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RARγ is a negative regulator of osteoclastogenesis

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ABSTRACT

Vitamin A is known to influence post-natal bone content, with excess intake being associated with reduced bone mineral density and increased fracture risk. Despite this, the roles retinoids play in regulating osteoclastogenesis, particularly *in vivo*, remain unresolved. This study therefore aimed to determine the effect of loss of retinoic acid receptors (RAR)α or RARγ on bone mass (analyzed by histomorphometry and dual-energy X-ray absorptiometry) and osteoclastogenesis in mice *in vivo*. RARγ null mice had significantly less trabecular bone at 8 weeks of age compared to wildtype littermates. In contrast, no change in trabecular bone mass was detected in RARα null mice at this age. Further histomorphometric analysis revealed a significantly greater osteoclast surface in bones from 8-week-old RARγ null male mice. This *in vivo* effect was cell lineage autonomous, and was associated with increased osteoclastogenesis *in vitro* from hematopoietic cells obtained from 8-week-old RARγ null male mice. The use of highly selective agonists in RANKL-induced osteoclast differentiation of wild type mouse whole bone marrow cells and RAW264.7 cells *in vitro* showed a stronger inhibitory effect of RARγ than RARα agonists, suggesting that RARγ is a more potent inhibitor of osteoclastogenesis. Furthermore, NFAT activation was also more strongly inhibited by RARγ than RARα agonists. While RARα and RARγ antagonists did not significantly affect osteoclast numbers *in vitro*, larger osteoclasts were observed in cultures stimulated with the antagonists, suggesting increased osteoclast fusion. Further investigation into the effect of retinoids *in vivo* revealed that oral administration of 5 mg/kg/day ATRA for 10 days protected against bone loss induced by granulocyte colony-stimulating factor (G-CSF) by inhibiting the pro-osteoclastogenic action of G-CSF. Collectively, our data indicates a physiological role for RARγ as a negative regulator of osteoclastogenesis *in vivo* and *in vitro*, and reveals distinct influences of RARα and RARγ in bone structure regulation.

1. Introduction

Skeletal development and maintenance of adult bone structure is a tightly regulated process relying on balanced actions of bone-resorbing osteoclasts and bone-forming osteoblasts [1]. When this balance is perturbed, clinical outcomes of osteopetrosis or osteoporosis can occur. High doses of the steroid hormone vitamin A (retinol) have been linked with increased bone resorption and bone loss. A recent meta-analysis of prospective clinical studies found that high intake of vitamin A as well as either low or high serum retinol levels are associated with greater risk of hip fracture [2]. Although the roles of retinoids in embryonic limb bud

Abbreviations: RAR, retinoic acid receptor; RXR, retinoid X receptor; ATRA, all-*trans* retinoic acid; BMM, bone marrow macrophages; G-CSF, granulocyte colony stimulating factor; RANKL, receptor activator of nuclear factor kappa-B; NFAT, nuclear factor of activated T cells; WBM, whole bone marrow; BMD, bone mineral density; DXA, dual X-ray absorptiometry; TNFα, tumour necrosis factorα.

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formation and skeletal patterning are well-defined, information on the effects of retinoids in post-natal skeletal development is very limited [3].

Retinoid signalling occurs *via* nuclear retinoic acid receptor (RAR) and retinoid X receptor (RXR) heterodimers. There are three subtypes each of RAR and RXR: α,β and γ. RARs are expressed in cells of the osteoclast and osteoblast lineage [4–7]. There is conflicting evidence regarding their direct roles in osteoblasts and osteoclasts, with numerous studies reporting both positive and negative effects on differentiation and activity. For example, all-*trans* retinoic acid (ATRA), the biological ligand for all three RARs, has been reported to both inhibit [8] and promote [9,10] differentiation and activity of osteoblasts. However, each study used different doses, cell lines and experimental techniques, making it difficult to discern the direct impact of retinoid exposure on osteoblasts. ATRA has been reported to stimulate osteoclast resorption in *ex vivo* bone cultures [11] but also to inhibit receptor activator of nuclear factor kappa-B (RANKL)-mediated osteoclastogenesis in osteoclast precursor cultures free of osteoblasts, specifically RAW264.7 cells, bone marrow macrophages (BMMs), bone marrow cells and spleen cells [12]. Thus, the direct effects or retinoids on individual cell types of the skeleton, and whether retinoids physiologically affect bone cell differentiation or activity remains unclear.

We previously reported that mice null for *Rarg* develop a myeloproliferative syndrome dependent on the bone marrow microenvironment [13]. In that study we noted that *Rarg* null mice had less trabecular bone, suggesting a role for RARγ in regulating bone mass. *Rarg*$^{-/-}$ mice also have an elevated frequency of axial skeleton defects [14,15], which occur at a much lower frequency in *Rara*$^{-/-}$ and *Rarb*$^{-/-}$ mice [15,16], indicating a role for RARs in skeletogenesis. The abnormalities exhibited by *Rara*$^{-/-}$ and *Rarg*$^{-/-}$ mice are more severe and result in early lethality [15], whereas *Rarb*$^{-/-}$ mice appear normal and have a full life expectancy [17]. Furthermore, previous studies have reported a role for RARα in regulating osteoclastogenesis *in vitro* [12]. Thus we investigated the roles of RARα and RARγ in post-natal bone *in vivo*.

2. Materials and methods

2.1. Mice

Rara [18] and *Rarg* [14] null mice were the kind gift of Professor Pierre Chambon. For ATRA/G-CSF studies, male C57BL/6 mice were obtained from Animal Resources Centre, Perth, WA, Australia. All experiments performed were approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee and were conducted in strict compliance to the regulatory standards of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Retinoid and G-CSF treatments

IRX5183 (RARα agonist), IXR4647 (RARγ agonist), IRX6996 (RARα antagonist), IRX5099 (RARγ antagonist), IRX4310 (RAR pan-antagonist) were kindly supplied by R. Chandraratna (Io Therapeutics, California) [19]. For *in vitro* studies, IRX ligands and ATRA (Sigma, St. Louis, MO) were dissolved in DMSO and diluted at 1/1000 for final concentrations in media. For *in vivo* studies, ATRA or an equal volume of DMSO was diluted in peanut oil and gavage fed to mice at 5 mg/kg/day for 10 days as previously described [20]. Recombinant human G-CSF (Filgrastim/Neupogen, Amgen, Thousand Oaks, CA) was diluted in sterile saline and s.c. injected at 125 μg/kg twice daily for 4 days from day 7–10 of ATRA treatment [20]. An equal volume of saline was injected in control mice.

2.3. Bone densitometry and histomorphometry

Mice were anesthetised with tribromoethanol (600 mg/kg) and bone densitometry was performed by dual-energy X-ray absorptiometry (DXA) on a Lunar PIXImus Densitometer (GE Medical Systems). Tibiae collected for histomorphometry were fixed in 4% paraformaldeyhyde and embedded in methylmethacrylate [21]. 5 μm sections were stained with toluidine blue or Xylenol Orange [22]. Histomorphometric analysis of undecalcified of trabecular bone of the secondary spongiosa of the proximal tibia and cortical bone of the antero-fibular tibial mid-diaphysis was performed using the Osteomeasure system (OsteoMetrics Inc., Decatur, GA) as previously described [21,23].

