



Estrogen-Related Receptors and the control of bone cell fate

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Abstract: Bone loss is naturally occurring in ageing males and females and exacerbated in the latter after menopause, altogether leading to cumulative skeleton fragility and increased fracture risk. Two types of therapeutic strategies can be envisioned to counteract age- or menopause-associated bone loss, aiming at either reducing bone resorption exerted by osteoclasts or, alternatively, promoting bone formation by osteoblasts. We here summarize data suggesting that inhibition of the Estrogen-Related Receptors α and/or γ could promote bone formation and compensate for bone loss induced by ageing or estrogen-deficiency.



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Lyon May 25th 2015,

Dear Editor

Please find a review entitled “Estrogen-Related Receptors and the control of bone cell fate” by Julie Carnesecchi and JM Vanacker, that is our tentative contribution to the special issue “Bone disorders: mechanisms and targets” of *Molecular and Cellular Endocrinology*.

In this review we tried to describe as exhaustively as possible the data concerning the effects of Estrogen-Related Receptors on the pathways and mechanisms of differentiation of mesenchymal cells. Although we focus on bone cells (osteoblasts and osteoclasts) as requested for this special issue, we indeed chose to extend the data we reviewed to mesenchymal cells, feeling a broader point of view would be useful to the reader.

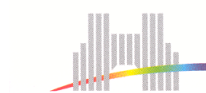
Hoping our review will be considered in a favorable manner.

Sincerely,

JM Vanacker

Possible reviewers of our manuscript include

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Estrogen-Related Receptors and the control of bone cell fate.

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ABSTRACT

Bone loss is naturally occurring in ageing males and females and exacerbated in the latter after menopause, altogether leading to cumulative skeleton fragility and increased fracture risk. Two types of therapeutic strategies can be envisioned to counteract age- or menopause-associated bone loss, aiming at either reducing bone resorption exerted by osteoclasts or, alternatively, promoting bone formation by osteoblasts. We here summarize data suggesting that inhibition of the Estrogen-Related Receptors α and/or γ could promote bone formation and compensate for bone loss induced by ageing or estrogen-deficiency.

MAIN TEXT

The ERRs as constitutively acting nuclear receptors

Nuclear receptors form a gene/protein family of 48 members in the human and have been defined as ligand-dependent transcription factors (Laudet and Gronemeyer, 2002; Perissi and Rosenfeld, 2005). With few exceptions, they share a common organization comprising two conserved, highly structured domains. A centrally located DNA-binding domain (DBD) encompassing two zinc finger modules mediates direct contact with DNA response elements located in the enhancer/promoter of their target genes. A C-terminal domain, containing up to 12 α -helices arranged in a globular structure, is responsible for ligand fixation (and is hence referred to as ligand-binding domain, LBD) as well as ligand-dependent transcriptional activity. These two domains are linked together by a poorly conserved, so-called “hinge” domain. In addition, some nuclear receptors comprise an additional N-terminally located domain that can mediate ligand-independent transcriptional activation. Schematically, the activity of nuclear receptors such as the Estrogen- or Thyroid hormone Receptors (ERs and TRs, respectively) is regulated *in vivo* by the fixation of a hormone (17 β -estradiol or tri-iodothyronine [T3], respectively) in a hydrophobic ligand-binding pocket, located in the heart of the LBD. This induces a conformational change in the LBD that relocates its C-terminal-most helix, thereby forming a contact surface for the recruitment of transcriptional co-factors that will ultimately trigger the expression of downstream target genes. In addition the recruitment of some NRs (such as ERs, but not TRs) to DNA can be induced by ligand-binding.

