Repression of Osteoblast Maturation by ERRα Accounts for Bone Loss Induced by Estrogen Deficiency

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Abstract

ERRα is an orphan member of the nuclear receptor family, the complete inactivation of which confers resistance to bone loss induced by ageing and estrogen withdrawal to female mice in correlation with increased bone formation vivo. Furthermore ERRα negatively regulates the commitment of mesenchymal cells to the osteoblast lineage ex vivo as well as later steps of osteoblast maturation. We searched to determine whether the activities of ERRα on osteoblast maturation are responsible for one or both types of in vivo induced bone loss. To this end we have generated conditional knock out mice in which the receptor is normally present during early osteoblast differentiation but inactivated upon osteoblast maturation. Bone ageing in these animals was similar to that observed for control animals. In contrast conditional ERRαKO mice were completely resistant to bone loss induced by ovariectomy. We conclude that the late (maturation), but not early (commitment), negative effects of ERRα on the osteoblast lineage contribute to the reduced bone mineral density observed upon estrogen deficiency.

Introduction

Bone remodeling is a dynamic process in which resorption exerted by osteoclasts is compensated for by formation exerted by osteoblasts. Although normally tightly regulated, this equilibrium can be disrupted under various circumstances [1,2]. For instance, during ageing mesenchymal cells (MCs, the osteoblast progenitors) become more prone to differentiate into adipocytes than into osteoblast, in fine leading to a relative excess osteoclast activity. In contrast estrogen deficiency can lead to post-menopausal women results in derespressed osteoblast differentiation and activity that is not compensated for by a similarly increased osteoblast activity. Ageing and menopause thus both lead to increased bone fragility (i.e. osteoporosis) and enhanced fracture risk. Given the global ageing of the world population, osteoporosis and its consequences now represent a major public health problem, in particular in females. Treatments against post-menopause osteoporosis have up to now generally aimed at reducing osteoclast activities, in particular via hormone replacement therapies [3–5]. Due to controversial side effects of the latter treatments, the possibility of enhancing osteoblast differentiation and/or activities (bone anabolism) could appear as a promising approach [6,7].

MCs differentiation into osteoblast has increasingly been characterized including at the level of the sequential expression of functional markers. Two transcription factors, Runx2 and Oxs, are instrumental in the first steps of this cascade and precede and/or regulate the expression of later markers, which reflect osteoblast maturation. These include collagen 1a (Col1a), alkaline phosphatase (ALP), osteocalcin and osteopontin [8].

ERRα belongs to the nuclear receptor family, the members of which act as transcription factors [9]. Although no natural ligand has been identified to date for ERRα (which is thus referred to as “orphan receptor”), synthetic compounds have been isolated that modulate its activities and/or protein stability [10–13]. High expression of ERRα in various human cancer types is correlated to poor prognosis (reviewed in [14]). Furthermore the receptor has been shown to promote tumorigenicity and angiogenesis in human carcinoma cells xenografted onto nude mice ([15–17], reviewed in [18]), suggesting that inhibition of ERRα could be beneficial in cancer treatment [19]. Several reports have demonstrated that ERRα is a key positive regulator of various metabolic functions, controlling lipid uptake, fatty acid oxidation, the tricarboxylic acid cycle, oxidative phosphorylation and mitochondrial biogenesis (reviewed in [20,21]). In addition, ERRα impacts on MCs differentiation, promoting commitment towards the adipocyte pathway [22] while inhibiting the osteoblast one [23,24]. Indeed, when set in differentiation ex vivo, MCs originating from ERRα knock-out (ERRαKO) mice display a higher and earlier expression of Runx2, Col1a, ALP and osteocalcin, associated with increased mineralization capacities. Mechanistically ERRα has been sug-
suggested to modulate Wnt signaling [25] whereas the closely related ERRγ receptor (which also represses osteoblast differentiation) impairs Runx2 transcriptional activities [26]. In addition to these early effects on MCs commitment, ERRα may impact on later steps of osteoblast differentiation. Indeed, the enhanced ex vivo differentiation of ERRα-KO-originating MCs can be reverted by re-expression of the receptor after the onset of differentiation, suggesting that ERRα-deficient MCs still display a certain level of plasticity [24]. Furthermore, the expression of osteopontin, an inhibitor of mineralization and late marker of osteoblast differentiation, is down regulated in ERRα-deficient osteoblasts, in contrast to other earlier markers. In vivo studies have shown that the bone mineral density of female ERRα-KO mice is not reduced upon ageing, in contrast to the situation in wild type animals [23,24]. Furthermore, these mice also resist to the bone loss resulting from estrogen withdrawal (obtained by ovariectomy).