2.4. Quantitation of osteoclast progenitor cells

CSF-1-responsive agar colony-forming unit (CFU) assays were performed as previously described [24]. Osteoclast assays were performed as previously described [25]. Immunophenotypical analysis of osteoclast progenitors was performed in spleen and bone marrow (BM) preparations using FITC-conjugated CD11b (BD Pharmingen, San Diego, California) and PE-conjugated F4/80 (Caltag, South San Francisco, CA) as previously described [26]. Isotype-matched antibodies were used to determine background staining. Cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA).

2.5. RNA extraction and quantitative RT-PCR

Spleens and whole marrow flushed from femurs was used for gene expression analysis. mRNA extraction, synthesis of cDNA and qRT-PCR were performed as previously described [27]. All primers used in these studies are given in Supplemental Table 1.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2015.03.005.

2.6. Osteoclast cultures with RAR ligands

Mouse WBM, spleen or RAW264.7 cells were cultured as previously described [28]. Cells were plated at 10,000 RAW264.7, 500,000 spleen or 100,000 WBM cells/well in 6 mm diameter tissue culture wells. Cells were stimulated to differentiate for 6 days with 50 ng/ml of RANKL (plus 25 ng/mg M-CSF for WBM) in the presence of 10 nM, 100 nM or 1 μM RAR ligands or an equal volume of DMSO. Osteoclasts were identified by tartrate-resistant acid phosphatase (TRAP) histochemical staining and >2 nuclei. NFAT reporter assay was performed using RAW264.7 cells transfected with a GL4.30 (luc2P/NFAT-RE/Hygro) reporter construct, as described previously [28].

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0. Results are expressed as mean ± SD for n samples. Data were analyzed using an unpaired t-test or one-way ANOVA and Tukey's multiple comparisons test. Data is considered statistically significant if $p < 0.05$.

3. Results

3.1. RARγ null mice have reduced trabecular bone mass

DXA analysis of *Rarg*$^{-/-}$ mice revealed significantly lower (~10%) humeral BMD compared to wild type controls. This was noted in both female and male 8-week-old *Rarg*$^{-/-}$ mice compared to wildtype (Fig. 1A and B). Male *Rarg*$^{-/-}$ mice also showed

significantly lower femoral BMD (Fig. 1A), but this was not significant in females (Fig. 1B).

Changes in trabecular bone volume were then assessed by histomorphometry of undecalcified tibial sections from 8-week-old male and female mice and 16-week-old females. Trabecular bone volume of *Rarg*$^{-/-}$ mice was significantly lower than *Rarg*$^{+/+}$ male and female mice (Fig. 1C–E). *Rarg*$^{-/-}$ mice exhibited lower trabecular number than *Rarg*$^{+/+}$ mice (Fig. 1F–H), accompanied by a greater trabecular separation (Fig. 1I–K). *Rarg*$^{-/-}$ males also had markedly reduced trabecular thickness (Fig. 1L–N). Histomorphometric analysis of bones from 8-week-old male *Rara*$^{-/-}$ mice found no significant difference compared to wildtype (Fig. S1), revealing a bone defect specific to loss of RARγ.

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While slightly reduced, the tibial cortical thickness was not significantly altered in 8-week-old RARγ$^{-/-}$ mice compared to wild type controls (males RARγ$^{+/+}$: 132 ± 20, RARγ$^{-/-}$: 110 ± 18; females RARγ$^{+/+}$: 140 ± 20, RARγ$^{-/-}$: 123 ± 21, data are mean (μm) ± SD).

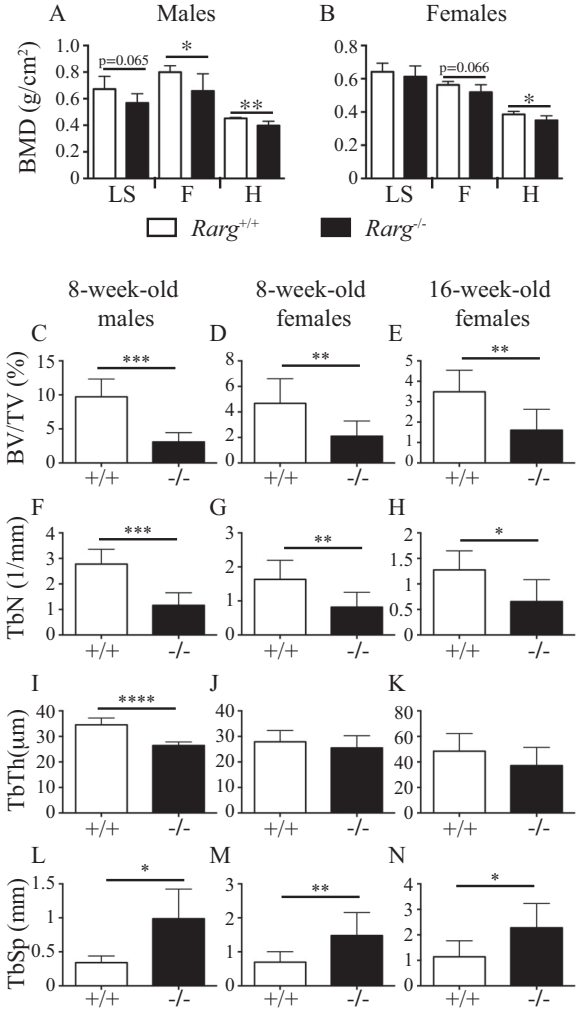

Fig. 1. Bone mineral density and trabecular bone is reduced in 8-week-old *Rarg*$^{-/-}$ mice. Bone mineral density was determined for the lumbar spine (LS), right femur (F) and right humerus (H) of 8-week-old *Rarg*$^{+/+}$ and *Rarg*$^{-/-}$ male (A) and female (B) mice. Histomorphometry analysis of tibiae from *Rarg*$^{+/+}$ and *Rarg*$^{-/-}$ 8-week-old male, 8- and 16-week-old female mice: (C–E) trabecular bone volume (BV/TV), (F–H) trabecular number (TbN), (I–K) trabecular thickness (TbTh), (L–N) trabecular separation (TbSp). Data are shown as the mean ± SD (n = 5–8/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

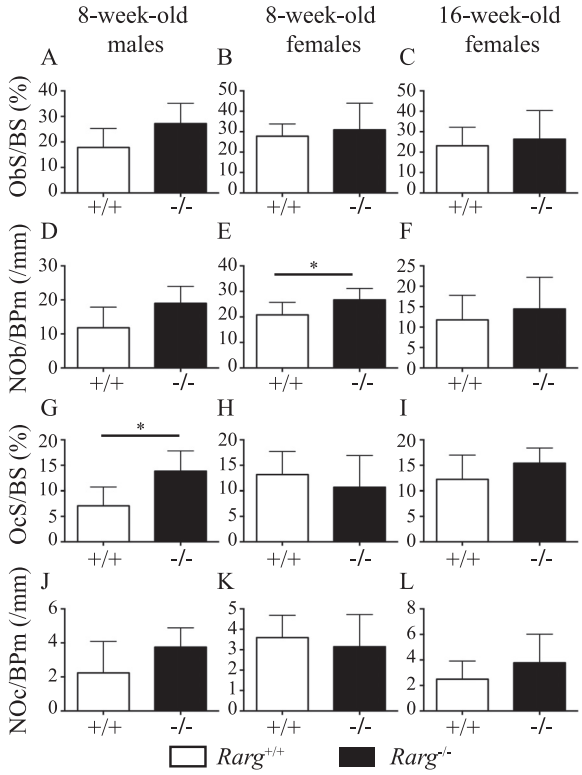

Fig. 2. *Rarg*$^{-/-}$ mice have altered bone remodelling favouring increased resorption. Histomorphometric indicators of bone formation in tibiae from *Rarg*$^{+/+}$ and *Rarg*$^{-/-}$ 8-week-old, 8-week-old female and 16-week-old female mice show: (A–C) bone surface occupied by osteoblasts (ObS/BS), (D–F) number of osteoblasts per bone perimeter (NOb/BPm), (G–I) bone surface occupied by osteoclasts (OcS/BS) and (J–L) number of osteoclasts per bone perimeter (NOc/BPm). Data are shown as the mean ± SD (n = 4–11/group), *$p < 0.05$.

