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Aryl hydrocarbon receptor catabolic activity in bone metabolism is osteoclast dependent *in vivo*

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ABSTRACT

Bone mass is regulated by various molecules including endogenous factors as well as exogenous factors, such as nutrients and pollutants. Aryl hydrocarbon receptor (AhR) is known as a dioxin receptor and is responsible for various pathological and physiological processes. However, the role of AhR in bone homeostasis remains elusive because the cell type specific direct function of AhR has never been explored *in vivo*. Here, we show the cell type specific function of AhR *in vivo* in bone homeostasis. Systemic AhR knockout (AhRKO) mice exhibit increased bone mass with decreased resorption and decreased formation. Meanwhile, osteoclast specific AhRKO ($AhR^{\Delta Oc/\Delta Oc}$) mice have increased bone mass with reduced bone resorption, although the mice lacking AhR in osteoblasts have a normal bone phenotype. Even under pathological conditions, $AhR^{\Delta Oc/\Delta Oc}$ mice are resistant to sex hormone deficiency-induced bone loss resulting from increased bone resorption. Furthermore, 3-methylcholanthrene, an AhR agonist, induces low bone mass with increased bone resorption in control mice, but not in $AhR^{\Delta Oc/\Delta Oc}$ mice. Taken together, cell type specific *in vivo* evidence for AhR functions indicates that osteoclastic AhR plays a significant role in maintenance of bone homeostasis, suggesting that inhibition of AhR in osteoclasts can be beneficial in the treatment of osteoporosis.

1. Introduction

Bone mass is regulated by various endocrine factors, such as sex steroid hormones and several pituitary gland hormones, and by exogenous factors, such as nutrients and pollutants. Among these regulators, fat-soluble bioactive substances are recognized as significant molecules. It is well known that fat-soluble bioactive substances target to various tissues and organs, and play significant roles in the maintenance of physiological homeostasis. Fat-soluble bioactive substances are diverse, and include fat-soluble vitamins such as vitamin A/D, steroid hormones such as androgen and estrogen, and environmental toxins such as dioxins.

Fat-soluble bioactive substances act as ligands and bind to nuclear receptors. Nuclear receptors bind directly to specific DNA elements in target gene promoters and/or enhancers, and positively or negatively control transcription [1]. For example, vitamin D binds to the vitamin D receptor (VDR) and targets small intestine and kidney, regulates calcium metabolism, and participates in bone homeostasis [2,3]. Likewise, estrogen, a sex steroid hormone, regulates development and maturation of reproductive organs, and also maintains bone homeostasis directly or indirectly [4]. The physiological significance of nuclear receptors in bone metabolism has been revealed by studies focused on the function of cell type specific nuclear receptors [5,6].

Notably, fat-soluble bioactive substances such as dioxins also exert physiological actions by binding to the aryl hydrocarbon receptor (AhR). AhR, which is expressed ubiquitously, is a member of a transcription factor superfamily characterized by structural motifs of basic helix-loop-helix (bHLH)/Per-AhR nuclear translocator (Arnt)-Sim (PAS) domains [7]. The toxicology and pharmacology of AhR has been studied for over a decade. With recent discoveries of novel AhR functions, however, AhR research has expanded into multiple aspects of physiology and pathology, such as immunoregulation [8,9], tumor suppression [10], liver development [11], reproduction [12], and vascular remodeling of the developing embryo [13]. However, few studies have addressed the regulation of bone homeostasis by AhR. Hitherto, the function of AhR on bone metabolism has been reported by two research

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groups [14,15], however, these studies were analyzed using systemic AhR knockout or transgenic mice expressing constitutively active AhR. Systemic mutant mice might influenced by indirect effects from tissues/organs except bone, therefore, AhR function might be clarified by more focused studies utilizing conditional AhR knockout mice that can elucidate the bone cell type specific functions of AhR *in vivo*.

In this study, we analyzed mice in which the AhR gene had been conditionally deleted from osteoclasts to reveal that osteoclastic AhR plays a significant role in the maintenance of bone. Mice lacking AhR in osteoclasts ($AhR^{\Delta Oc/\Delta Oc}$) were resistant to bone loss induced by ovariectomy (OVX), orchidectomy (ORX), or treatment with 3MC, an AhR agonist, suggesting that control of AhR activity in the osteoclast might be a promising therapeutic strategy for osteoporosis.

