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Mineralocorticoid receptor function in bone metabolism and its role in glucocorticoid-induced osteopenia

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ABSTRACT

Although the mineralocorticoid receptor (MR) is expressed in osteoblasts and osteocytes and frequently co-localizes with the glucocorticoid receptors (GR), its pathophysiological functions in bone remain elusive. We report here that pharmacologic inhibition of MR function with eplerenone resulted in increased bone mass, with stimulation of bone formation and suppression of resorption, while specific genetic deletion of MR in osteoblast lineage cells had no effect. Further, treatment with eplerenone as well as specific deletion of MR in osteocytes ameliorated the cortical bone thinning caused by slow-release prednisolone pellets. Thus, MR may be involved in the deleterious effects of glucocorticoid excess on cortical bone.

1. Introduction

Hypertension and osteoporosis are among the most prevalent disorders associated with aging, and emerging evidence suggests that the renin–angiotensin–aldosterone system (RAAS), which plays a central role in the regulation of fluid and electrolyte balance as well as blood pressure, is closely linked to bone metabolism. We have previously demonstrated that activation of RAAS in double transgenic mice expressing both the human renin and angiotensinogen genes induces osteopenia due to excessive bone resorption [1], while genetic deletion of the angiotensin II receptor subtype AT1a in mice results in a high bone mass phenotype in association with elevated bone formation [2].

In primary aldosteronism, the most common cause of secondary hypertension, aldosterone excess has been associated with cardiovascular and renal injury as well as disordered calcium and bone metabolism [3]. In fact, in a rat model mimicking aldosteronism by means of chronic aldosterone infusion and salt loading, increased urinary calcium excretion and elevated PTH levels were observed along with a concomitantly compromised bone strength, all of which were reversed by treatment with spironolactone, a mineralocorticoid receptor (MR) antagonist [3,4]. These observations led to the hypothesis that renal calcium leak and secondary hyperparathyroidism underlie the bone abnormalities associated with aldosterone excess. While MR was detected in bone samples taken from human specimens, especially osteoblasts and osteocytes [5], the pathophysiological functions of MR in bone remain to be elucidated.

Glucocorticoid-induced osteoporosis is the most common form of secondary osteoporosis, and glucocorticoids are believed to impact calcium and bone metabolism through the glucocorticoid receptor (GR) in bone, intestine and kidney [6–8]. Glucocorticoid activity is locally controlled by the activity of the 11β-hydroxysteroid dehydrogenase (11β-HSD) isoenzymes 11β-HSD type 1 (11β-HSD1) and type 2 (11β-HSD2), which catalyze the interconversion of active glucocorticoids (cortisol in humans, corticosterone in rodents) and the inert 11-keto forms (cortisone and 11-dehydrocorticosterone or 11-DOC) [9,10]. 11β-HSD1 is widely distributed and has been suggested to regenerate active glucocorticoids from circulating inert 11-keto steroids, thereby amplifying glucocorticoid activity locally [10], while 11β-HSD2 is expressed in aldosterone target organs, such as distal nephron, colon and sweat glands, where it protects the MR from becoming occupied by glucocorticoids, thereby allowing aldosterone-selective access to the otherwise nonselective MR in target cells [9,11]. In view of the fact that glucocorticoids bind to the MR with high affinity [11], there exists the possibility that at least a portion of the glucocorticoid effect on bone is mediated through the MR, although this has never been reported. In the present study we have examined the

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pathophysiological functions of MR in bone metabolism using pharmacologic as well as genetic approaches. In doing so, we have focused on the functional interaction between the MR and GR in the setting of glucocorticoid excess.

2. Materials and methods

2.1. Reagents

Eplerenone was provided by Pfizer, Inc. (Groton, CT). 60-day release pellets respectively containing 5 mg prednisolone and placebo were purchased from Innovative Research of America (Sarasota, FL). Calcein and tetracycline for bone labeling were purchased from Sigma (St. Louis, MO).