Further analysis of *Rarg*$^{-/-}$ mice detected no significant change in osteoblast surface (Fig. 2A–C) or osteoblast number in 8-week old males (Fig. 2D) or 16-week-old females (Fig. 2F), although there were higher osteoblast numbers in 8-week-old female *Rarg*$^{-/-}$ mice compared to wildtype (Fig. 2E). In contrast, osteoclast surface was significantly elevated in *Rarg*$^{-/-}$ males (Fig. 2G), but not females (Fig. 2H and I). The number of osteoclasts was not significantly different between *Rarg*$^{-/-}$ and wildtype mice (Fig. 2J–L). Hence increased osteoclast size is likely contributing to the elevated surface of bone covered by osteoclasts in male mice.

3.2. RARγ null spleen cell populations have increased numbers of osteoclast precursors and increased ex vivo osteoclast formation

We previously reported that *Rarg*$^{-/-}$ BM contains significantly more osteoclast precursor cells (granulocyte/macrophage progenitors) than wild type BM [13,27]. Thus we determined whether the elevated osteoclastogenesis observed in *Rarg*$^{-/-}$ mice was due to increased numbers of osteoclast progenitors. After 7 days in culture the number of CSF-1-responsive CFUs, which contain osteoclast progenitors, formed from *Rarg*$^{-/-}$ BM was significantly higher than from wildtype BM (Fig. 3A).

We performed further immunophenotypical analysis of the two cell types known to be capable of differentiating into osteoclasts [29], monocyte/macrophages (F4/80$^+$/CD11b$^+$) and more immature pre-osteoclasts (F4/80$^+$/CD11b$^-$) in BM and spleen of *Rarg* mutants. *Rarg*$^{-/-}$ BM had similar content of both of these populations compared to wildtype BM (Fig. 3B). In contrast, spleens of 8-week-old RARγ$^{-/-}$ mice contained more F4/80$^+$/CD11b$^+$ and F4/80$^+$/

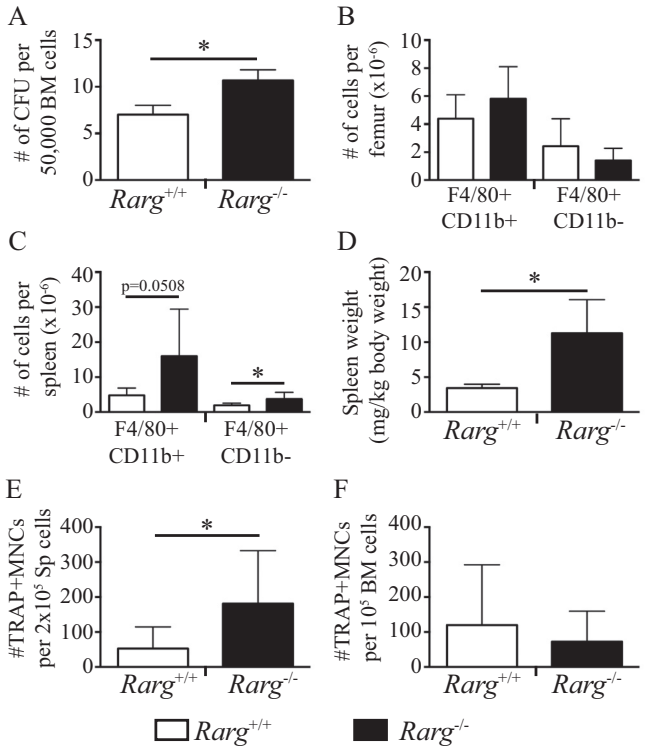

Fig. 3. Increased osteoclast precursors and *in vitro* spleen osteoclastogenesis in 8-week-old *Rarg*$^{-/-}$ mice. (A) Numbers of M-CSF-responsive CFUs produced per 50,000 bone marrow (BM) cells from 8 week old *Rarg*$^{+/+}$ and *Rarg*$^{-/-}$ mice ($n = 4$/group). (B) Spleen weights (mg/g body weight) of 8-week-old male *Rarg*$^{+/+}$ and *Rarg*$^{-/-}$ mice ($n = 4$–6/group). (C) Numbers of monocyte/macrophages (CD11b$^+$/F4/80$^+$) and immature pre-osteoclasts (CD11b−/F4/80$^+$) per spleen from 8-week-old *Rarg*$^{+/+}$ and *Rarg*$^{-/-}$ mice ($n = 7$/group). (D) Numbers of monocyte/macrophages (CD11b$^+$/F4/80$^+$) and immature pre-osteoclasts (CD11b$^-$/F4/80$^+$) per femur from 8-week-old *Rarg*$^{+/+}$ and *Rarg*$^{-/-}$ mice ($n = 7$/group). (E) Numbers of TRAP$^+$ MNCs per 200,000 spleen (Sp) cells ($n = 9$–10/group). (F) Numbers of TRAP$^+$ MNCs per 100,000 BM cells ($n = 9$–10/group). Data are shown as the mean ± SD, $^*p < 0.05$.

CD11b$^-$ osteoclast precursor populations than *Rarg*$^{+/+}$ mice (Fig. 3C). RARγ$^{-/-}$ mice also had splenomegaly, with male spleens being approximately 3.3-fold larger than *Rarg*$^{+/+}$ mice when corrected for body weight (Fig. 3D).

We next assessed *in vitro* osteoclastogenic potential of BM and spleen cells from *Rarg*$^{-/-}$ and *Rarg*$^{+/+}$ mice. Consistent with the increased numbers of monocyte/macrophages and pre-osteoclasts in *Rarg*$^{-/-}$ spleens, we observed significantly more osteoclasts from spleen cultures of *Rarg*$^{-/-}$ mice. This amounted to ~3.5-fold elevated osteoclastogenesis in M-CSF and RANKL-stimulated spleen cultures from *Rarg*$^{-/-}$ mice compared to wild type (Fig. 3E). In contrast, there were no differences in osteoclast numbers in cultures of M-CSF and RANKL-stimulated *Rarg*$^{-/-}$ BM (Fig. 3F). Furthermore, *in vitro* osteoclastogenesis was not altered in *Rara*$^{-/-}$ BM or spleen cultures compared to wildtype littermate cultures (data not shown).