2. Materials and Methods

2.1. Mice

Systemic AhR KO mice [16] and AhR floxed mice were provided by Dr. Y Fujii-Kuriyama. The $\alpha_1(I)$-*Collagen-Cre* mice (*Col1a1-Cre*$^{tg/0}$) were kindly provided by G. Karsenty Laboratory [17]. Osteoblast-specific AhR KO mice ($AhR^{\Delta Ob/\Delta Ob}$: *Col1a1-Cre*$^{tg/0}$; $AhR^{flox/flox}$) were generated by breeding *Col1a1-Cre*$^{tg/0}$; $AhR^{flox/+}$ male mice and $AhR^{flox/flox}$ female mice, and $AhR^{flox/flox}$ mice were considered as controls. The *Cathepsin K Cre* (*Ctsk-Cre*) mice (*Ctsk*$^{Cre/+}$) [5] were back-crossed into C57BL6 more than ten times. Osteoclast-specific AhR KO mice ($AhR^{\Delta Oc/\Delta Oc}$: *Ctsk*$^{Cre/+}$; $AhR^{flox/flox}$) were generated by breeding *Ctsk*$^{Cre/Cre}$; $AhR^{flox/+}$ male mice and *Ctsk*$^{+/+}$; $AhR^{flox/+}$ female mice, and *Ctsk*$^{Cre/+}$; $AhR^{+/+}$ were considered as controls. All mice were housed in a specific-pathogen-free (SPF) facility under climate-controlled conditions with a 12-h light/dark cycle and were provided with water and standard diet (CE-2, CLEA, Japan) *ad libitum*. They were euthanized at 12 or 24 weeks of age. All animals were maintained and examined according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo and Ehime University.

2.2. Micro-computed tomography (μCT) analysis

Micro-CT analysis was performed as described using a μCT system (μCT35, SCANCO Medical, Bruttisellen, Switzerland) [2,6]. Briefly, 466 slices were acquired, starting just beneath the end of the growth plate, thus including both the primary and secondary spongiosa, and a region 1.8 mm in length of the distal metaphyseal secondary spongiosa (300 slices) was selected for analysis. Three-dimensional reconstructions were generated and analyzed according to the guideline [18].

2.3. Bone histomorphometric analyses

Bone histomorphometry was performed as previously described [5,6]. Bone histomorphometric analyses were performed using the OsteoMeasure analysis system (OsteoMetrics Inc., GA, USA) according to ASBMR guidelines [19].

2.4. AhR-ligand treatment

Female control ($AhR^{+/+}$: *Ctsk*$^{Cre/+}$ $AhR^{+/+}$) and osteoclast-specific AhR KO ($AhR^{\Delta Oc/\Delta Oc}$: *Ctsk*$^{Cre/+}$; $AhR^{flox/flox}$) littermates were treated with 3-methylcholanthrene (3MC, Wako, Japan) or a placebo of corn oil starting when they were eight weeks old. 3MC was dissolved in corn oil and injected at 0.01 mg/g body weight, twice a week for four weeks. Mice were analyzed at 12 weeks of age.

2.5. Mice orchidectomy and ovariectomy model

Eight-week-old control mice ($AhR^{+/+}$: *Ctsk*$^{Cre/+}$ $AhR^{+/+}$) and osteoclast-specific AhR KO mice ($AhR^{\Delta Oc/\Delta Oc}$: *Ctsk*$^{Cre/+}$; $AhR^{flox/flox}$) were sham operated, orchidectomized, or ovariectomized under anesthesia. Four weeks after surgery, all of the mice were euthanized and subjected to micro-computed tomography and bone histomorphometry.

2.6. Elisa

ELISAs were performed following the manufacturers' protocols using the Estradiol EIA Kit (Cayman Chemical Company) for estradiol, Testosterone EIA Kit (Cayman Chemical Company) for testosterone, and Insulin-like Growth Factor-l (IGF-1) ELISA TEST (Endocrine Technologies) for IGF-1.

2.7. Osteoclast culture

Osteoclastogenesis *in vitro* was studied by plating bone-marrow cells from 8-week-old mice in culture dishes containing α-MEM (GIBCO) with 10% FBS (MP Biomedicals). After incubation for 8 h, nonadherent cells were collected, and cells were seeded (3×10^5 -cells/dish) in 6 cm suspension dishes containing α-MEM with 10 ng/ml M-CSF (R&D Systems). After 2 days (about 80% confluent), adherent cells were used as osteoclast precursor cells after washing out the nonadherent cells. Cells were cultured in the presence of 10 ng/ml M-CSF and 234 ng/ml GST-RANKL (Oriental yeast, Japan) for three days to generate osteoclast like cells.

2.8. Real-time PCR analysis

Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using PrimeScript RT Master Mix (Takara Bio Inc) and subjected to real-time PCR using KAPA SYBR Fast qPCR Kits (Kapa Biosystems) with Thermal Cycler Dice (Takara Bio Inc) according to the manufacturers' instructions. Expression levels were normalized by *Gapdh*. The following primers were used: *AhR*, 5′-TTCTATGCTTCCTCCACTATCCA-3′ and 5′-GGCTTCGTCCAC TCCTTGT-3′; *Gapdh*, 5′-AAATGGTGAAGGTCGGTGTG-3′ and 5′-TG AAGGGGTCGTTGATGG-3′.