The existence and (at least part) of the physiological activities of hormones such as 17 β -estradiol or thyroid hormone had been explored for decades before the identification, in the mid 80s, of their corresponding receptors (Evans and Mangelsdorf, 2014; Gustafsson, 2015). In contrast, other nuclear receptors have been later isolated (starting in the late 80s),

based on their sequence similarity to “classical” receptors. Since no hormone had been previously identified that promoted their activities, these newly isolated receptors were referred to as “orphan” (Giguère, 1999). Subsequent research efforts have allowed to “de-orphanize” a number of these receptors, by identifying natural compounds that specifically bind to them and regulate their activities *in vivo*. However some orphan nuclear receptors are still reluctant to adoption. Strikingly this is the case of the first orphan nuclear receptors identified in 1988 (Giguère et al.), namely the Estrogen-Related Receptor α and β (ERR α and β), which, together with ERR γ (identified in 1999, Hong et al.), form a defined subfamily (Tremblay and Giguère, 2007). Interestingly these receptors display the above-mentioned domain organization, with a DBD that displays an elevated level of sequence identity to the one of the ERs, and a more moderately conserved LBD (Horard and Vanacker, 2003). As evidenced by structural analysis, the latter includes a putative ligand-binding pocket, which is much smaller than that of the ERs and is crossed by the side-chain of a phenylalanine residue (Chen et al., 2001; Greschik et al., 2002; Kallen et al., 2004). This is thought to confer a certain level of rigidity to the LBD and to lock the receptor in an active conformation where the helix 12 is constitutively positioned so as to contact co-activators, even in the absence of compound filling the ligand-binding pocket (Takacs et al., 2013). In other terms (and although structural data have so far only been obtained for ERR α and γ , not for β), it is likely that the ERRs regulate transcription in constitutive, ligand-independent manner, thereby betraying the current definition of nuclear receptors. However, one cannot exclude the existence of *bona fide* ligands that could over-promote or reduce the activities of these receptors *in vivo*, by inducing conformational changes. In this respect, synthetic ligands have been identified that modulate the activities of the ERRs. For instance 4-OH-tamoxifene (a selective estrogen receptor modulator [SERM] that is widely used in breast cancer therapy) decreases the activities of ERR γ (Coward et al., 2001; Tremblay et al., 2001) whereas DY131 increases the activities of both ERR β and γ (Yu and Forman, 2005). A special mention should be made of Bisphenol A (BPA, an endocrine disruptor) that, on its own, appears innocuous toward ERR γ but prevents the inactivation of the receptor by compounds such as 4-OH-tamoxifene in cell culture based assays (Takayanagi et al., 2006; Matsushima et al., 2007). In contrast to ERR β and γ , only deactivating compounds have been identified for ERR α to date (Yang and Chen, 1999; Willy et al., 2004; Chisamore et al., 2009a; Chisamore et al., 2009b; Duellman et al., 2010). This is for instance the case of XCT790 which enters the ligand binding pocket, disrupt the organization of the LBD and prevents co-activator recruitment, in addition to inducing proteasome-dependent degradation of the receptor (Willy et al., 2004; Kallen et al., 2007; Lanvin et al., 2007). Few of these compounds have been evaluated *in vivo* for a specific ERR-dependent effect, although recent data show

that BPA induces otolith aggregation in the zebrafish embryo in an ERR γ -dependent manner (Tohmé et al., 2014). In spite of the lack of clear *in vivo* data, the identification of (de)activating ligands for the ERRs suggests that they could be targeted through a pharmacological approach in order to modulate their activities. The identification of ERR-ligands would open promising avenues for the treatment of given pathologies.

In addition, diverse levels of possible regulation of ERRs' activities have been suggested. For instance, ERK8 relocates ERR α in the cytoplasm, thereby inhibiting its DNA binding and transactivation capabilities (Rossi et al., 2011). Moreover, ERR α and γ are subjected to post translational modifications (sumoylation, phosphorylation, acetylation), some which having been shown to impact on the receptor activity (Barry and Giguère, 2005; Vu et al., 2007; Tremblay et al., 2008; Wilson et al., 2010; Kim et al., 2014). One of the key levels of regulations seems however to be represented by the capacity of contacting given co-activators, which can be expressed in a cell-type specific manner. For instance members of the PGC-1 family of transcriptional co-activators have been shown to be instrumental in promoting ERR-based response (at least ERR α and γ), in particular in metabolism-related processes (Villena and Kralli, 2008; Giguère, 2008).