3.3. RARγ null hematopoietic organs have elevated mRNA expression of factors that promote osteoclastogenesis

We previously reported that the pro-osteoclastic factor TNFα is upregulated in the BM and spleen of 8-week-old *Rarg*$^{-/-}$ mice [13]. We thus examined mRNA levels of other osteoclastogenic factors in hematopoietic organs of *Rarg*$^{-/-}$ and *Rarg*$^{+/+}$ mice. There were no changes in mRNA levels of *Tnfsf11* in any hematopoietic organ in *Rarg*$^{-/-}$ compared to *Rarg*$^{+/+}$ mice (Fig. 4A). However, *Socs3* mRNA levels were markedly higher in both BM and spleen of *Rarg*$^{-/-}$ mice (Fig. 4B). mRNA levels of *Csf1* and *Il6* were significantly higher in *Rarg*$^{-/-}$ spleens (Fig. 4C and D). *Il7* mRNA levels were significantly lower in *Rarg*$^{-/-}$ BM than wildtype (Fig. 4E).

3.4. RAR agonists inhibit osteoclastogenesis and RAR antagonists increase osteoclast size

To investigate the effects of RAR specific ligands on osteoclast formation *in vitro*, we treated cells from whole mouse BM (WBM) from wildtype mice and the osteoclast/macrophage progenitor cell line RAW264.7 with RARα and RARγ selective and RAR pan-agonists and antagonists. The pan-agonist ATRA and the RARα and RARγ selective agonists, IRX5183 and IRX4647, respectively, dose-dependently inhibited RANKL-induced osteoclast formation in WBM. Partial inhibition of osteoclastogenesis in WBM cultures was observed at 10 nM of ATRA, IRX5183 and IRX4647 (Fig. 5A), and this inhibition was more pronounced at higher doses (Fig.S2A and B). This inhibitory effect was more prominent in RAW264.7 cells treated with retinoid agonists. 10 nM–1 μM doses of ATRA and IRX4647 completely inhibited RANKL-induced osteoclastogenesis by RAW264.7 cells (Fig. 5B, E and G; Fig. S2C and D). The effect of the RARα agonist IRX5183 was not as potent as the RARγ agonist IRX4647 or the pan-agonist ATRA, but significant inhibition was observed at 100 nM and 1 μM doses of IRX5183 (Fig. 5F; Fig. S2E and G). Treatment with the RAR antagonists did not alter the numbers of TRAP$^+$ multinucleated cells (MNCs) formed (Fig. 5B and I–K, Fig. S2C and D). Interestingly, however, cultures treated with the pan-antagonist, IRX4310, RARα antagonist, IRX6996, or RARγ antagonist, IRX5099, formed osteoclasts that were substantially larger than those observed in the DMSO control treatment group after 6 days of culture (Fig. 5H–K).

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3.5. Retinoid signalling indirectly inhibits NFAT activation

Nuclear factor of activated T cells c1 (NFATc1) is an osteoclastic transcription factor that is activated following RANK activation by RANKL and promotes osteoclast differentiation from precursor cells. We further investigated the *in vitro* effects of retinoids on osteoclasts using RAW264.7 cells transfected with a *luciferase2P/*

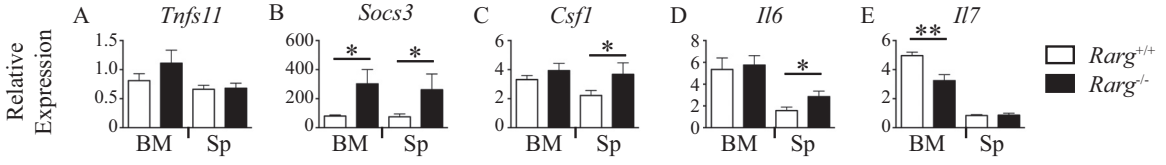

Fig. 4. *Rarg*$^{-/-}$ hematopoietic organs have altered expression of factors affecting osteoclastogenesis. qRT-PCR analysis of relative gene expression compared to β_2M in BM and spleen (Sp) of (A) *Tnfsf11*, (B) *Socs3*, (C) *Csf1* (D) *Il6*, and (E) *Il7*. Data are shown as the mean ± SD ($n = 3$). $^*p < 0.05$ and $^{**}p < 0.01$.

NFAT-RE/Hygro GL4.30 reporter construct [28]. Stimulation of cells with RANKL resulted in a significant increase in the luciferase signal compared to the unstimulated cells, indicating elevated NFAT expression (Fig. 5C). Addition of 10 nM ATRA or IRX4647 to cultures produced a significant repression of RANKL-stimulated NFAT activation compared to the DMSO control treated cells (Fig. 5C). No change was observed with 10 nM IRX5183, however significant inhibition of NFAT activity was detected at 100 nM and 1 μM doses of IRX5183 (Fig. S2). Inhibition of RARs did not elucidate a change in NFAT activation in response to RANKL, except for treatment with 1 μM IRX4310, which increased the NFAT-luciferase signal (Fig. S2, $p < 0.05$). Additionally, no induction of NFAT was observed with any retinoids in the absence of RANKL (Fig. 5C and Fig. S2).

3.6. ATRA treatment stimulates bone formation and reduces the increased bone resorption associated with in vivo G-CSF treatment

The cytokine granulocyte-colony stimulating factor (G-CSF) is used clinically for many purposes, including mobilization of hematopoietic stem cells. G-CSF is known to stimulate osteoclast formation and bone resorption *in vivo* [30] and is significantly elevated in *Rarg*$^{-/-}$ serum [31]. Given the potent RAR agonist inhibition of osteoclastogenesis *in vitro,* we hypothesised that ATRA treatment might prevent the increased osteoclastogenesis observed in response to G-CSF treatment *in vivo*.

Based on our previous studies [20], mice were gavage fed ATRA or an equal volume of DMSO for 10 days and from day 7 were injected with G-CSF or saline twice daily for 4 days. G-CSF treatment significantly reduced trabecular bone volume and trabecular number compared to saline + DMSO controls (Fig. 6A and B). Trabecular thickness was reduced and trabecular separation was elevated in G-CSF-treated mice, although not significantly (Fig. 6C and D). Bone loss observed with G-CSF treatment was accompanied by a marked increase in osteoclast surface (Fig. 6G and H) and reduced osteoblast surface (Fig. 6E and F). ATRA treatment alone did not significantly alter trabecular bone structure (Fig. 6A–D). However, there was a dramatic increase in all parameters associated with bone formation (osteoblast surface, osteoid volume, osteoid thickness (Fig. 6E–F and I–K)) compared to control mice. There was no significant change in osteoclast surface between ATRA-treated mice and controls (Fig. 6G and H). Strikingly, administration of ATRA prevented the increase in osteoclast surface observed in G-CSF-treated mice, and partially restored the trabecular bone volume, trabecular number, trabecular thickness and osteoclast number and surface levels such that they were no longer significantly different to control (Fig. 6A–C, G and H).