2.9. Statistical analysis

Data were analyzed by a two-tailed student's *t*-test. For all graphs, data are represented as mean ± SD. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Increased bone mass in AhR KO ($AhR^{-/-}$) mice

We characterized the bone phenotype of mice in which AhR was systemically knocked out (AhRKO, $AhR^{-/-}$). We performed micro-computed tomography (μCT) on the distal femur to measure BMD and analyze three-dimensional trabecular architecture to determine whether there were differences between $AhR^{-/-}$ and $AhR^{+/+}$ mice. As shown in Fig. 1A–C, the distal femurs of both male and female $AhR^{-/-}$ mice exhibited greater trabecular bone volume (BV/TV), trabecular number (Tb.N), connectivity density (Conn.D), and bone mineral density (BMD), and smaller trabecular separation (Tb.Sp) and structure model index (SMI) when compared with $AhR^{+/+}$ mice. Next we performed Von Kossa/Van Gieson staining in the L3 and L4 lumbar vertebrae to assess mineralized bone

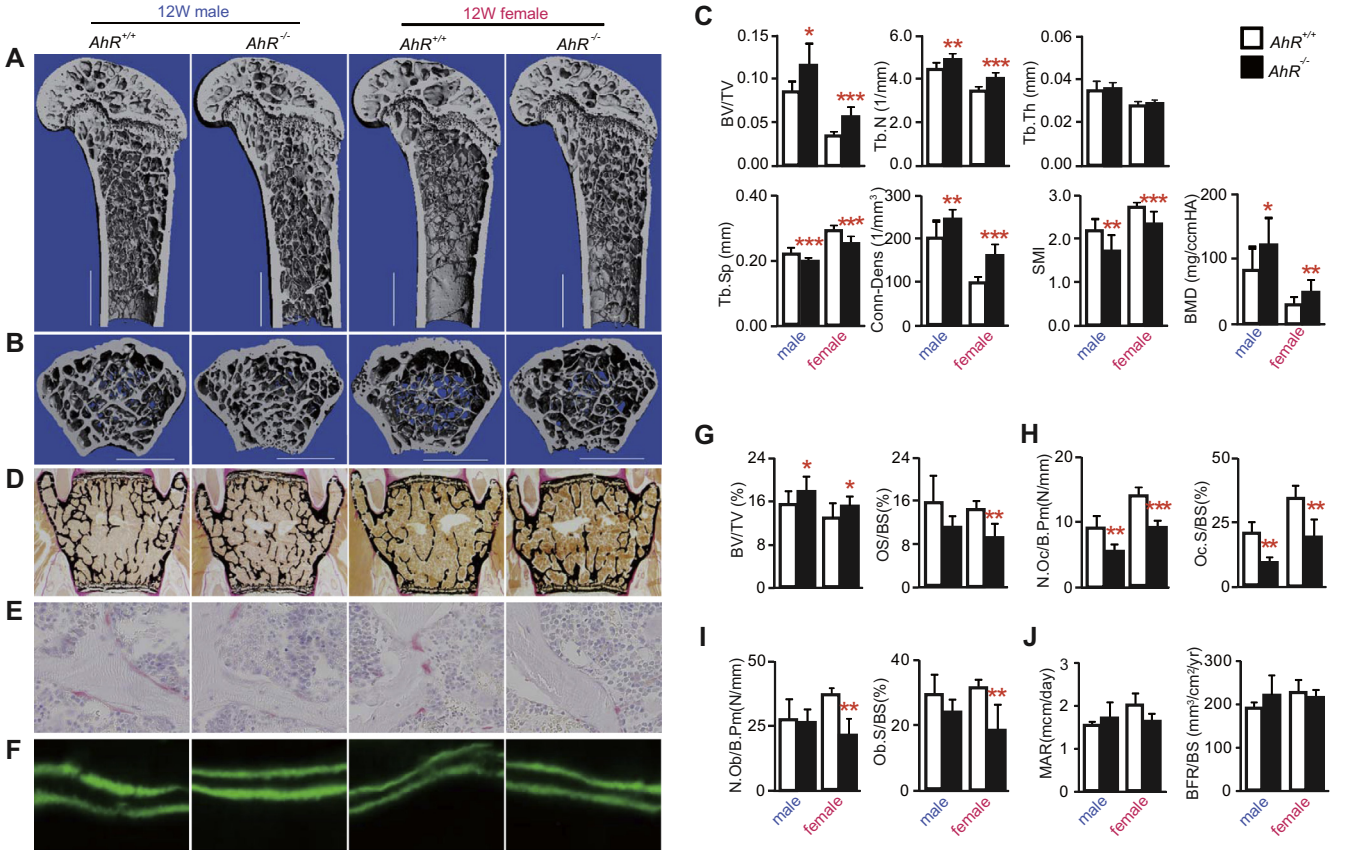

Fig. 1. $AhR^{-/-}$ mice exhibit high bone mass. (A–C) Micro-computed tomography (μCT) analysis of the distal femurs from 12-week-old $AhR^{-/-}$ mice and their wild-type littermates ($AhR^{+/+}$). Representative images of the distal femurs (A) and axial sections of the distal metaphysis (B). Scale bars indicate 1.0 mm. μCT parameters are shown in (C) (n = 7–13 animals per group). (D–J) Bone histomorphometric analysis of lumbar vertebrae. Sections were stained with Von Kossa/Van Gieson stain (D), tartrate-resistant acid phosphatase (TRAP) stain (E) or left unstained to evaluate calcein labeling (F). Trabecular bone volume and osteoid surface (G), osteoclast number and surface (H), osteoblast number and surface (I) and dynamic histomorphometric parameters (J) are shown. Scale bars indicate 1.0 mm. Results are shown as mean ± SD (n = 5–7 animals per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

volume in vertebral trabecular bone of $AhR^{-/-}$ mice. Consistent with the femur, histomorphometric analysis of vertebrae also demonstrated that both male and female $AhR^{-/-}$ mice displayed significantly greater vertebral BV/TV (Fig. 1D and G).