2.2. Mice

MR flox mice have been described [12]. Mice hemizygous for the *Osterix-GFP::Cre* transgene were obtained from The Jackson Laboratory (Bar Harbor, ME) [13]. A transgenic cassette carrying a 7.4 kb mouse DMP1 gene promoter, including intron 1, Cre, IRES and an enhanced GFP (eGFP), was constructed. The transgenic construct was excised and purified using standard techniques. Founder DMP1-Cre mice were generated by microinjection of the DNA into fertilized eggs of C57BL/6 mice. Three independent transgenic founders were mated to wild-type C57BL/6 mice, and the F1 offspring were analyzed. All three lines produced pups at the expected ratio. These pups appeared normal, grew indistinguishably from wild-type mice, and were fertile. The results presented here are from line #2. RT-PCR with the RNA extracted from various tissues revealed that cre mRNA was expressed only in the bone of DMP1-Cre mice (Fig. 4A). Integration of the transgene was screened by PCR and then confirmed by Southern analysis of genomic DNA extracted from the tail. CAG-CAT-Z reporter mice were kindly provided by Dr. Junichi Miyazaki (Osaka Univ.) [14].

Mice were raised under standard laboratory conditions at 24 ± 2 °C and 50–60% humidity, and were allowed free access to tap water and standard rodent chow (CE-2, Clea Japan) containing 1.20% calcium, 1.08% phosphate and vitamin D_3 (240 IU per 100 g). For the p.o. administration of eplerenone (approximately 400 mg/kg/day by calculation), AIN-76A chow containing eplerenone (2.5 g/kg) obtained from the ESG Co., Ltd. (Tokyo, Japan) was used. Slow-release prednisolone pellets were implanted s.c. (2.8 mg/kg/day for 2 months) into 6-month-old male C57BL/6 mice according to the manufacturer's instructions. Control mice were implanted with placebo pellets from the same supplier. Experiments were performed on male mice unless indicated otherwise. All experiments were performed in accordance with NCGG ethical guidelines for animal care, and the experimental protocols were approved by the animal care committee.

2.3. Gene expression analysis

Total RNA was isolated with TRIzol reagent, purified with an RNeasy Mini Kit (Qiagen, Valencia, CA) and subjected to RT using a high-capacity cDNA RT kit (Applied Biosystems, Carlsbad, CA). Quantitative PCR analysis was performed using PowerSYBR Green PCR master mix and an ABI7300 real-time PCR system (Applied Biosystems). Semiquantitative RT-PCR was conducted using GoTaq (Promega, Madison, WI). RNAs from osteoblast- and osteocyte-rich fractions were isolated from the tibia and femur, as described previously [15]. In brief, after the bone marrow was flushed out with PBS, the diaphysis was cut longitudinally and the cells on the endocortical surface were collected in TRIzol reagent as the osteoblast-rich fraction. The residual bone pieces were crushed in liquid nitrogen to yield osteocyte-rich fraction, from which total RNA was isolated with the use of TRIzol. The sequences of the PCR primers (forward and reverse, respectively) were as follows: *Cre*, 5′-AGGTTCGTTCACTCATGGA-3′ and 5′-TCGACCA GTTTAGTTACCC-3′; *Gapdh*, 5′-ACTTTGTCAAGCTCATTTCC-3′ and