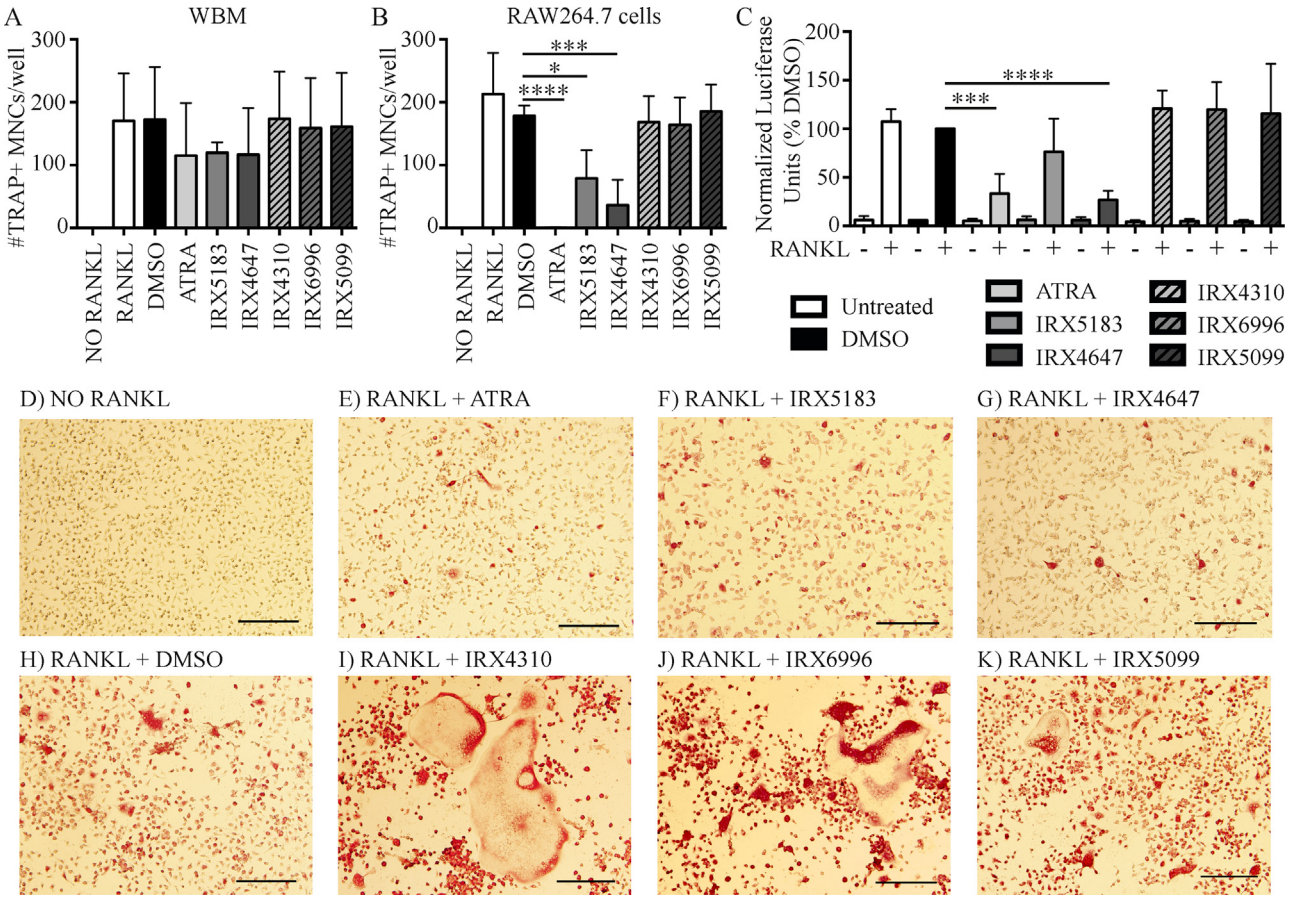

Fig. 5. RAR agonists inhibit RANKL-induced osteoclastogenesis and NFAT activation. Shown are the effects of 10 nM ATRA, IRX5183, IRX4647, IRX4310, IRX6996, IRX5099 or an equal volume of DMSO on formation of TRAP$^+$ MNCs 6 days after 50 ng/ml RANKL induction of (A) WBM or (B) RAW264.7 cells. (C) NFAT reporter activation following RANKL stimulation in the presence of 10 nM ATRA, IRX5183, IRX4647, IRX4310, IRX6996, IRX5099 or DMSO. Images of TRAP stained (red) RAW264.7 cultures induced to form osteoclasts with (D) no RANKL or RANKL plus 1 μM (E) ATRA, (F) IRX5183, (G) IRX4647, (H) RANKL alone, (I) IRX4310, (J) IRX6996 or (K) IRX5099. Images photographed at 10X magnification, bars = 200 μm. Data shown are the mean ± SD ($n = 3$), $^*p < 0.05$ $^{***}p < 0.001$ and $^{****}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

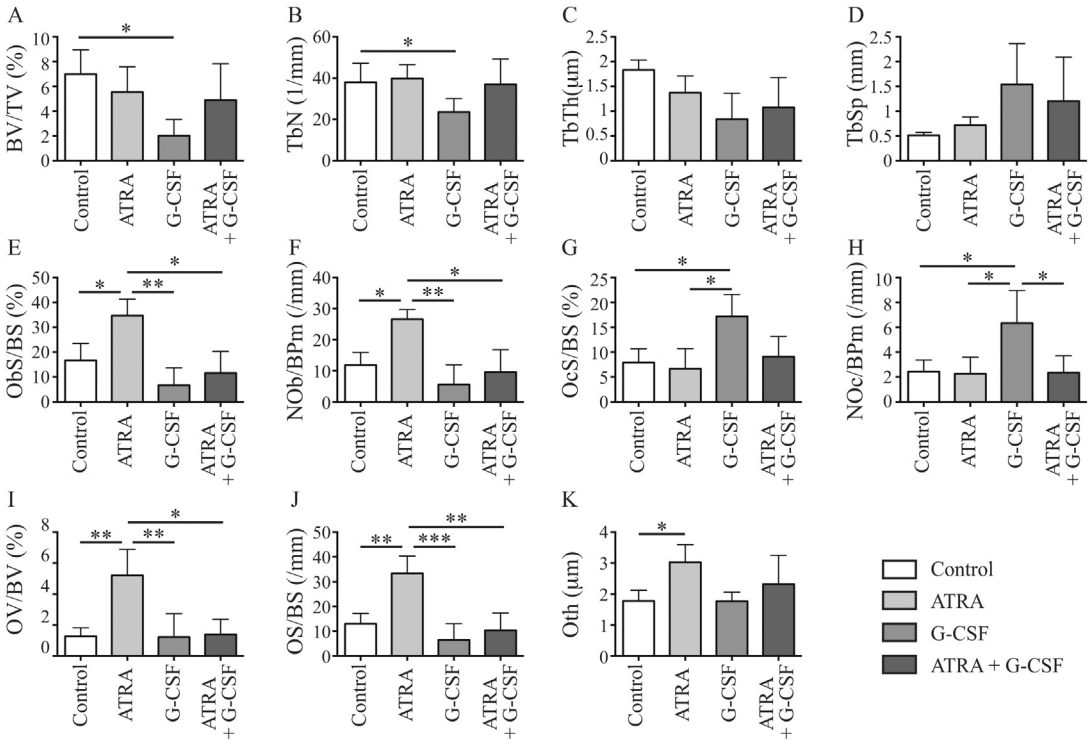

Fig. 6. ATRA prevents G-CSF-mediated increase in osteoclastogenesis *in vivo*. Shown are histomorphometric analysis of tibial structure and indicators of bone formation for mice fed DMSO/saline injected (control), fed ATRA/saline injected (ATRA), fed DMSO/G-CSF injected (G-CSF) or fed ATRA/G-CSF injected (ATRA + G-CSF). (A) BV/TV, (B) TbN, (C) TbTh, (D) TbSp, (E) ObS/BS, (F) NOb/BPm, (G) OcS/BS, (H) NOc/BPm, (I) OV/BV, (J) OS/BS and (K) Oth. Data are mean ± SD (n = 3–5/group), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

4. Discussion

Retinoids and their receptors have well characterised roles in regulating embryonic limb bud formation and skeletal development [3], but their roles in regulating post-natal skeletal growth and maintenance of bone structure has not been fully elucidated. Here we show that lack of RARγ resulted in osteopenia in young adult mice. In contrast, *Rara*$^{-/-}$ mice had no detectable bone defect, demonstrating that the key physiological role of retinoids in maintaining post-natal bone mass is mediated *via* RARγ signaling. While *Rarb*$^{-/-}$ mice have not been analyzed in this study, to date there have been no reports of post-natal bone defects in these mice. Analysis of 8-week-old *Rarg*$^{-/-}$ males indicated increased osteoclastogenesis. This anti-osteoclastogenic action of RARγ signaling was supported by potent inhibition of osteoclastogenesis *in vitro* by ATRA or the RARγ specific agonist (IRX4647), and inhibition of G-CSF-induced osteoclastogenesis by ATRA *in vivo*.