Functions of the ERRs

A number of physiopathological functions have been ascribed to the ERR receptors. ERR β is mainly expressed in the embryo and regulates placental formation, the maintenance of pluripotency of embryonic stem cells, and the specification of epithelial cells in the inner ear (Luo et al., 1997; Chen and Nathans, 2007; Chen et al., 2008). ERR α and γ are strongly expressed in tissues with a high energy demand (e.g. liver, heart, muscle and fat) where they govern energetic metabolism, regulating processes such as lipid uptake, fatty acid oxidation, neoglucogenesis, tricarboxylic acid cycle, oxidative phosphorylation, and mitochondrial biogenesis and activities (Villena and Kralli, 2008; Giguère, 2008; Deblois and Giguère, 2011). These functions strongly depend on the PGC-1 co-activators and are also exerted during somatic reprogramming to pluripotency (Kida et al., 2015) as well as in cancer cells (reviewed in Chang and McDonnell, 2012; Bianco et al., 2012; Deblois and Giguère, 2013; Deblois et al., 2013). For instance, ERR α induces, in advanced breast cancers and in a PGC-1 β -dependent manner, a transcriptional cascade leading to the switch from oxidative to glycolytic metabolism referred to as the Warburg effect which is instrumental in cancer progression (Eichner et al., 2010; Chang et al., 2011; Cai et al., 2013). ERR γ apparently displays converse activities, promoting oxidative rather than glycolytic metabolism thus potentially reducing cancer aggressiveness (Eichner et al., 2010). The activities of the ERRs

in cancer cells however extend beyond the regulation of metabolism. Opposite activities of $ERR\alpha$ and γ have been documented in cancer-related processes such as cell proliferation and epithelial-to-mesenchymal transition (EMT), which are promoted by $ERR\alpha$ and reversed by $ERR\gamma$ (Yu et al., 2007; Tiraby et al., 2011; Bianco et al. 2012; Lam et al., 2014). In addition $ERR\alpha$ promotes cell migration and invasion as well as the establishment of distant metastasis in a manner that apparently does not directly rely on energy metabolism (Dwyer et al., 2010; Fradet et al., 2011; Sailland et al., 2014). In line with these functions, high expression of $ERR\alpha$ is considered as a factor of poor prognosis in diverse cancers (including from the breast), whereas $ERR\gamma$ constitutes a favorable biomarker (Ariazi et al., 2002; Suzuki et al., 2004; reviewed in Bianco et al., 2012).

ERRs and mesenchymal cell fate

Mesenchymal stem cells can differentiate into various lineages including myocytes, adipocytes, chondrocytes and osteoblasts. Strikingly an array of data indicates that $ERR\alpha$ and/or γ modulate the differentiation of all these lineages, suggesting that they may be key regulators of mesenchymal stem cell fate. $ERR\alpha$ expression increases during the transition from myoblasts to myotubes (Bonnelye et al., 1997) and stimulates the expression of Dusp1/MKP1, which in turn dephosphorylates ERK1/2 (Murray and Huss, 2011). In the absence of $ERR\alpha$, ERK1/2 signaling is therefore aberrantly increased and results in sustained proliferation and delayed differentiation. Interestingly $ERR\gamma$ expression is also strongly enhanced when myoblasts differentiate into myotubes (Murray and Huss, 2011). The receptor increases mitochondrial content and promotes oxidative programs *in vivo*, including in mouse model of muscular dystrophy suggesting that it may constitute a promising target against muscle wasting (Narkar et al., 2011; Murray et al., 2013; Matsakas et al., 2013).