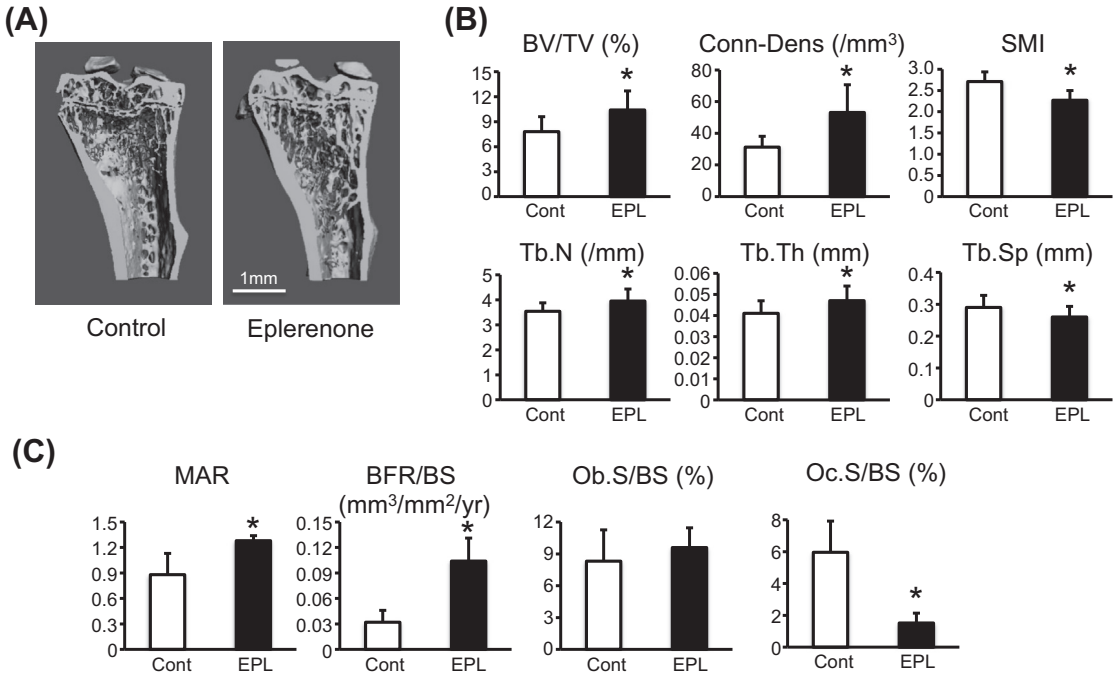

Fig. 1. Pharmacological inhibition of MR function with epleronone. (A) Representative micro-CT images of the tibia in control and eplerenone-treated mice. (B) Quantitation of the 3D bone volume fraction (BV/TV) and trabecular structure by micro-CT imaging as in (A). Conn-Dens, connectivity density; SMI, structure model index; Tb.N, Tb.Th, and Tb.Sp, trabecular number, thickness, and separation, respectively. Data are means ± SD for 9–10 mice of each group. $^*P < 0.05$. (C) Histomorphometric analysis of the tibial metaphysis of the control and eplerenone-treated mice. Mineral apposition rate (MAR), bone formation rate (BFR), osteoblast surface (Ob.S) and osteoclast surface (Oc.S) were corrected for the bone surface (BS). Data are means ± SD for 4 mice of each group. $^*P < 0.05$.

