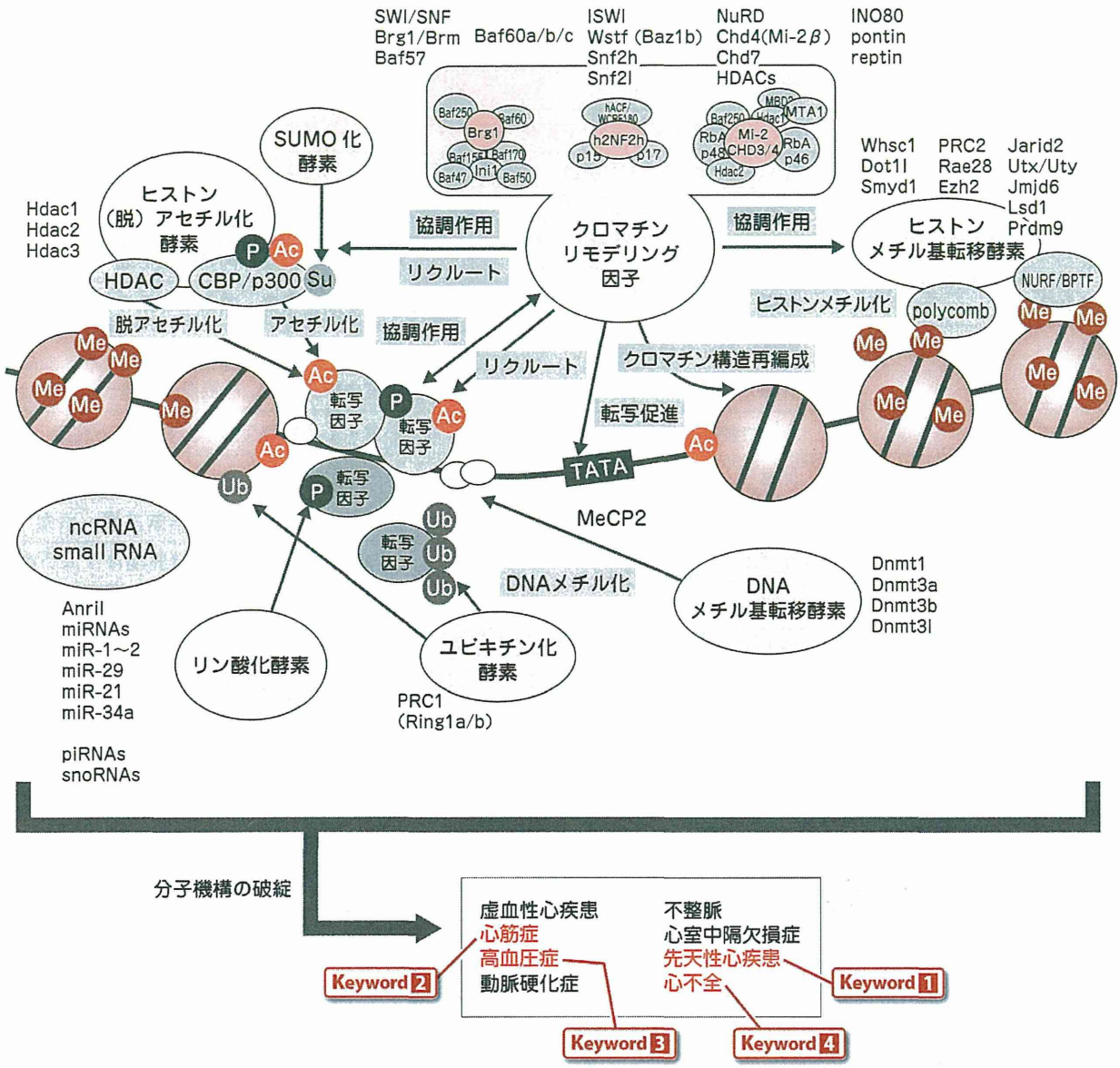
1）遺伝的要因と外的要因

過剰な塩の摂取が高血圧の主な原因であるなど，心血管疾患の多くは生活習慣と強い関係があることが知られている．本態性高血圧を例に取ると，その発症機序は複数の遺伝的要因やさまざまな外的要因が絡み合って生じるとするモザイク説がよく知られている．実際に胎児期の断水が食塩感受性高血圧の原因となるなど，外的要因がエピジェネティクスとして固定されることで疾患が起きやすい遺伝的環境をつくっていることを示唆する実験結果は複数存在し，この説をエピジェネティクスの方面から支持しているといえる．

二次大戦中のオランダの飢饉の際に母親の胎内にいた子供の出生後における追跡調査では，*IGF2*のプロモーター領域が低メチル化状態になっていることが判明するなど，外因がどの遺伝子座に影響を与え，病因となっているかということまでも明らかになっている例も存在する[1]．しかしながら，現状としては発症の分子的メカニズムを具体的に与える研究の多くが，遺伝子改変マウスの表現型を解析し，既知の心血管疾患との関連性を見出すといったものである．

全身疾患とのかかわりなども強く，成人心血管疾患に関しては単独の原因に帰するのは難しい場合が多い．一方で先天性心疾患に関しては，近年の発生学の大きな進歩により，心臓発生に重要な転写因子，分泌因子さらに

イラストマップ　心臓発生・心疾患発症に関与するエピジェネティック因子群



心臓発生，ストレス応答においては他の臓器と同様にヒストンのアセチル化，メチル化，DNAのメチル化などが一般的な転写調節因子として働いているほか，SWI/SNFやNuRDなどのクロマチンリモデリング複合体が選択的な遺伝子発現において中心的な機能を果たしている（文献6を改変して転載）