These phenomena are associated with an increased bone formation rate without any modification in bone resorption, indicating that osteoblasts, not osteoclasts, are the major cellular effectors mediating these resistances. In contrast, male ERRα-KO mice were indistinguishable from wild type animals both in terms of bone ageing and sensitivity to androgen deficiency. Inactivating ERRα may thus be beneficial in females to protect bone against the deleterious impacts of ageing and estrogen deficiency by promoting bone anabolism [reviewed in [27]].

It is unknown whether only one or both the early and late (as defined above) effects of ERRα on osteoblast differentiation contribute to which in vivo resistance. To investigate this question, we have used the Cre-Lox technology to engineer mice in which ERRα is specifically inactivated during the late phase of osteoblast differentiation. We here report that female conditional ERRα-KO mice resist to bone loss induced by ovariectomy but not to the one induced by natural ageing. ERRα thus contributes to estrogen deficiency-induced bone loss through its inhibitory activities on osteoblast maturation. Moreover, our results describe an animal model in which age-related and hormone-deficiency-related bone loss in females can be clearly uncoupled.

Results

To generate a conditional knock-out mouse model for ERRα, we used ERRαfl/fl mice in which ERRα exon 2 was flanked by loxP sites. These animals were crossed with Col1a-Cre mice [28]. The latter animals express the Cre recombinase under the control of a collagen 1α promoter fragment, which is selectively active in early osteoblasts, only after the onset of differentiation. The resulting mice (ERRαfl/fl, Col1aCre/−) are thus predicted to be inactivated for ERRα only in maturing osteoblasts and not in mesenchymal stem cells or early committed osteoblast progenitors (Fig. 1A). Genotyping using specific primer sets detected the floxed allele in all tissues tested, whereas recombination only occurred in bone (long bone and skull) but not in "soft" (i.e. bone-free) tissues (Fig. 1B). This recombination appears only partial at the organ level likely due to the unavoidable presence of osteoclasts and non-bone contaminating cells (e.g. blood cells) as well as to the heterogeneity in the stages of maturation in which osteoblasts are in bone. ERRαfl/fl and ERRαΔfl/fl,Col1aCre/− mice are hereafter referred to as control and conditional knock-out (cKO) mice, respectively.

Due to the above-mentioned tissue heterogeneity, a decrease of ERRα expression could not be measured in bone in vivo. We thus turned to an ex vivo model in which pre-osteoblasts from mice calvaria (cranial vault) were set in culture and allowed to differentiate into osteoblasts. At the beginning of differentiation,
global enzymatic activity (Fig. 2C). Mineralizing activity was also dramatically enhanced in cKO cultures as compared to control ones (Fig. 2D). In the absence of ERRα, the expression (measured by real time PCR) of middle to late differentiation markers such as Col1a, ALP and osteocalcin (Ocn) was transiently enhanced (after 5 days of differentiation) and thereafter normalized, suggesting a time-dependent effect of the receptor (Fig. 3A). In contrast, expression of osteopontin, an ERRα direct target gene [30], was reduced after 10 days of differentiation. Interestingly these variations of expression are identical to those observed when analyzing cells originating from complete (i.e. not conditional) ERRα KO mice [24]. Reintroduction of ERRα by lentiviral infection reversed the expression of the differentiation markers (Fig. 3B). However, the expression of Runx2, an early differentiation inducer (the expression of which precedes that of Col1a; [8]) was not modified in cKO-originating cells as compared to control ones nor upon reintroduction of ERRα. Since Runx2 activates the expression of Col1a, ALP and Ocn [8], this may altogether suggest that ERRα counteracts Runx2 activity. In this hypothesis, ERRα would act similarly to the closely related ERRγ [26]. To investigate this possibility, we performed cotransfection experiments in C3H10T1/2 (mesenchymal) cells (Fig. 3C). Runx2 activated expression driven by its cognate response element (OSE; [31]). However coexpression of ERRα or ERRγ completely blunted this effect. As a control, we verified that both ERRγ and ERRα were capable of activating transcription from their common response element (ERRE), even if ERRα requires the presence of the PGC-1α coactivator in these cells. Noteworthy, Runx2 did not impact on ERR activities under these conditions. Similar results were obtained in C2C12 cells, which are more committed than C3H10T1/2 (data not shown).