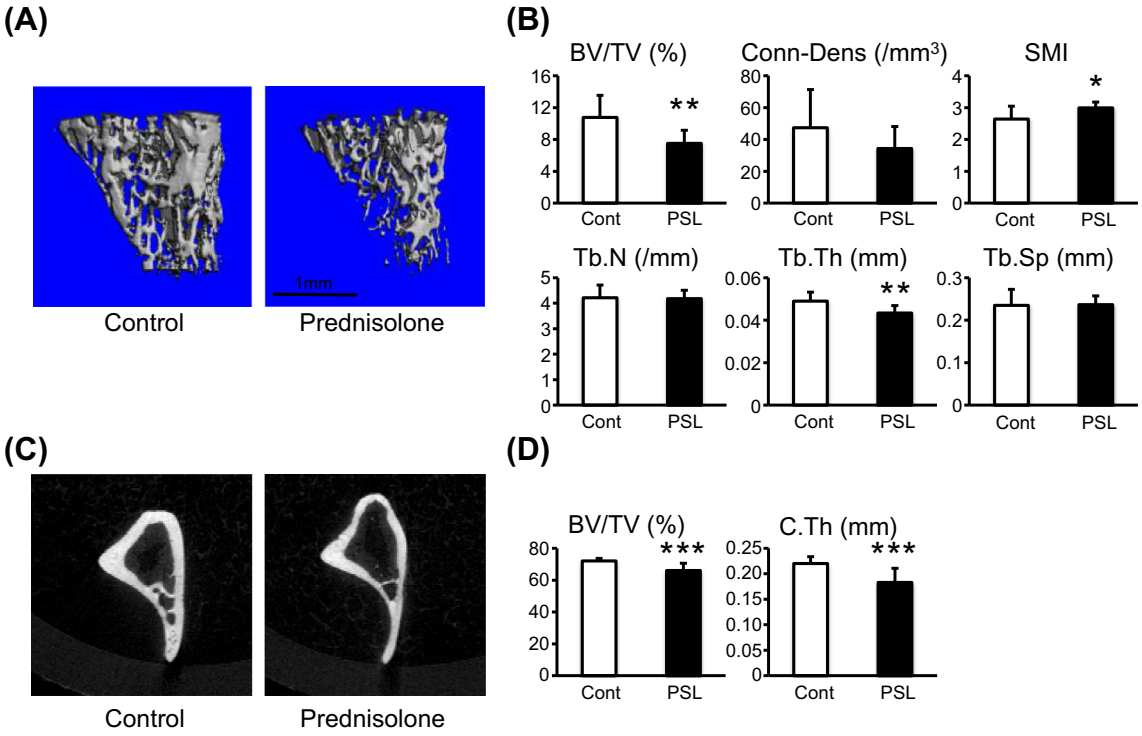

Fig. 2. Trabecular and cortical bone atrophy by slow-release prednisolone pellets. (A) Representative micro-CT images of the tibia of the control and prednisolone-treated mice. (B) Quantitation of the 3D bone volume fraction (BV/TV) and trabecular structure by micro-CT imaging as in (A). Conn-Dens, connectivity density; SMI, structure model index; Tb.N, Tb.Th, and Tb.Sp, trabecular number, thickness, and separation, respectively. Data are means ± SD for 10–12 mice of each group. $^{**}P < 0.01$, $^{*}P < 0.05$. (C) Cross-sectional micro-CT images of the tibial diaphysis. Note the thin cortex in the prednisolone-treated mouse. (D) Quantitation of cortical thickness (C.Th) and the cortical bone volume fraction (BV/TV) by micro-CT. Data are means ± SD for 10–12 mice of each group. $^{***}P < 0.001$.

5′-TGCAGCGAACTTTATTGATG-3′; *MR*, 5′-CGAAGTGTTTCTACTGGA TCCTCAA-3′ and 5′-TGACACCCAGAAGCCTCATCT-3′; and *Gapdh* (for qPCR), 5′-AGCTTGTCATCAACGGGAAG-3′ and 5′-TTTGATGT-TAGTGGGGTCTCG-3′. The abundance of target mRNA was normalized by that of *Gapdh* mRNA.

2.4. Bone analysis

Micro-CT scanning was performed with a μCT-40 device (SCANCO Medical, Bassersdorf, Switzerland) at a resolution of 12 μm, and the 3D microstructural parameters were calculated as described previously [16]. Bone samples were fixed in 70% ethanol for the determination of histomorphometric parameters using nondecalcified sections at the Ito Bone Science Institute (Niigata, Japan); double labeling with calcein and tetracycline was performed by the consecutive s.c. administration of the labels with a 2-day interval. The nomenclature used for the micro CT and histomorphometry follows the recommendations of the published guidelines [17,18].

2.5. Statistical analysis

Quantitative data are presented as means ± SD and were analyzed with Student's *t* test. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. MR function in bone metabolism

In an attempt to address the functions of MR *in vivo*, we first adopted a pharmacological approach and examined the effects of eplerenone on bone remodeling. As shown in Fig. 1A, eplerenone treatment increased the trabecular bone at the proximal tibia. Detailed 3D analysis following micro CT scanning of the metaphysis of the proximal tibia revealed that the 3D bone volume fraction (BV/TV) as well as the trabecular connectivity, number and thickness were all significantly increased following eplerenone treatment compared with the control group (Fig. 1B). Bone histomorphometry at the proximal tibia revealed that the mineral apposition rate (MAR) and bone formation rate (BFR) were increased, indicating the stimulation of bone formation by eplerenone, while the osteoclast surface (Oc.S) was reduced (Fig. 1C). Collectively, it was suggested that MR exerts a negative effect on bone mass regulation.