The increased osteoclast surface in *Rarg*$^{-/-}$ male mice was in part due to increased osteoclast size, as osteoclast numbers were not significantly elevated. An inhibitory role of RARs in regulating osteoclast size was also observed *in vitro* where RAR antagonist-treated RAW264.7 cultures developed giant osteoclasts without an obvious change in osteoclast numbers. The reason for formation of very large osteoclasts is unclear, but must involve increased osteoclast progenitor fusion, that may be mediated by CSF-1 [32], which was significantly elevated at the mRNA level in spleens of these mice (Fig. 4C). Furthermore, while *Csf1* levels were not significantly increased in the BM of the *Rarg*$^{-/-}$ mice (Fig. 4C), we did detect increased numbers of CSF-1-responsive colonies from BM obtained from *Rarg*$^{-/-}$ mice (Fig. 3A), which may have contributed to the increased osteoclast size observed in these mice. Increases in osteoclast size *in vivo* have been associated either with reduced osteoclast resorptive activity and osteopetrosis [33], or with increased osteoclast activity [34]. Since trabecular bone mass is lower in the *Rarg* null mice, the greater osteoclast size associated both with absence of *Rarg* and with RAR antagonist treatment indicates greater osteoclast activity.

Osteoclasts are derived from hematopoietic stem cells, which differentiate along the granulocyte/macrophage lineage to form macrophage/osteoclast precursors, which reside in the BM and extramedullary organs such as the spleen, and can be detected in low levels in the peripheral blood [35]. In this study the *Rarg*$^{-/-}$ mice exhibited splenomegaly, which was accompanied by an increase in the number of osteoclast precursor cells (CD11b^{+}/F4/80^{+} and CD11b^{-}/F4/80^{+} cells) per spleen. The higher osteoclast precursor numbers detected in spleens could reflect population expansions that also lead to increased spleen size. However, splenic cell cultures from *Rarg*$^{-/-}$ mice had greater osteoclastogenic potential than controls. Interestingly, in the BM there was no change in osteoclast precursor numbers or osteoclastogenic potential *in vitro*. This may suggest that the osteoclast precursor populations within the spleen and BM are distinct. This has been reported previously: osteoclast precursors in the spleen and BM have different responses to ligands for toll-like receptors [36]. Splenic CD11b^{+} cells have been identified as a source of osteoclast precursors recruited to the joint synovia that contribute to bone destruction [37] and splenic osteoclast precursors can be recruited through the circulation to the bone. In *Rarg*$^{-/-}$ mice, the elevated numbers of osteoclast precursor cells in spleens plus increased osteoclast potential of the spleen cells, as observed *in vitro*, may therefore contribute to the increased resorptive activity and osteopenia.

Rarg$^{-/-}$ BM and spleen showed elevated mRNA levels of factors that enhance osteoclastogenesis or expand osteoclast precursor populations, and may contribute to the pro-osteoclastic phenotype of osteoclast precursor cells from the spleen in *Rarg*$^{-/-}$ mice, including *Tnf*, *Il6*, and *Csf1* [13,38–40]. We also detected elevated mRNA levels of suppressor of cytokine signalling 3 (*Socs3*) in *Rarg*$^{-/}$

$^{-}$ splenic cells. This is perplexing since SOCS3 is an inhibitor of JAK/STAT signaling that negatively regulates the action of pro-inflammatory cytokines, including many pro-osteoclastic cytokines [41]. Furthermore, genetic deletion of *Socs3* in hematopoietic and endothelial cells, including osteoclast precursors, led to elevated osteoclastogenesis in adult mice [42]. We suggest that the increased *Socs3* mRNA could be a response to increased TNFα, reported to stabilise *Socs3* mRNA in mouse BMMs [43]. Thus, it is not a mechanism for increased osteoclastogenesis, but reflects increased levels of pro-osteoclastogenic cytokines within the hematopoietic organs. In contrast to the *Rarg*$^{-/-}$ mice, *Rara*$^{-/-}$ mice had no detectable bone defect at 8 weeks of age, demonstrating that RARγ signaling plays a more vital role in regulating bone mass *in vivo*.

We similarly found RARγ had the more potent effect on osteoclast formation *in vitro*. The pan-agonist ATRA and the RARγ agonist IRX4647 completely blocked osteoclastogenesis in RAW264.7 cells and significantly reduced TRAP^{+} MNCs in WBM. In contrast, the RARα agonist IRX5183 was not as effective at lower doses, indicating a more potent effect of RARγ activation over RARα. This contrasts with previous studies by Conaway et al., who reported RARα as a more potent inhibitor of osteoclastogenesis in bone marrow cells, RAW264.7 cells and BMMs [12]. Comparable responses were observed to ATRA treatment in these studies, but there are differences in subtype-specific responses, which may be explained by the use of different RARα and RARγ selective ligands with variations in binding affinity and selectivity. However, our *in vitro* data reflects our observations in *in vivo* knockout models. This also implies that RARα and RARγ play similar roles in osteoclastogenesis *in vitro* but that *in vivo* RARα may be functionally redundant to RARγ in regulating bone resorption, as loss of *Rarg* alone was sufficient to produce an aberrant bone phenotype. Whether loss of *Rara* is functionally compensated by RARγ cannot be directly tested in these knockout models as the double knockout is embryonic lethal [15].

A major regulator of osteoclastogenesis induced by RANKL treatment of osteoclast precursors is the transcription factor NFATc1, which binds to and exerts its influence through NFAT binding gene promoter motifs. RAR agonists inhibited RANKL-induced activation of NFAT, with more potent inhibition occurring with the RARγ agonist IRX4647 than the RARα agonist IRX5183, similarly to observations in the osteoclast differentiation assay. The RAR antagonists did not elucidate a change in RANKL-induced NFAT activation, except at the highest IRX4310 dose. This indicates that retinoids inhibit NFAT activation and osteoclast differentiation induced by RANKL. As no change in osteoclastogenesis was detected with RAR ligands in the absence of RANKL-stimulation, it is likely that retinoids do not directly control NFAT expression, consistent with previous findings [12]. RANK expression by osteoclast precursors has been shown to be downregulated by ATRA [4]. This does not appear to be the mechanism behind ATRA inhibition of RANKL-induced osteoclastogenesis, as cells over-expressing RANK have been reported to be similarly blocked from differentiating into osteoclasts when treated with ATRA [44].