Altogether this shows that i) ERRα is specifically inactivated in cKO cells only after the onset of osteoblast differentiation ex vivo, ii) the absence of the receptor under these conditions promotes late osteoblast differentiation without impacting on early commitment and differentiation steps. This validates the present cKO model as a tool to study the in vivo effects of ERRα on osteoblast maturation (as opposed to early MSC commitment).

The absence of ERRα in complete KO mice protects female (but not male) animals against age-induced as well as against ovariectomy-induced bone loss [24]. We thus first investigated whether cKO mice were also protected from age-related bone loss. To this end bone structural parameters were determined by X-ray microtomography (microCt) comparing 14 wk and 24 wk old females (Fig. 4). We observed an equal reduction of bone volume (BV/TV, Fig. 4A), bone mineral density (BMD, Fig. 4B) and trabecular number (Tb N, Fig. 4C) in both genotypes upon ageing. Trabecular spacing (Tb Sp, Fig. 4D) was also equally enhanced, reflecting the decreased number of trabeculae, in spite of constant trabecular thickness (Tb Th, Fig. 4E), not expected to decrease upon ageing. Noteworthy all these parameters displayed identical values between control and cKO mice at a given age, indicating

![Figure 2](image-url)
that ERRα inactivation after the onset of osteoblast differentiation does not protect against age-induced bone loss.

We next studied the bone structural parameters two weeks after gonadectomy on control and cKO animals (Fig. 5). Female mice of both genotype responded identically to ovariectomy (ovx) in terms of reduced uterine thickness (Fig. 5A). In control female mice, we observed the expected decrease in bone volume (Fig. 5B), bone mineral density (Fig. 5C) and trabecular thickness (Fig. 5F) upon ovx. As also expected the number of trabeculae was unchanged (Fig. 5D) and a non-significant trend toward enhanced spacing between trabeculae was observed (Fig. 5E). Strikingly, none of these parameters were modified in ovariectomized cKO mice as compared to sham-operated animals. The divergent response to ovx of control and cKO mice can also be viewed on the reconstructed 3D structures of trabecular bones (see Movies S1, S2, S3, and S4). This situation is in full contrast with the one prevailing in male mice in which orchidectomy (orx) led to dramatically reduced bone volume (Fig. 5G) and mineral density (Fig. 5H) both in control and cKO mice. We concluded that inactivating ERRα during osteoblast maturation completely protects against bone loss induced by hormonal deficiency, selectively in females.

Discussion

Amongst other, ageing is associated with bone loss, leading to osteoporosis (understood as bone fragility syndrome) and increased fracture risk. Due to the global ageing of the human population worldwide, osteoporosis is now a major health problem, being the most common metabolic disorder of old age in humans [1,2]. During ageing bone formation by osteoblasts is impaired due a decreased number and activity of osteoblasts, and concomitantly bone resorption by osteoclasts is increased. Whereas both males and females are subjected to age-associated osteoporosis, the situation is further aggravated in females after menopause. The cessation of ovarian function, resulting in highly decreased circulating levels of estrogens, indeed leads to unimpaired osteoblast differentiation and activity which is not compensated for by an equivalent rise in osteoblast activity [32]. Although age-related- and estrogen-deficiency-related bone loss essentially originates in different cell compartments, it is expected that enhancing bone anabolism (i.e. promoting bone formation by osteoblasts) could be an efficient mean to counteract osteoporosis in general [6,7].

In this line, we and others have shown that genetic inactivation of the orphan nuclear receptor ERRα in mice leads to resistance to bone loss induced by ovariectomy (used as a model for estrogen-deficiency, thus mimicking menopause) or ageing [23,24]. Interestingly these phenotypes were not associated with a decrease in osteoclast activity suggesting that ERRα does not modulate bone resorption at least under the above-mentioned challenging conditions. In contrast, ERRαKO animals displayed a higher bone formation rate as compared to wild type ones, altogether strongly suggesting that ERRα contributes to bone loss exclusively through the effects it exerts in osteoblasts. It should however be mentioned that ERRα has been shown to be required for
osteoclast differentiation and/or activities in response to bone loss induced by rosiglitazone, a thiazolidinedione prescribed for the treatment of insulin resistance and diabetes [33].

The actual role of ERRα in osteoblast differentiation in vitro (inducer or inhibitor of differentiation?) is controversial and has been thoroughly discussed in our recently published review [27]. However, the absence of ERRα has been associated to an increased capacity of mesenchymal cells (MCs) to differentiate in osteoblasts ex vivo [23,24]. Conversely MCs were less prone to differentiate into adipocytes in the absence of the receptor [22] leading to reduced fat marrow in ERRα KO animals [23] suggesting that ERRα is an early switch factor influencing MCs differentiation towards the adipocyte pathway at the expense of the osteoblastic one. ERRα has been suspected of additional effects in later steps of osteoblast maturation which may be mediated by osteopontin (opn), a downstream positive target of the receptor [30]. Indeed opn has been shown to reduce bone mineralization and its absence in KO mice confers resistance to ovx-induced bone loss [34], as does inactivation of ERRα.