We next generated mice with targeted deletion of the MR in osteoblast lineage cells, osteoblasts and osteocytes, by crossing MR floxed mice [12] with Osterix-Cre mice [13], based on a previous immunohistochemical study that reported potent MR antibody staining in osteoblasts and osteocytes [5]. We have shown that Osterix-Cre is capable of deleting a target gene by more than 80% [15]. Micro CT analysis of the tibial metaphysis revealed that the 3D bone volume fraction and other microstructural parameters were not different from those of the control group (Supplemental Fig. 1).

3.2. MR function in glucocorticoid excess

In view of the cross talk between MR and GR and also the previous finding that MR and GR, especially the β isoform, are co-localized in bone sections [5], we addressed the issue of functional interaction between the MR and GR in bone metabolism. Based on a previous report, we first generated a mouse model of glucocorticoid-induced osteoporosis by subcutaneously implanting slow-release prednisolone pellets in mature (6-month-old) male mice [19]. Micro CT analysis showed that the trabecular bone volume

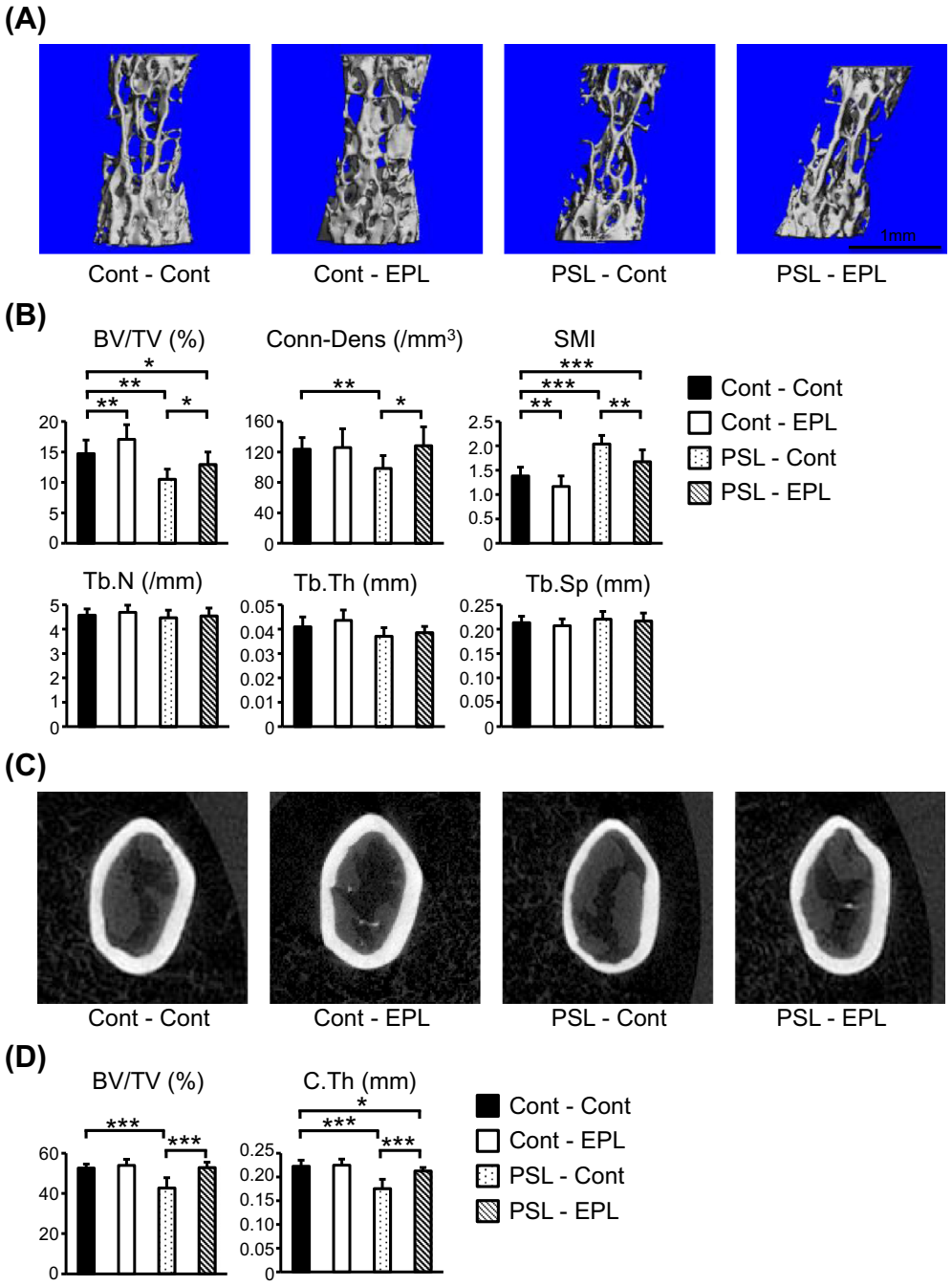

Fig. 3. MR in glucocorticoid-induced osteopenia. (A) Representative micro-CT images of the third lumbar vertebra of the control (Cont) and eplerenone (EPL)- and/or prednisolone (PSL)-treated groups. (B) Quantitation of the 3D bone volume fraction (BV/TV) and trabecular structure by micro-CT imaging as in (A). Conn-Dens, connectivity density; SMI, structure model index; Tb.N, Tb.Th, and Tb.Sp, trabecular number, thickness, and separation, respectively. Data are means ± SD for 10–15 mice of each group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. (C) Cross-sectional micro-CT images of the femoral diaphysis. Note the thin cortex in the prednisolone-treated mouse (PSL-Cont) and amelioration with eplerenone (PSL–EPL). (D) Quantitation of cortical thickness (C.Th) and the cortical bone volume fraction (C.BV/TV) by micro-CT as in (C). $^{***}P < 0.001$, $^{*}P < 0.05$.