ATRA also inhibited G-CSF-stimulated osteoclastogenesis *in vivo*. G-CSF is highly elevated in the serum of *Rarg*$^{-/-}$ mice [31] and strongly stimulates osteoclastogenesis/bone resorption while reducing bone formation [30]. In our study ATRA did not affect basal levels of osteoclast formation but did reduce the increased osteoclastogenesis caused by G-CSF treatment. Interestingly, ATRA treatment also markedly elevated bone formation and rescued the G-CSF-induced reduction in osteoblasts, suggesting an additional effect on osteoblasts. While we have not yet investigated the impact of ATRA on other models of osteoclastogenesis-induced bone loss, such as sex steroid deficiencies, this would be of interest in future studies.

We found that a short time course of a low dose of 5 mg/kg/day ATRA (10 days orally) did not produce a significant change in bone volume, but stimulated bone formation and protected against G-CSF-induced osteoclastogenesis. In contrasts, high doses of vitamin A have been shown to reduce bone mass and increase bone fragility in rodent studies by stimulating osteoclastogenesis at some sites, but not others [45,46] and impairing bone formation [47]; this suggests that in the rodent, there are not only site-dependent, but also dose-dependent effects of retinoids on bone. Human studies show an associative link between high intake of vitamin A or both low and high serum retinol levels with reduced BMD [48] and increased hip fracture risk [2]. It remains unknown whether any of these conditions cause an increase in osteoclast formation or activity. The reduced BMD and hip fracture risk may also be caused by a lower level of bone formation, or, as suggested by the rodent studies, retinoid effects on osteoclastogenesis may be dependent on dose, duration of treatment and site.

Collectively, our data suggest that RARγ is a key negative regulator of osteoclastogenesis both *in vivo* and *in vitro*. These data therefore reveal previously unrecognised roles for RARγ in the regulation of post-natal skeletal structure.

Disclosures

RASC is the president and Chief Scientific Officer of IO Therapeutics, which developed the RAR ligands used in this study. All other authors have no conflicts of interest or competing financial interests to disclose.

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References

[1] T.J. Martin, N.A. Sims, Osteoclast-derived activity in the coupling of bone formation to resorption, Trends Mol. Med. 11 (2) (2005) 76–81.
[2] A.M. Wu, C.Q. Huang, Z.K. Lin, N.F. Tian, W.F. Ni, X.Y. Wang, et al., The relationship between vitamin a and risk of fracture: meta-analysis of prospective studies, J. Bone Miner. Res. 29 (9) (2014) 2032–2039.
[3] K.W. Ng, H. Zhou, S. Manji, T.J. Martin, Regulation and regulatory role of the retinoids, Crit. Rev. Eukaryot. Gene Expr. 5 (3–4) (1995) 219–253.
[4] H.H. Conaway, A. Pirhayati, E. Persson, U. Pettersson, O. Svensson, C. Lindholm, et al., Retinoids stimulate periosteal bone resorption by enhancing the protein RANKL, a response inhibited by monomeric glucocorticoid receptor, J. Biol. Chem. 286 (36) (2011) 31425–31436.
[5] A. Inoue, E. Otsuka, Y. Hiruma, S. Hirose, M. Furuya, S. Tanaka, et al., Stimulation by retinoids of the natriuretic peptide system of osteoblastic MC3T3-E1 cells, Biochem. Biophys. Res. Commun. 228 (1) (1996) 182–186.
[6] A. Kindmark, H. Torma, A. Johansson, S. Ljunghall, H. Melhus, Reverse transcription-polymerase chain reaction assay demonstrates that the 9-*cis* retinoic acid receptor alpha is expressed in human osteoblasts, Biochem. Biophys. Res. Commun. 192 (3) (1993) 1367–1372.
[7] E. van Beek, C. Lowik, M. Karperien, S. Papapoulos, Independent pathways in the modulation of osteoclastic resorption by intermediates of the mevalonate biosynthetic pathway: the role of the retinoic acid receptor, Bone 38 (2) (2006) 167–171.
[8] T. Lind, A. Sundqvist, L. Hu, G. Pejler, G. Andersson, A. Jacobson, et al., Vitamin a is a negative regulator of osteoblast mineralization, PLoS One 8 (12) (2013) e82388.
[9] I. Fernandez, D.M. Tiago, V. Laize, M. Leonor Cancela, E. Gisbert, Retinoic acid differentially affects in vitro proliferation, differentiation and mineralization of two fish bone-derived cell lines: different gene expression of nuclear receptors and ECM proteins, J. Steroid Biochem. Mol. Biol. 140 (2014) 34–43.