The conditional KO approach described in the present report allows us to discriminate between the early (MCs commitment) and late (osteoblast maturation) effects of ERRα. To inactivate a floxed ERRα allele, we indeed expressed the Cre recombinase

![Figure 4](image-url) **Figure 4.** Conditional inactivation of ERRα in vivo does not impact bone ageing. Bone volume fraction (BV/TV; A), bone mineral density (BMD; B), trabecular number (Tb N; C), trabecular spacing (Tb Sp; D) and trabecular thickness (Tb Th; E) were determined by microCT-scan at 14 (white bars) and 24 wk (black bars) in the femur of female control (c) and conditional ERRα knock-out (cKO) mice (n=6 to 11 per group). Error bars represent s.e.m. *: p<0.05; ***: p<0.005; ns: non significant.

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![Figure 5](image-url) **Figure 5.** Conditional inactivation of ERRα in vivo protects against ovariectomy-induced bone loss. A. Photograph of uteri in sham-operated or ovariectomized (ovx) control (c) or cKO mice. B–F. Same parameters as in Fig. 3 were determined in females either sham-operated (white bars) or two weeks after ovariectomy (ovx; black bars). BV/TV (G) and BMD (H) were determined in male mice either sham-operated (white bars) or four weeks after orchidectomy (orx; black bars). All mice (n=6 to 10 per group) were 14 wk old at the end of the experiment. Error bars represent s.e.m. *: p<0.05; ***: p<0.005; ns: non significant.

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Dual Effects of ERRα on Resistance to Bone Loss
under the control of a Colla promoter fragment, driving expression during mid stages of osteoblast differentiation, i.e. well after MCs commitment to the osteoblastic lineage [28]. As a consequence we observed a decrease in ERRα expression only during mid to late stages of osteoblast maturation ex vivo, whereas the receptor is normally present in pre-osteoblasts. Consistently the expression of Runx2, an early differentiation marker and inducer [9], was normally regulated during cKO pre-osteoblast differentiation in contrast to what observed in complete ERRαKO mice, where a marked increase of Runx2 expression had been observed [23,24]. However, the expression of mid- to late markers of osteoblast differentiation was deregulated in cKO-originating cells, in a manner similar to that observed in complete ERRαKO animals. In this respect our data suggest that ERRα may reduce osteoblast differentiation by antagonizing Runx2 transactivation capacities, in a manner similar to that demonstrated for the closely related ERRγ receptor [26].

Age-induced bone loss in cKO mice was similar to the one measured in control animals. This excludes that the activities of ERRα during osteoblast maturation may mediate age-dependent bone loss. It is tempting to rather speculate that the activities of ERRα as a repressor of MCs commitment to the osteoblastic lineage are involved in this phenomenon. However, we cannot formally exclude the impact of a yet uncharacterized primary activity of ERRα outside the bone compartment that would result in age-related bone loss. Investigating the bone response of ERRα cKO animals to gonadectomy revealed that males were normally sensitive whereas females were completely protected from bone loss. This situation is actually identical to the one observed in complete ERRαKO mice and confirms a gender-dependent effect of the receptor in bone. More importantly our results demonstrate that ERRα contributes to ovariecytomy-induced bone loss via its activities on osteoblast maturation, and not through the regulation of MCs commitment. These results are summarized on Figure 6.

Together with data published by other laboratories, our results suggest that ERRα could be a promising target for the design of innovative therapies against bone loss, specifically in females. In this respect, we previously demonstrated that expression of the receptor in bone is not modified according to the estrogen status in mice [35]. Although ERRα is an orphan receptor, several synthetic compounds have been identified that modulate its activities and impact on its stability [10–14]. A pharmacological approach could thus be considered to specifically impact on the receptor. However complete inhibition of the receptor can be expected to lead to various undesired side effects on metabolism. For instance, given the role of ERRα as a switch factor in MCs commitment, its inhibition, while promoting osteoblast differentiation, would likely affect adipogenesis and thereby lipid storage and consumption. A more reliable approach would consist in impacting only the late osteoblast activities of the receptor, although this would solely be efficient against estrogen deficiency-induced bone loss. Such a compound, modulating a specific subset of the receptor’s activities, is still to be identified.