Furthermore, to define the cellular bases of the increased bone mass phenotype observed in $AhR^{-/-}$ mice, we evaluated parameters of bone formation and resorption. Histomorphometric analyses of lumbar vertebrae revealed that both male and female $AhR^{-/-}$ mice had significantly fewer osteoclasts, evaluated by osteoclast number and osteoclast surface (Fig. 1E and H). Although female $AhR^{-/-}$ mice had fewer osteoblasts, evaluated by osteoblast number and osteoblast surface (Fig. 1I), no significant effect was observed on mineral apposition rate (MAR) or bone formation rate (BFR) (Fig. 1F and J). These results demonstrate that the increased bone mass phenotype of $AhR^{-/-}$ mice is caused by reduced bone resorption. Collectively, our findings support those reported by Iqbal et al. [14]. Still, the physiological function of AhR in bone metabolism *in vivo* might be influenced by secondary effects on bone from other tissues rather than direct effects on bone cells.

3.2. Normal bone mass in osteoblast-specific AhR KO ($AhR^{\Delta Ob/\Delta Ob}$) mice

We generated and analyzed cell type specific AhR KO mice to determine whether AhR is directly or indirectly involved in bone metabolism *in vivo*. The analyses of the systemic AhRKO mice showed that high bone mass was the result of fewer osteoclasts and presumably less bone resorption. RANKL, which is mainly derived from osteoblast lineage cells, is critical for osteoclast differentiation. Therefore, it is necessary to assess the cell type specific function of AhR in both osteoblasts and osteoclasts to evaluate the direct function of AhR in bone metabolism *in vivo*.

We relied on osteoblast-specific gene inactivation experiments in the mouse to determine whether osteoblast-specific AhR signaling directly affects bone mass. Crossing mice harboring a floxed allele of AhR with α_1(I)-Collagen-*Cre* mice ($Col1a1\text{-}Cre^{tg/0}$), a transgenic mouse line expressing the Cre recombinase specifically in osteoblasts, allowed us to generate osteoblast-specific AhR KO mice ($AhR^{\Delta Ob/\Delta Ob}$: $Col1a1\text{-}Cre^{tg/0}$; $AhR^{flox/flox}$). Likewise, μCT analyses of the distal femurs in 12-week-old $AhR^{\Delta Ob/\Delta Ob}$ mice, a time when $AhR^{-/-}$ mice have significantly greater bone mass, failed to detect any difference between groups (Supplemental Fig. S1A–C). In addition, bone histomorphometric analyses of L3 and L4 lumbar vertebrae in 12-week-old $AhR^{\Delta Ob/\Delta Ob}$ mice indicated that bone mass, bone formation and bone resorption parameters were all normal (Supplemental Fig. S1D–J).

These results indicate that the absence of AhR in osteoblasts is not the cause of the high bone mass observed in $AhR^{-/-}$ mice, and suggest that expression of AhR in osteoblasts plays little role in bone mass regulation.

3.3. Increased bone mass and decreased bone resorption in osteoclast-specific AhR KO ($AhR^{\Delta Oc/\Delta Oc}$) mice

We next asked whether a deletion of AhR in osteoclasts would affect bone mass. We crossed mice harboring a floxed allele of AhR

with Ctsk-Cre mice ($Ctsk^{Cre/+}$), a knock-in mouse line expressing the Cre recombinase specifically in differentiated mature osteoclastic cells [5]. Real-time RT-PCR analyse demonstrated that we had effectively deleted AhR in osteoclasts of osteoclast-specific AhR KO mice ($AhR^{\Delta Oc/\Delta Oc}$: $Ctsk^{Cre/+}$; $AhR^{flox/flox}$) (Supplemental Fig. S2A).

12-week-old $AhR^{\Delta Oc/\Delta Oc}$ mice showed greater BV/TV, Tb.N, BMD, and Conn.D, and smaller Tb.Sp and SMI in the distal femurs compared with littermate controls ($Ctsk^{Cre/+}$; $AhR^{+/+}$), regardless of gender (Fig. 2A–C). In addition, bone histomorphometric analyses revealed significantly greater bone mass in lumbar vertebrae of $AhR^{\Delta Oc/\Delta Oc}$ mice (Fig. 2D and G), without significant alterations in serum levels of IGF-1 or sex hormones (Supplemental Fig. S2B and C). Static and dynamic histomorphometric evaluation showed lower bone resorption parameters (N.Oc/B.Pm and Oc.S/BS) (Fig. 2E and H), but bone formation parameters (N.Ob/B.Pm, Ob.S/BS, MAR and BFR/BS) were not different (Fig. 2F, I and J). This phenotype also was observed in 24-week-old male mice, but female $AhR^{\Delta Oc/\Delta Oc}$ mice did not exhibit significantly greater bone mass although bone resorption parameters were significantly lower (Supplemental Fig. S3). This might be caused by static sexual maturation in older mice as previously described [15]. Taken together, these results indicate that AhR in osteoclasts, not in osteoblasts, plays a significant role in bone homeostatic maintenance.