Human and mouse mesenchymal cells in which $ERR\alpha$ has been inactivated display reduced adipocytic differentiation capacities (Ijichi et al., 2007; Delhon et al., 2009; Rajalin et al., 2010; Carnesecchi and Vanacker, 2013). This is consistent with the reduced fat deposit and the decreased number and size of adipocytes as well as reduced marrow fat volumes that have been observed in $ERR\alpha^{-/-}$ mice (Luo et al., 2003; Delhon et al., 2009). $ERR\alpha$ expression increases during human adipocyte differentiation supporting the hypothesis of a contribution of the receptor to this process (Delhon et al., 2009). Interestingly, it has recently been shown that miR125a directly targets $ERR\alpha$ to inhibit its expression in porcine preadipocytes (Ji et al., 2014). Furthermore, miR125a expression decreases during adipocytic differentiation, suggesting a possible mechanism for the regulation of $ERR\alpha$

expression during this phenomenon. $ERR\gamma$ expression also increases during preadipocyte differentiation *in vitro* and the receptor promotes the expression of markers of adipocytic differentiation as well as intracellular fat accumulation (Kubo et al., 2009).

$ERR\alpha$ is expressed in rat cartilage *in vivo* and chondrogenic cell lines *in vitro* and has been suggested to positively modulate chondrocytic differentiation *in vitro* through a possible effect on the expression of Sox9 (the master gene for chondrocyte differentiation) (Bonnelye et al., 2007). A recent report addressed the *in vivo* effect of $ERR\gamma$, which was specifically overexpressed in the cartilage of transgenic mice (Cardelli et al., 2013). The resulting animals displayed a mild dwarfism attributed to a reduced thickness of the proliferative zone of long bones. This is likely due to a disequilibrium of the balance between proliferation and differentiation of the chondrocytes of the growth plate. Indeed, $ERR\gamma$ overexpression was found to result in increased expression of the p27 cell cycle inhibitor thereby reducing chondrocyte proliferation. It is possible that this reduced proliferation is the primary event leading to increased differentiation, accompanied by enhanced expression of functional chondrocyte markers such as Pth1 receptor and Aggrecan.

ERRs and bone cell fate

$ERR\alpha$ is strongly expressed in the ossification zones during mouse embryonic development (Bonnelye et al., 1997). This observation is valid for the long bones as well as for the flat bones of the skull, suggesting that the receptor may contribute to both endochondral and intramembranous ossification. $ERR\alpha$ is also highly expressed in osteoblastic cell lines and normal human bones. The receptor has initially been thought to promote osteoblast differentiation when overexpressed in *in vitro* experiments (Bonnelye et al., 2001). However analysis of young (8 weeks old) $ERR\alpha^{-/-}$ mice showed that these animals did not display an overt bone phenotype (Delhon et al., 2009; Teyssier et al., 2009; reviewed in Gallet and Vanacker, 2010). Nevertheless, no bone loss was observed in $ERR\alpha^{-/-}$ mice upon ageing or when females were ovariectomized, in sharp contrast to wild type littermates. Consistently *in vivo* markers of osteoblastic activity as well as bone formation rate were found increased in $ERR\alpha^{-/-}$ animals as compared to wild type counterparts. This altogether suggests that bone loss induced by ageing or estrogen-deficiency is compensated for by a maintained or increased osteoblast activity in $ERR\alpha^{-/-}$ mice. Pre-osteoblasts from originating from these animals indeed displayed an enhanced differentiation capacity *ex vivo*, as evidenced by increased expression of Runx2 (the master gene for osteoblast differentiation) and downstream functional markers (e.g. alkaline phosphatase, collagen I and osteocalcin). Cotransfection studies have suggested a complex effect of $ERR\alpha$ on Runx2 promoter activity

(Kammerer et al., 2013). Indeed the receptor may act positively or negatively depending on the presence of PGC-1 α or β as well as on the expression and activity of the Estrogen Receptor. A similar non-monotonous effect of ERR α has been proposed on Wnt signaling, which could be repressed or activated, depending on the expression of co-factors (e.g. PGC-1 family members) (Auld et al., 2012). Although these results have not been verified *in vivo*, they together suggest a bimodal effect of the receptor which could fine-tune osteoblast differentiation (far from an all-or-nothing effect). This might depend on the specific expression and/or mode of co-factor recruitment that will drive the target-specific activity of ERR α . In addition, it is possible that the *in vivo* consequences of these subtle effects are to be observed only in the long term, consistent with the appearance of a bone phenotype in ageing, but not young, ERR α ^{-/-} mice.