エピジェネティック因子が多数同定され，その原因遺伝子や発症機序が解明されつつある（表）．ほとんどのヒストン修飾酵素（第1部-4～8参照）やDNAメチル化酵素（第1部-1～3参照）のノックアウトマウスは心臓発生に重篤な異常を生じ，その多くが胎生致死となることが知られている．

2）明らかになりつつあるエピジェネティクス因子

さらに，さまざまなエピジェネティック因子の改変マウスが先天性心疾患のみならず心筋症などの成人心疾患と酷似した表現型を示すということがわかってきた．そのなかでも心疾患の理解に特に重要と考えられるエピジェネティック因子が，哺乳類SWI/SNF型クロマチン

表 心疾患を発症するエピジェネティック因子群

エピジェネティック因子・遺伝子	遺伝的改変・異常	表現型
Jmjd6	欠損	両大血管右室起始症，心室中隔欠損
Jarid2	欠損	両大血管右室起始症，肉柱の過発達
Utx	欠損	ルーピング異常，心筋の菲薄化
Mll2	Kabui症候群患者において変異	心室・心房中隔欠損症，大動脈縮窄症
Whsc1	Nkx2-5とのダブルヘテロ	心室・心房中隔欠損症
Chd7	ヘテロ欠損	大動脈弓の発達異常
Wstf	欠損	心室中隔欠損症，肉柱の低形成
miR-1-2	欠損	心室中隔欠損症，刺激伝導系の異常
Brg1	成体における高発現	肥大型心筋症
	欠損	心室中隔欠損症，心筋層の菲薄化，胎生致死
	ヘテロ欠損	心室・心房中隔欠損症，拡張型心筋症
Dot1l	欠損	拡張型心筋症
Rae28	欠損	拡張型心筋症
Hdac1，Hdac2	ダブルノックアウト	拡張型心筋症，不整脈
Hdac3	欠損	肥大型心筋症
Dnmt1	親の低タンパク質食による発現レベル低下	高血圧症
Lsd1	ヘテロ欠損	食塩感受性高血圧症