3.4. Mice lacking AhR in osteoclasts are resistant to bone loss induced by sex hormone deficiency

However, it is important to stress that these are metabolically normal animals. The pathophysiological significance of AhR under conditions of altered bone metabolism, such as osteoporosis, remains to be clarified. To address this question, 8-week-old control and $AhR^{\Delta Oc/\Delta Oc}$ mice were gonadectomized or sham-operated, and analyzed four weeks after surgery.

In female mice, μCT revealed that ovariectomized mice of both genotypes exhibited significant trabecular bone loss in the distal femur when compared with sham-operated mice, as expected. However, whereas BMD and BV/TV were reduced by about 37% and 51% respectively in control mice after ovariectomy (OVX), BMD and BV/TV were decreased in femoral trabecular bone in OVX $AhR^{\Delta Oc/\Delta Oc}$ mice by only 26% and 32% respectively (Fig. 3A, B and C). In addition, bone histomorphometric analyses revealed that trabecular bone loss in vertebrae induced by OVX in $AhR^{\Delta Oc/\Delta Oc}$ mice (24%) was significantly less than that in control mice (47%) (Fig. 3D, E). Furthermore, it was revealed that the increase of N.Oc/B.Pm and Oc.S/BS induced by OVX was suppressed in $AhR^{\Delta Oc/\Delta Oc}$ mice compared to controls (Fig. 3F, G), but without any alteration in static bone formation parameters (Fig. 3H). These results indicate that $AhR^{\Delta Oc/\Delta Oc}$ mice are more resistant to bone loss induced by OVX because there are fewer osteoclasts. Likewise in males, orchidectomy (ORX) also reduced bone loss and bone resorption in $AhR^{\Delta Oc/\Delta Oc}$ mice compared to control male mice (Supplemental Fig. S4).

3.5. $AhR^{\Delta Oc/\Delta Oc}$ mice are tolerant to bone loss induced by the AhR-agonist, 3MC

Environmental pollutants such as dioxins affect bone health by inducing osteopenia [20]. Low concentrations of AhR-agonists,