fraction was significantly decreased in the proximal tibia and lumbar vertebra (Fig. 2A and B, and data not shown) of the prednisolone-treated group, and that cortical thickness as well as bone volume were also reduced compared with control mice implanted with placebo pellets (Fig. 2C and D).

In order to gain some insight into the function of MR in the pathogenesis of glucocorticoid-induced osteoporosis, eplerenone was administered p.o. into mice implanted with slow-release prednisolone pellets. Micro CT analysis of the third lumbar vertebra revealed that the decreased 3D bone volume fraction and connectivity induced by the prednisolone pellets were significantly ameliorated by eplerenone treatment (Fig. 3A and B). The thinning of cortical bone by chronic prednisolone release was also significantly ameliorated by eplerenone (Fig. 3C and D).

Finally, in order to examine the role of the osteocytic MR in glucocorticoid-induced osteopenia, we attempted to generate mice specifically lacking MR in osteocytes using DMP1-Cre transgenic mice in which Cre expression in bone is targeted (Fig. 4A). Gene expression analysis using RNA extracted from the osteocyte- and osteoblast-enriched fractions demonstrated that MR was more

abundant in the osteocyte- than bone surface osteoblast-rich fraction, and that the expression of MR in the osteocyte fraction was selectively decreased by mating with the DMP1-Cre transgenic mice (Fig. 4B). Again, the slow-release prednisolone pellets induced significant decreases in cortical thickness and bone volume in the control group, whereas a specific deletion of MR in osteocytes attenuated these changes caused by prednisolone administration (Fig. 4C and D), suggesting that the MR in osteocytes mediates the deleterious effects of glucocorticoids on cortical bone.