The above results were obtained using “complete” knock out models, *i.e.* where ERR α is inactivated in all cells including in mesenchymal stem cells and progenitors, suggesting an effect of the receptor during the early phases of osteoblast differentiation. The role of ERR α in late osteoblast differentiation has also been addressed using a conditional knock out (cKO) model in which the receptor is only inactivated after the onset of osteoblast maturation (Gallet et al., 2013). *Ex vivo*, Runx2 activity (but not expression) is enhanced in differentiating ERR α cKO pre-osteoblasts as compared to wild type, resulting in increased terminal differentiation, including in terms of mineralizing capacities. Interestingly ERR α cKO females resist to bone loss induced by estrogen-deficiency but not by ageing. The molecular mechanisms through which osteoblasts maturation is affected may also involve osteopontin (*opn*), a positive direct target of ERR α in various cell types, including osteoblasts (Vanacker et al., 1998, 1999; Delhon et al., 2009; Teyssier et al., 2009; Zirnbigl et al., 2008, 2013; Boudjadi et al., 2013). Indeed *opn* inhibits mineralization and its inactivation *in vivo* leads to resistance to the bone loss induced by estrogen deficiency (Yoshitake et al., 1999; Boskey et al., 2002).

Together these data show that ERR α negatively impacts osteoblast differentiation at two different defined stages (onset and maturation), resulting in two distinct modes of resistance to bone loss (ageing and estrogen-deficiency). However, future studies are needed to precisely tackle the molecular mechanisms driving these time-specific activities in osteoblasts.

Due to perinatal death ERR γ ^{-/-} mice cannot be studied for their post-embryonic bone phenotype (Alaynick et al., 2007). However a recent publication showed that ERR γ ^{+/-} displayed increased trabecular bone as compared to wild type animals (Cardelli and Aubin, 2014). Strikingly this phenotype only affected males but not females. Intriguingly an opposite

gender-dependent effect has been observed in the case of $ERR\alpha^{-/-}$ mice, where only females, but not males, are resistant to ageing (Teyssier et al., 2009). Although these phenotypic differences have not been confirmed by an independent study using a different $ERR\alpha^{-/-}$ mouse strain (Delhon et al., 2009), these data altogether suggest intricate levels of interferences between ERRs and sex-hormone signaling. Increased trabecular bone in $ERR\gamma^{+/-}$ mice was readily observed in 8 week-old animals (in contrast to $ERR\alpha^{-/-}$) and aggravated with age (Cardelli and Aubin, 2014). Similar to $ERR\alpha^{-/-}$ this phenotype is associated with increased osteoblast number and activity *in vivo* and enhanced differentiation capacities of $ERR\gamma^{+/-}$ *ex vivo*. Interestingly the negative effects of $ERR\gamma$ on osteoblast differentiation appear to rely on inhibition of Runx2 signaling. Indeed $ERR\gamma$ physically interacts with Runx2 and represses its transactivation capacities (Jeong et al., 2009; Cardelli and Aubin, 2014). An alternative mechanism of action has also been suggested where $ERR\gamma$ stimulates the expression of miR-433 (at least in *in vitro* cell cultures), which in turn, induces the degradation of Runx2 mRNA (Kim et al., 2013).