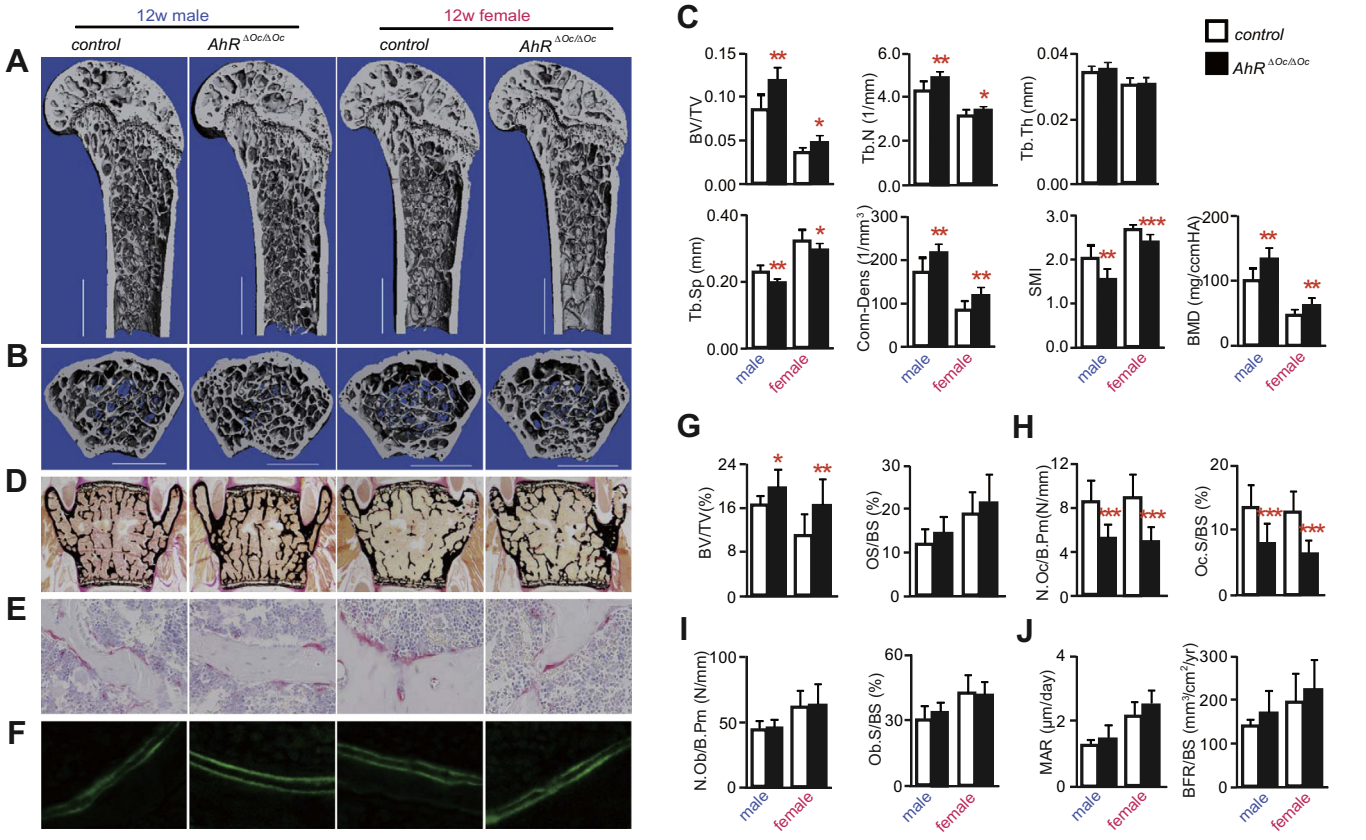

Fig. 2. Osteoclastic AhR regulates bone mass *in vivo*. (A–C) μCT analysis of the distal femurs from 12-week-old $AhR^{\Delta Oc/\Delta Oc}$ mice and their littermate controls ($Ctsk^{Cre/+}$; $AhR^{+/+}$). Representative images of the distal femurs (A) and axial sections of the distal metaphysis (B). Scale bars indicate 1.0 mm. μCT parameters are shown in (C) (n = 8–11 animals per group). (D–J) Bone histomorphometric analysis of lumbar vertebrae from 12-week-old $AhR^{\Delta Oc/\Delta Oc}$ mice and their littermate controls. Sections were stained with Von Kossa/Van Gieson stain (D), TRAP stain (E) or left unstained to evaluate calcein labeling (F). Trabecular bone volume and osteoid surface (G), osteoclast number and surface (H), osteoblast number and surface (I) and dynamic histomorphometric variables (J) are shown. Results are shown as means ± SD (n = 10–12 animals per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