[10] W. Zhang, Z.L. Deng, L. Chen, G.W. Zuo, Q. Luo, Q. Shi, et al., Retinoic acids potentiate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells, PLoS One 5 (7) (2010) e11917.
[11] B.A.A. Scheven, N.J. Hamilton, Retinoic acid and 1,25-dihydroxyvitamin D3 stimulate osteoclast formation by different mechanisms, Bone 11 (1) (1990) 53–59.
[12] H.H. Conaway, E. Persson, M. Halen, S. Granholm, O. Svensson, U. Pettersson, et al., Retinoids inhibit differentiation of hematopoietic osteoclast progenitors, FASEB J. 23 (10) (2009) 3526–3538.
[13] C.R. Walkley, G.H. Olsen, S. Dworkin, S.A. Fabb, J. Swann, G.A. McArthur, et al., A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency, Cell 129 (6) (2007) 1097–1110.
[14] D. Lohnes, P. Kastner, A. Dierich, M. Mark, M. LeMeur, P. Chambon, Function of retinoic acid receptor gamma in the mouse, Cell 73 (4) (1993) 643–658.
[15] D. Lohnes, M. Mark, C. Mendelsohn, P. Dolle, A. Dierich, P. Gorry, et al., Function of the retinoic acid receptors (RARs) during development (I): craniofacial and skeletal abnormalities in RAR double mutants, Development 120 (10) (1994) 2723–2748.
[16] N.B. Ghyselinck, V. Dupe, A. Dierich, N. Messaddeq, J.M. Garnier, C. Rochette-Egly, et al., Role of the retinoic acid receptor beta (RARbeta) during mouse development, Int. J. Dev. Biol. 41 (3) (1997) 425–447.
[17] C. Mendelsohn, M. Mark, P. Dolle, A. Dierich, M.P. Gaub, A. Krust, et al., Retinoic acid receptor beta 2 (RAR beta 2) null mutant mice appear normal, Dev. Biol. 166 (1) (1994) 246–258.
[18] T. Lufkin, D. Lohnes, M. Mark, A. Dierich, P. Gorry, M.P. Gaub, et al., High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice, Proc. Natl. Acad. Sci. U. S. A. 90 (15) (1993) 7225–7229.
[19] S.M. Thacher, J. Vasudevan, R.A. Chandraratna, Therapeutic applications for ligands of retinoid receptors, Curr. Pharm. Des. 6 (1) (2000) 25–58.
[20] K.E. Herbert, C.R. Walkley, I.G. Winkler, J. Hendy, G.H. Olsen, Y.D. Yuan, et al., Granulocyte colony-stimulating factor and an RARalpha specific agonist, VTP195183, synergize to enhance the mobilization of hematopoietic progenitor cells, Transplantation 83 (4) (2007) 375–384.
[21] N.A. Sims, K. Brennan, J. Spaliviero, D.J. Handelsman, M.J. Seibel, Perinatal testosterone surge is required for normal adult bone size but not for normal bone remodeling, Am. J. Physiol. Endocrinol. Metab. 290 (3) (2006) E456–E462.
[22] D. Horn, P. Rivas, B. McCluskey, G.R. Mundy, G. Gutierrez, A new staining technique for undecalcified bone sections that enhances visualization of fluorochromes, J. Bone Mine. Res. 17 (2002) S416.
[23] N.A. Sims, P. Clement-Lacroix, F. Da Ponte, Y. Bouali, N. Binart, R. Moriggl, et al., Bone homeostasis in growth hormone receptor-null mice is restored by IGF-I but independent of Stat5, J. Clin. Invest. 106 (9) (2000) 1095–1103.
[24] C.R. Walkley, Y.D. Yuan, R.A. Chandraratna, G.A. McArthur, Retinoic acid receptor antagonism in vivo expands the numbers of precursor cells during granulopoiesis, Leukemia 16 (9) (2002) 1763–1772.
[25] I.J. Frew, N.A. Sims, J.M. Quinn, C.R. Walkley, L.E. Purton, D.D. Bowtell, et al., Osteopenia in Siah1a mutant mice, J. Biol. Chem. 279 (28) (2004) 29583–29588.
[26] L.E. Purton, I.D. Bernstein, S.J. Collins, All-trans retinoic acid delays the differentiation of primitive hematopoietic precursors (lin-c-kit+Sca-1(+)) while enhancing the terminal maturation of committed granulocyte/monocyte progenitors, Blood 94 (2) (1999) 483–495.
[27] L.E. Purton, S. Dworkin, G.H. Olsen, C.R. Walkley, S.A. Fabb, S.J. Collins, et al., RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation, J. Exp. Med. 203 (5) (2006) 1283–1293.
[28] P.P. Singh, A.G. van der Kraan, J. Xu, M.T. Gillespie, J.M. Quinn, Membrane-bound receptor activator of NFkappaB ligand (RANKL) activity displayed by osteoblasts is differentially regulated by osteolytic factors, Biochem. Biophys. Res. Commun. 422 (1) (2012) 48–53.
[29] S. Takeshita, K. Kaji, A. Kudo, Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts, J. Bone Miner. Res. 15 (8) (2000) 1477–1488.
[30] Y. Takamatsu, P.J. Simmons, R.J. Moore, H.A. Morris, L.B. To, J.P. Levesque, Osteoclast-mediated bone resorption is stimulated during short-term administration of granulocyte colony-stimulating factor but is not responsible for hematopoietic progenitor cell mobilization, Blood 92 (9) (1998) 3465–3473.
[31] L.C. Chee, J. Hendy, L.E. Purton, G.A. McArthur, The granulocyte-colony stimulating factor receptor (G-CSFR) interacts with retinoic acid receptors (RARs) in the regulation of myeloid differentiation, J. Leukoc. Biol. 93 (2) (2013) 235–243.
[32] H. Amano, S. Yamada, R. Felix, Colony-stimulating factor-1 stimulates the fusion process in osteoclasts, J. Bone Miner. Res. 13 (5) (1998) 846–853.
[33] E.C. Walker, N.E. McGregor, I.J. Poulton, S. Pompolo, E.H. Allan, J.M. Quinn, et al., Cardiotrophin-1 is an osteoclast-derived stimulus of bone formation required for normal bone remodeling, J. Bone Miner. Res. 23 (12) (2008) 2025–2032.
[34] R.L. Lees, V.K. Sabharwal, J.N. Heersche, Resorptive state and cell size influence intracellular pH regulation in rabbit osteoclasts cultured on collagen-hydroxyapatite films, Bone 28 (2) (2001) 187–194.
[35] Y. Fujikawa, J.M. Quinn, A. Sabokbar, J.O. McGee, N.A. Athanasou, The human osteoclast precursor circulates in the monocyte fraction, Endocrinology 137 (9) (1996) 4058–4060.
[36] S. Hayashi, T. Yamada, M. Tsuneto, T. Yamane, M. Takahashi, L.D. Shultz, et al., Distinct osteoclast precursors in the bone marrow and extramedullary organs characterized by responsiveness to Toll-like receptor ligands and TNF-alpha, J. Immunol. 171 (10) (2003) 5130–5139.
[37] B. De Klerck, I. Carpentier, R.J. Lories, Y. Habraken, J. Piette, G. Carmeliet, et al., Enhanced osteoclast development in collagen-induced arthritis in interferon-gamma receptor knock-out mice as related to increased splenic CD11b^{+} myelopoiesis, Arthritis Res. Ther. 6 (3) (2004) R220–R231.
[38] B.F. Boyce, P. Li, Z. Yao, Q. Zhang, I.R. Badell, E.M. Schwarz, et al., TNF-alpha and pathologic bone resorption, Keio J. Med. 54 (3) (2005) 127–131.
[39] H. Kodama, M. Nose, S. Niida, A. Yamasaki, Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells, J. Exp. Med. 173 (5) (1991) 1291–1294.
[40] T. Tamura, N. Udagawa, N. Takahashi, C. Miyaura, S. Tanaka, Y. Yamada, et al., Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6, Proc. Natl. Acad. Sci. U. S. A. 90 (24) (1993) 11924–11928.
[41] R. Starr, T.A. Willson, E.M. Viney, L.J. Murray, J.R. Rayner, B.J. Jenkins, et al., A family of cytokine-inducible inhibitors of signalling, Nature 387 (6636) (1997) 917–921.
[42] P.K. Wong, P.J. Egan, B.A. Croker, K. O'Donnell, N.A. Sims, S. Drake, et al., SOCS-3 negatively regulates innate and adaptive immune mechanisms in acute IL-1-dependent inflammatory arthritis, J. Clin. Invest. 116 (6) (2006) 1571–1581.
[43] C. Ehlting, W.S. Lai, F. Schaper, E.D. Brenndorfer, R.J. Matthes, P.C. Heinrich, et al., Regulation of suppressor of cytokine signaling 3 (SOCS3) mRNA stability by TNF-alpha involves activation of the MKK6/p38MAPK/MK2 cascade, J. Immunol. 178 (5) (2007) 2813–2826.
[44] H.H. Conaway, P. Henning, U.H. Lerner, Vitamin a metabolism, action, and role in skeletal homeostasis, Endocr. Rev. 34 (6) (2013) 766–797.
[45] C.E. Hotchkiss, J. Latendresse, S.A. Ferguson, Oral treatment with retinoic acid decreases bone mass in rats, Comp. Med. 56 (6) (2006) 502–511.
[46] M. Kneissel, A. Studer, R. Cortesi, M. Susa, Retinoid-induced bone thinning is caused by subperiosteal osteoclast activity in adult rodents, Bone 36 (2) (2005) 202–214.
[47] T. Lind, P.M. Lind, A. Jacobson, L. Hu, A. Sundqvist, J. Risteli, et al., High dietary intake of retinol leads to bone marrow hypoxia and diaphyseal endosteal mineralization in rats, Bone 48 (3) (2011) 496–506.
[48] J.H. Promislow, D. Goodman-Gruen, D.J. Slymen, E. Barrett-Connor, Retinol intake and bone mineral density in the elderly: the Rancho Bernardo Study, J. Bone Miner. Res. 17 (8) (2002) 1349–1358.