リモデリング複合体のコア因子であり，ATPase活性をもつ*Brg1*（*Smarca4*）と心臓特異的サブユニットである*Baf60c*（*Smarcd3*）である[2]．*Brg1*は転写因子などと協調して働くことで，心臓発生に重要であるだけでなく，心筋症などの成人心疾患発症にも大きくかかわることが示唆されており，近年盛んに研究されている分子であるので，**1**と**2**で詳細にその解説を述べた．

イラストマップに示すように心臓においては他のタイプのクロマチンリモデリング因子も重要な働きを有しており，NuRD（nucleosome remodeling and histone deacetylase）複合体のコア因子である*Chd7*は大動脈弓の発達を制御することが報告されており[3]，また，ISWI（imitation-SWI）複合体に属するWICH〔Wstf（Williams syndrome transcription factors）-ISWI chromatin remodeling〕複合体の構成因子である*Wstf*（*Baz1b*）欠損マウスは肉柱の低形成や心室・心房中隔欠損を発症することが報告されている[4]．

3. 臨床応用とこれからの展望

心筋がほとんど細胞分裂を起こさないことは有名で，傷ついた心臓が再生することは基本的にない．そのため心血管疾患は予防が第一に重要であり，心血管疾患のリスクファクターとなる環境要因やその下流のエピジェネティックな変異などを特定し，予防に役立てることは重要なテーマといえる．

1）モデル動物解析の問題点

心疾患とエピジェネティクスの関係に関しては，研究が進みつつあるとはいえ未解明な点が多く，マウスやゼブラフィッシュなどモデル生物での知見がどの程度ヒトに適応できるか，という点も明らかでないことが多い．臨床データやヒトサンプルを用いた研究を，実験動物や細胞などを用いた基礎研究と付き合わせ，実際に心血管疾患の発症や進行にどのようにエピジェネティクスがかかわっているかを明らかにすることが，これからの課題といえるだろう．

2）再生医療への期待

また，特定遺伝子群などの強制発現によって幹細胞を介す，または介さない心筋細胞へのリプログラミングが注目されている．われわれの研究によって，胎児性中胚葉細胞から心筋細胞を誘導するためには*Tbx5*や*Gata4*といった転写因子のみならず，SWI/SNF型クロマチンリモデリング複合体の心臓特異的サブユニットである*Baf60c*が必須であるということが明らかになった[5]．これはクロマチン因子がダイレクトリプログラミングに必要であることを示した最初の事例であり，クロマチン状態の細胞運命の決定における重要性を強く示唆している．このことから，どのようなエピジェネティックな変化が心筋へのリプログラミング，分化に際して起こっているのかを明らかにすることが，効率よく心筋細胞を作製する方法の開発とその現象の理解に必須であると考えられる．また，現在知られている方法で誘導された心筋細胞はあくまでも胎児性の心筋であり，移植に使えるような生理的，機能的に成熟した心筋を誘導する方法というのはいまだ開発されていない．そのため，心筋前駆細胞から成熟心筋に至る過程をエピジェネティクスのレベルで詳細に解明することが，移植治療にも使用可能な心筋を*in vitro*でつくるという再生医療の目標のため不可欠であると考えられる．

さらに，患者から採取した細胞をもとにiPS細胞を作製し，これを心筋細胞などに分化させるという方法を取ることで，疾患の*in vitro*モデル系をつくることが可能である．これはドラッグスクリーニングなど，治療法の開発のために非常に有用であり，これから幅広く用いられる手法と考えられる．しかしながら，特に遺伝的なリスク因子が必ずしも関係する疾患を引き起こさない，いわゆるpenetranceが低い場合にはその発症にエピジェネティクスが大きく関係していると考えられ，適切なモデル系を立ち上げるためには患者のエピゲノムを詳細に解析し，どのような条件下で病的な表現型を示すのかという点を明らかにする必要があると考えられる．

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