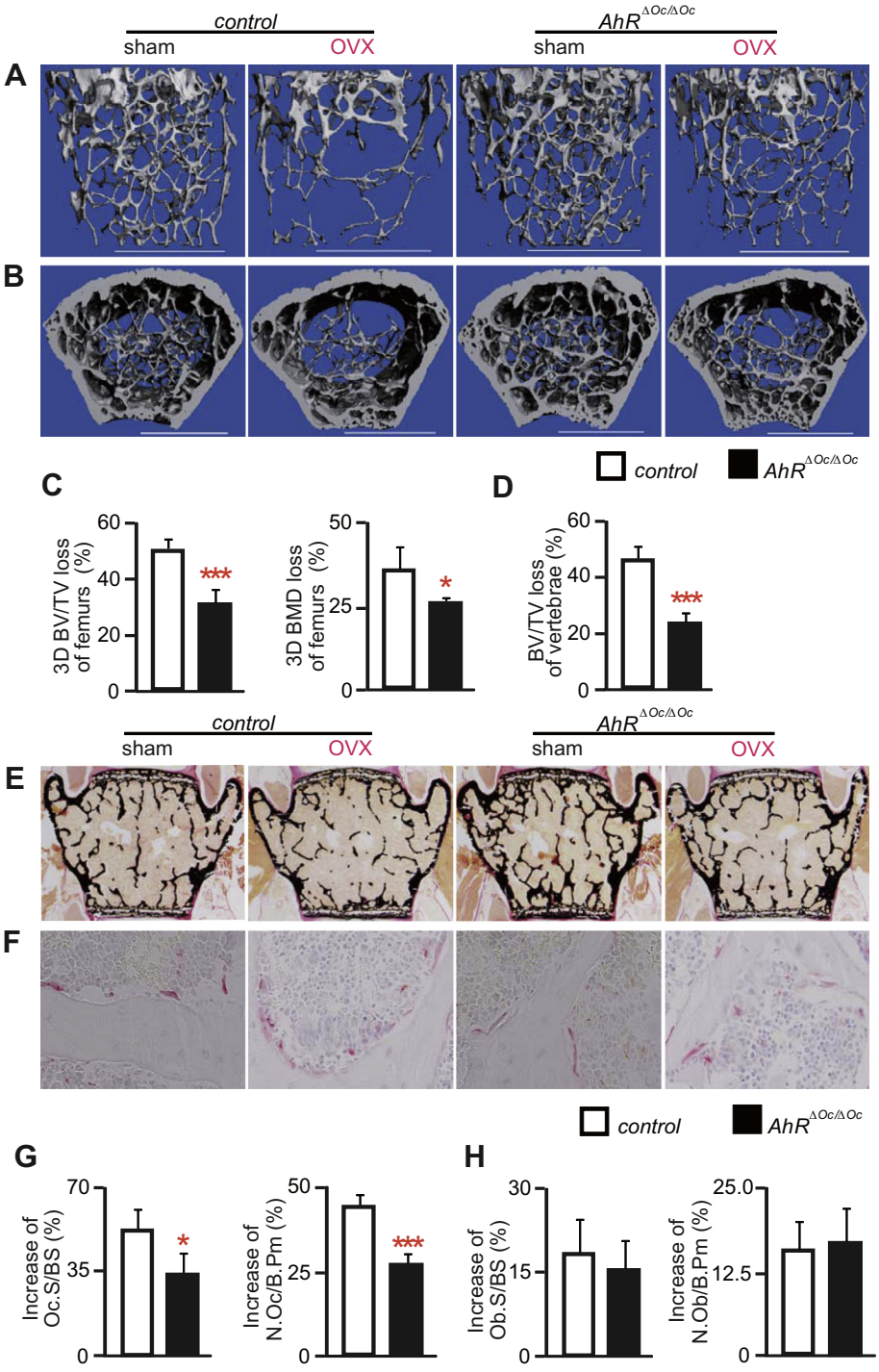

Fig. 3. $AhR^{\Delta Oc/\Delta Oc}$ mice are resistant to bone loss induced by OVX. (A–C) μCT analysis in the distal femurs from 12-week-old $AhR^{\Delta Oc/\Delta Oc}$ mice and their littermate controls ($Ctsk^{Cre/+}$; $AhR^{+/+}$) following ovariectomy (OVX) or sham operation. Representative images of trabecular bone of the distal femurs (A) and axial sections of the distal metaphysis (B). Scale bars indicate 1.0 mm. μCT parameters are shown (C). (D–H) Bone histomorphometric analysis of lumbar vertebrae. Sections were stained with Von Kossa/Van Gieson stain (E), or TRAP stain (F). The parameters of trabecular bone volume (D), osteoclast number and surface (G) and osteoblast number and surface (H) are shown. Results are shown as means ± SD ($n = 4$ animals per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo[a]-pyrene (BaP), stimulate osteoclastogenesis and bone resorption, and result in bone loss [21,22]. However, it is unclear whether AhR ligand-associated bone loss is directly mediated by osteoclastic AhR or not. To answer this question, $AhR^{\Delta Oc/\Delta Oc}$ and control mice were treated with a well-characterized AhR agonist, 3MC. Similar to the effects of other AhR agonists, 3MC induced bone loss in the distal femur of control mice by decreasing BV/TV, Tb.N, BMD and Conn.D, and increasing Tb.Sp and SMI compared to vehicle treated controls. However, bone mass of $AhR^{\Delta Oc/\Delta Oc}$ mice was not impaired by 3MC treatment (Fig. 4A and B). Furthermore, bone histomorphometric analyses showed that 3MC treatment increased bone resorption in control mice, but not in $AhR^{\Delta Oc/\Delta Oc}$ mice (Fig. 4C–I).

4. Discussion

It has been considered that environmental pollutants impair human health. Among them, dioxins induced osteopenia and AhR agonists facilitated the bone loss [20–22]. The function of AhR in bone metabolisms has been recently reported using systemically engineered genetic mouse models with either loss-of-function or gain-of-function of AhR [14,15]. In these studies, both systemic over-expression of constitutively active AhR and systemic knockout of AhR resulted in greater bone mass, although there were slight differences in the phenotypes depending on gender. These results might be due to systemic alterations of the AhR gene in mice, which may cause secondary effects from other tissues and/or organs. Our study revealed that cell type specific AhR knockout mice clearly showed that AhR in osteoclasts, not in osteoblasts, regulates bone metabolism.

Considering the possible mechanisms underlying the function of AhR in osteoclasts, Iqbal et al. concluded that *Cyp1a1* and *Cyp1a2*, which are transcriptional target genes of AhR, play roles in maintaining bone homeostasis mediated by AhR function [14]. Moreover, it was recently reported that B lymphocyte-induced maturation protein 1 (*Blimp1*) is one of the target genes for AhR [23]. The mice lacking *Blimp1* in osteoclasts exhibited greater bone mass, like $AhR^{\Delta Oc/\Delta Oc}$ mice [24,25], via suppressing the expression of Bcl6, which is a negative regulator of osteoclastogenesis [24]. This indicates that the bone catabolic function of AhR in osteoclasts may be mediated by expression of its target gene, *Blimp1*. Taken together, these data show that AhR plays a significant role in bone metabolism via an intricate molecular network.

AhR has been recognized as dioxin receptor, which is receptor for exogenous pollutants. However, recent studies revealed that endogenous AhR ligand, tryptophan derivatives such as kynurenine, play important roles in pathophysiological processes in various tissues [26,27]. This series of evidence enable us to recognize AhR as a significant receptor contributing in the maintenance of biological homeostasis, binding with endogenous ligands as well as exogenous pollutants. Also, this suggests that the modulation of AhR function can be a possible therapeutic strategy for various kinds of diseases because fat-soluble bioactive substances work as ligands for AhR. Current study provide evidence that the pathological effects of AhR ligands are directly mediated by osteoclastic AhR and support the results of previous studies utilizing systemic AhR null mice [14]. In contrast, it has been reported that mice treated with an AhR antagonist, 3,5,4′-trihydroxystilbene (resveratrol), showed increased BMD and bone mass [28]. This supports the suggestion that even under pathological conditions, AhR plays an important role in osteoclastic bone resorption, and the alteration of osteoclastic AhR activity by AhR ligands may be a promising novel therapy to reduce bone loss in osteoporosis or osteodystrophy.