**Materials and Methods**

**Animals**

Col1a-Cre mice have been described elsewhere [28]. ERRα<tm1ICS> (Errα<tm1ICS> mouse line) animals have been generated in the Institut Clinique de la Souris (Illkirch-Graffenstaden). For genotyping, DNA extracted from organs using conventional methods was PCR amplified using Eurobio kit. PCR cycle used: 94°C, 30 sec; 62°C, 30 sec; 72°C, 1 min. PCR products were analysed on 1.2% agarose gels.

**Primers Used for Genotyping**

a: 5’-GCCCCCCTTGCCGCCGCCCTTTAGCCCCCTCCC-3’
b: 5’-CCCTGTCTGCTGTGCCTTTGC-3’
c: 5’-CCACCACGTGCCAGCCCTAC-3’

For surgery, animals were anesthetized with sodium pentobarbital. Testes were ligatured and cut through an incision in the scrotum. Ovaries were removed through an incision in the flanks. Animals were sacrificed 2 wk after operation.

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*Figure 6. In vivo and ex vivo effects of ERRα on bone.* ERRα impairs the later steps of osteoblastic differentiation. The results presented here show that these activities are involved in bone loss induced by estrogen deficiency and may involve the repression of Runx2-driven transcriptional activities. In contrast, these activities are not involved in bone sensitivity to ageing. Data published by other show that ERRα also promotes the early commitment of mesenchymal stem cells (MSCs) toward the adipocytic pathway while restricting the osteoblastic one. We hypothesize that these early activities are responsible, at least in part, for bone loss induced by ageing. See text for details and references.

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All animal experiments were performed in the Plateau de Biologie Expérimentale de la Souris (PBES; ENS Lyon) under animal care procedures, conducted in accordance with the guidelines set by the European Community Council Directives (86/609/EEC) and approved by the ENS Lyon ethical committee. Animals were in C57black6 background and had access to food and water ad libitum. Mice were sacrificed by cervical dislocation at 10 a.m.

X-ray Microcomputed Tomography Analysis

3D microarchitecture of the femur was evaluated using a high-resolution (8 μm) microtomographic imaging system (eXplore Locus, GE, USA). A 3D region within the secondary spongiosa in the proximal metaphysis of the femur was reconstructed, beginning 500 μm proximal to the growth plate and extending to 1.5 mm. Cortical bone was reconstructed from a 1 mm thick region of interest centered on the diaphysis, 5 mm distal from the proximal growth plate. Morphometric parameters were computed using the Advanced Bone Analysis Micrview Software (GE).

Calvarial Cell Cultures

Calvariae were isolated from 1–5 day old mice and digested for 1 hr in collagenase (Sigma). After centrifugation, cell pellet was set in culture in six-well plates in αMEM supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin. Cells were induced to differentiate into osteoblasts with 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate. Culture medium was replaced every 2 to 3 days. For immunoﬂuorescence, cells were fixed in 4% paraformaldehyde for 10 min, then permeabilized with 0.5% Triton, 0.3% BSA in PBS for 1 hr at RT. Hoescht staining was diluted 1/20,000 and secondary antibodies from Jackson (anti-rabbit-Alexa647 diluted 1/1000) or Novotech diluted 1/200) were added or 3 hrs at RT. Secondary antibodies from Santa Cruz diluted 1/50; anti-collagen1 from Novotech diluted 1/200 and reporter activities were determined using standard methods. All animal experiments were performed in the Plateau de Biologie Expérimentale de la Souris (PBES; ENS Lyon) under animal care procedures, conducted in accordance with the guidelines set by the European Community Council Directives (86/609/EEC) and approved by the ENS Lyon ethical committee. Animals were in C57black6 background and had access to food and water ad libitum. Mice were sacrificed by cervical dislocation at 10 a.m.

Statistical Analysis

Statistical significance was analyzed using one-way ANOVA.

Supporting Information

Movie S1 3D reconstruction of trabecular bone originating from control sham female mice. (AVI)

Movie S2 3D reconstruction of trabecular bone using control ovx mice. (AVI)

Movie S3 3D reconstruction of trabecular bone using eKO sham mice. (AVI)

Movie S4 3D reconstruction of trabecular bone using eKO ovx mice. (AVI)

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Author Contributions

Conceived and designed the experiments: OC MCS JMV. Performed the experiments: MG SS JP CM JS JC VT BB CF. Analyzed the data: OC MCS JMV. Contributed reagents/materials/analysis tools: MCB TS. Wrote the paper: OC MCS JMV.
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