In addition to osteoblasts as bone-forming cells, bone remodeling involves another major cellular component, the osteoclasts, which are bone-resorbing cells of hematopoietic origin. Data have been published concerning the functions of the $ERR\alpha$ on osteoclasts. *In vitro* $ERR\alpha$ does not appear to exert any effect on osteoclast differentiation but promotes spreading and migration of the cells (Bonnelye et al., 2010). However analysis of $ERR\alpha^{-/-}$ mice provided contradictory results: deletion of $ERR\alpha$ *in vivo* may or may not reduce osteoclast differentiation and activity (Delhon et al., 2009; Teyssier et al., 2009; Wei et al., 2010). The reason of these discrepancies is unknown. However $ERR\alpha$ may also exert indirect effects on osteoclasts. Indeed overexpression of $ERR\alpha$ in human breast cancer cells induces the expression of osteoprotegerin (an inhibitor of osteoclastogenesis). Xenografting these cells in immunodeficient mice results in decreased osteoclast differentiation (Fradet et al., 2011). No study has, to date, addressed the potential role of $ERR\gamma$ on osteoclasts, but it should be mentioned that $ERR\gamma^{+/-}$ mice do not show any variation in osteoclast activity, as compared to wild type animals (Cardelli and Aubin, 2014).

Conclusions and future prospects.

In cancer cells $ERR\alpha$ and γ appear to act in an opposite manner, respectively promoting and repressing traits of cancer progression. In contrast, the above-mentioned results strongly suggest that $ERR\alpha$ and γ exert rather similar effects on cells from mesenchymal origin, which are summarized on Figure 1. Both receptors indeed promote

adipocyte differentiation and inhibit osteoblast differentiation. This suggests that they may act as switch factors in the choice of differentiation exerted by progenitor cells to engage into one or the other lineage. The inhibition of osteoblastic commitment appears to be exerted in a Runx2-dependent manner, impacting on the expression and/or the activity of this transcription factor. However, this view is challenged by data indicating a possible co-factor dependent effect and non-monotonous effect of ERR α on Runx2 and/or Wnt-targets expression. This last explanation possibly accounts for the delay with which ERR α inactivation in mice leads to an observable bone phenotype. Nevertheless, a general hypothesis that may be proposed from published data is that inactivating ERR α and/or ERR γ should increase bone mass *in vivo* and therefore protect from bone loss, whether originating from ageing or from estrogen-deficiency (*i.e.* after menopause in humans). However, it should be noted that the majority of these data (outside few studies using cultures of human cells) were obtained using rodent models. In the present state of the art it would be therefore hazardous to claim that deactivation of ERR receptors would be beneficial in human bones. Nonetheless it is worth noting that deactivating ERR α , in contrast to ERR γ , is unlikely to lead to adverse effects in cancers, given that receptor rather promotes cancer aggressiveness.

Most studies on the functions of the ERRs on bone have, up to now, focused on the investigating of autonomous effects. The ERRs are however well known as instrumental regulators of energy metabolism. How the metabolic activities of the ERRs in “energetic tissues” impact on bone status and how potential metabolic activities of ERRs within bone cells influence bone physiology remain to be investigated although hypotheses have started to emerge (Bonnelye and Aubin, 2013).

The vast majority of the data describing the effects of the ERR receptors in bone have used a genetic approach to overexpress or inhibit the expression of the receptors and examine the consequences of such a modification. However, if ERR functions in bone formation are well described, how these effects are exerted needs to be understood, including in a temporal manner. Targeting the receptors using a pharmacological approach would however be more convenient. Such a strategy can be envisioned since small compounds have been identified that deactivate or overactivate the ERRs. The effects of such compounds in bone *in vivo* remain to be evaluated.

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LEGEND TO FIGURE

Figure 1: ERRs and mesenchymal cell fate, a summary.

ERR α and γ positively or negatively contribute to the differentiation of mesenchymal cells into the myocyte, adipocyte, chondrocyte and osteoblast lineages. Green and red arrows represent positive and negative actions, respectively, on the considered process or gene.

See text for details and references.