A limitation of this study is that the validation of AhR ligand treatment for $AhR^{\Delta Oc/\Delta Oc}$ mice was performed using only exogenous agonist, 3MC. Future studies are required to whether endogenous agonist such as kynurenine or AhR antagonists can effect in bone metabolism under both physiological and pathological conditions. In addition, precise molecular mechanisms still remain elusive even our study directly elucidated that AhR plays a significant role in osteoclast with *in vivo* evidence using cell-type specific AhR knockout mice. Further studies are needed to deeply comprehend the detailed molecular basis of AhR function in bone metabolism.

In summary, we illustrated that osteoclast specific AhR knockout mice exhibited increased bone mass, recapitulating systemic AhRKO mice bone phenotype, and are resistant to sex hormone deficiency or exogenous AhR ligand induced bone loss. This evidence is important to understanding the significance of fat-soluble

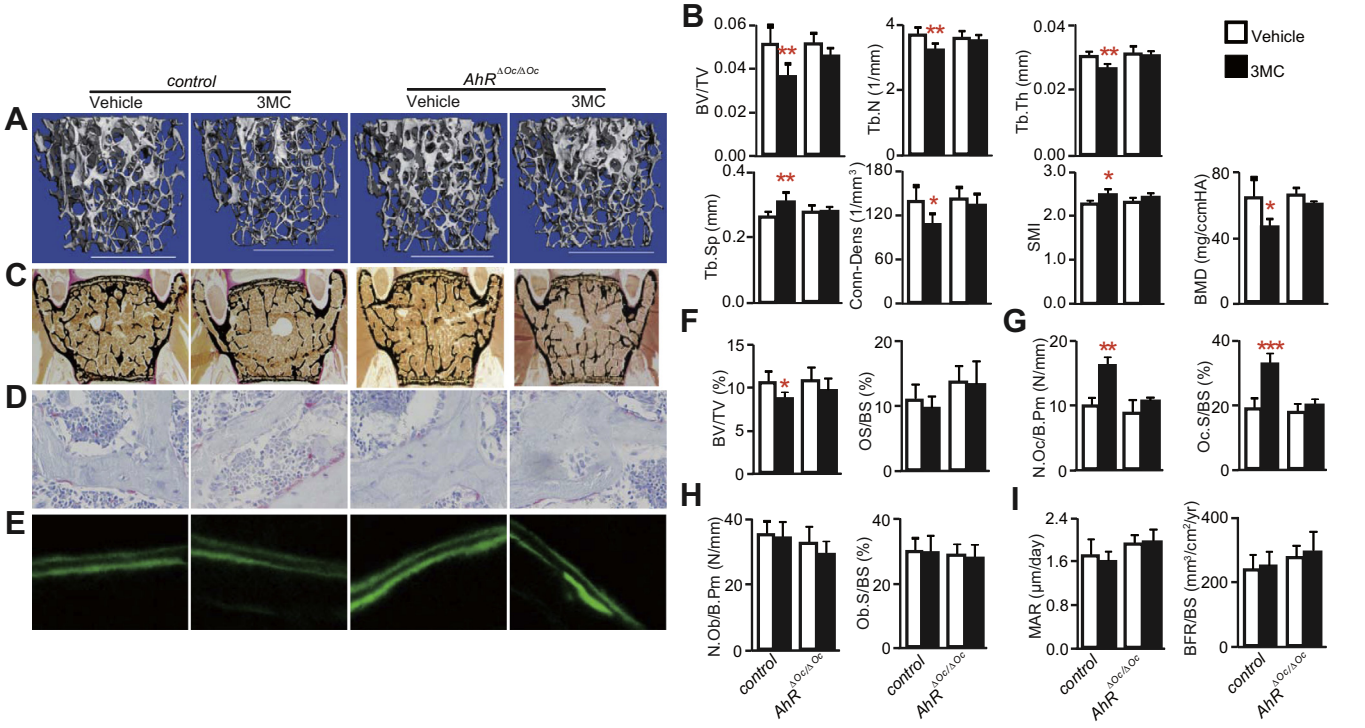

Fig. 4. AhR agonist, 3MC, did not reduce bone mass in $AhR^{\Delta Oc/\Delta Oc}$ mice. (A and B) μCT analysis of the femurs from 12-week-old $AhR^{\Delta Oc/\Delta Oc}$ mice and their littermate controls treated with or without 3MC. Representative images of trabecular bone in the distal femurs (A). Scale bars indicate 1.0 mm. μCT parameters are shown in (B). (C–I) Bone histomorphometric analysis of lumbar vertebrae from 12-week-old $AhR^{\Delta Oc/\Delta Oc}$ mice and their littermate controls treated with or without 3MC. Sections were stained with Von Kossa/Von Gieson stain (C), TRAP stain (D) or left unstained to evaluate calcein labeling (E). Trabecular bone volume and osteoid surface (F), osteoclast number and surface (G), osteoblast number and surface (H) and dynamic histomorphometric parameters (I) are shown. Results are shown as means ± SD (n = 6–8 animals per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

bioactive substances in bone metabolism under both physiological and pathological conditions.

Conflict of interest

All authors state that they have no conflicts of interest.

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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.2014.05.114.

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