4. Discussion

Glucocorticoid-induced osteoporosis (GIO) is the most common form of secondary osteoporosis and affects patient quality of life by predisposing to fragility fracture [6–8]. It is recognized that GIO afflicts essentially all patients undergoing glucocorticoid treatment, with little inter-individual variation, even at low doses. Fracture due to GIO can occur quickly, sometimes within just a few months of the initiation of treatment, when the bone mineral density (BMD) is still above the fracture threshold defined for the most common involutional form of osteoporosis. Thus, it is conceivable that glucocorticoids compromise bone quality preferentially rather than by a direct impact on bone quantity [20], although the underlying pathogenic process remains poorly understood [7,21,22].

In bone, glucocorticoids are known to inhibit the differentiation and function of osteoblasts and to promote the apoptosis of osteoblasts and osteocytes, thereby leading to a profound suppression of bone formation, a central feature of GIO [19]. Although the physiologic functions of osteocytes are not fully understood [23], accumulating evidence points to their having a critical role in the regulation of both osteoblastic and osteoclastic function as well as mineral homeostasis. These activities are carried out by specific secretory products, such as fibroblast growth factor (FGF)-23, sclerostin and RANKL [24]. Using a targeted ablation model of osteocytes in transgenic mice, we previously demonstrated that osteocytes play important physiological roles in bone remodeling through the regulation of both osteoblastic and osteoclastic activities, mainly via mechanotransduction in bone [25]. In the absence of functional osteocytes, bone becomes fragile, with both impaired bone formation and proliferation of adipose tissue in the bone marrow [25]. Taken together with the findings that glucocorticoids induce apoptosis in osteocytes as well as osteoblasts [19], we hypothesized that osteocyte dysfunction is involved in the development of fragile bone that is associated with glucocorticoid use. In the current model of chronic glucocorticoid excess, we did not observe a decrease in osteocyte number, but nevertheless, did observe a thinning of cortical bone, which is a major determinant of long bone fragility. Intriguingly, the thinning of cortical bone caused by the prednisolone pellets was ameliorated, at least in part, through an inhibition of MR function by eplerenone on the one hand, and by genetic deletion of MR in osteocytes on the other, pointing to there being a cross-talk between the MR and GR in osteocytes. At baseline, without glucocorticoid excess, however, the pharmacologic inhibition of MR with eplerenone had substantial anabolic effects on bone, while the genetic deletion of MR in osteoblasts and osteocytes with Osx-Cre had no effect. This may be due to the fact that eplerenone treatment was started after skeletal maturity, while genetic deletion of MR was in place even before birth, when osterix started to be expressed. Alternatively, the skeletal effects of eplerenone may not be mediated through

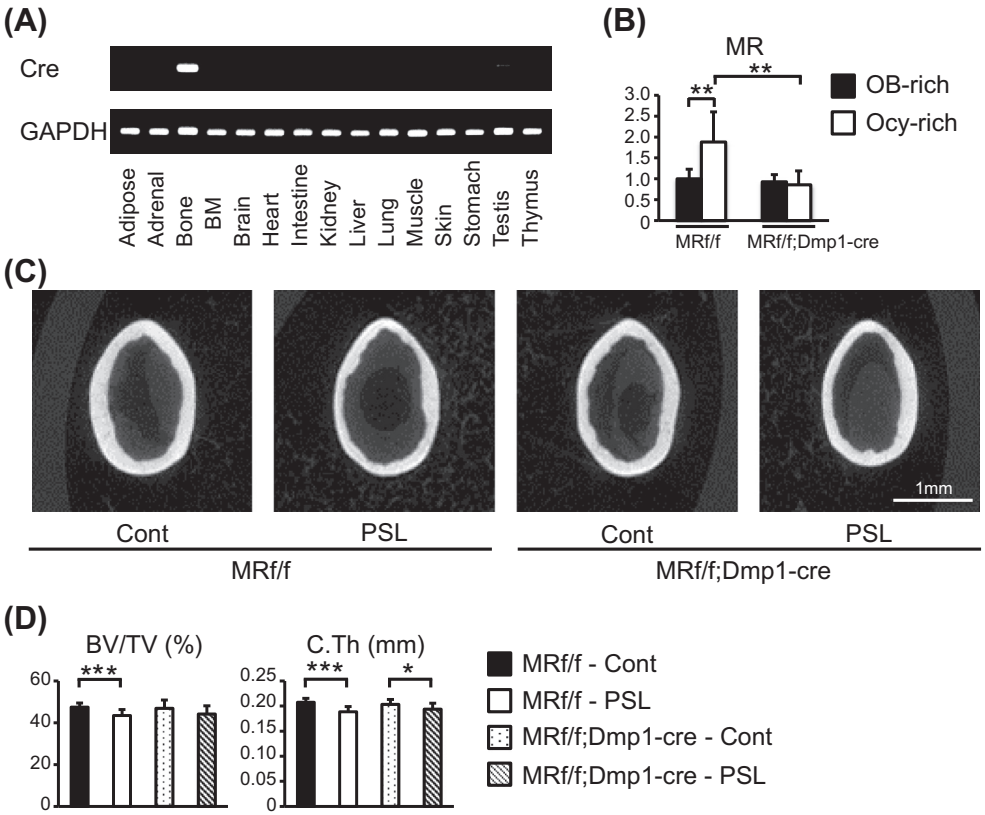

Fig. 4. Mice lacking MR in osteocytes. (A) Specific expression of Cre mRNA in the bone of DMP1-Cre mice at 12 weeks of age. (B) Quantitative RT-PCR analysis of MR mRNA in osteoblast- (OB) and osteocyte (OCy)-rich fractions isolated from the femur of female mice of the indicated genotypes at 12 weeks of age. Data are expressed relative to the corresponding value for the control (MRf/f) group (OB-rich) and are means ± SD for eight mice of each group. $^{**}P < 0.01$. (C) Cross-sectional micro-CT images of the femoral diaphysis. Note the thin cortex in the prednisolone-treated control (PSL MRf/f) mouse and amelioration in MRf/f;DMP1-Cre mouse. (D) Quantitation of cortical thickness (C.Th) and the cortical bone volume fraction (BV/TV) by micro-CT. Data are means ± SD for 11–13 mice of each group. $^{***}P < 0.001$, $^{*}P < 0.05$.

MR in the osteoblast lineage, but through MR in tissues other than bone.

Local glucocorticoid activity is regulated by 11β-hydroxysteroid dehydrogenase (11β-HSD), which interconverts active and inactive glucocorticoids [10]: 11β-HSD type 1 (11β-HSD1) is widely expressed in glucocorticoid target organs and converts inactive cortisone (11-dehydrocorticosterone in mice) to active cortisol (corticosterone in mice). In contrast, 11β-HSD2 is expressed highly in a few aldosterone target organs, such as the kidney, where it protects the otherwise nonselective MR from being occupied by cortisol or corticosterone, thereby conferring target cell specificity to MR [26]. Bone cells express both 11β-HSD1 and 11β-HSD2. Mice deficient in 11β-HSD1 exhibit a normal bone mass with an absence of bone marrow adipocytes, suggesting that 11β-HSD1 is required for adipocyte formation, but not for bone formation [27]. The reported observation that the inactivation of glucocorticoids in bone through transgenic expression of 11β-HSD2 in mature osteoblasts and osteocytes resulted in reduced bone mass led the investigators to conclude that physiologic levels of endogenous glucocorticoids are required for the maintenance of normal bone mass, at least in female mice [28]. Since glucocorticoids also bind to the MR [11], another interpretation might be that the local inactivation of glucocorticoids and the resultant activation of MR function by aldosterone may have caused the low bone mass phenotype, and our findings are consistent with this scenario.

In conclusion, MR is suggested to be involved in the deleterious actions of glucocorticoids on bone, especially cortical thinning.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.149.

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