Aryl hydrocarbon receptor (AhR)-mediated signaling as a critical regulator of skeletal cell biology

Dima W Alhamad1, Husam Bensreti1, Jennifer Dorn1, William D Hill2,3, Mark W Hamrick1 and Meghan E McGee-Lawrence1,4

1Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta University, Augusta, Georgia, USA
2Department of Pathology, Medical University of South Carolina, Charleston, South Carolina, USA
3Ralph H Johnson VA Medical Center, Charleston, South Carolina, USA
4Department of Orthopaedic Surgery, Augusta University, Augusta, Georgia, USA

Correspondence should be addressed to M E McGee-Lawrence: mmcgeelawrence@augusta.edu

Abstract

The aryl hydrocarbon receptor (AhR) has been implicated in regulating skeletal progenitor cells and the activity of bone-forming osteoblasts and bone-resorbing osteoclasts, thereby impacting bone mass and the risk of skeletal fractures. The AhR also plays an important role in the immune system within the skeletal niche and in the differentiation of mesenchymal stem cells into other cell lineages including chondrocytes and adipocytes. This transcription factor responds to environmental pollutants which can act as AhR ligands, initiating or interfering with various signaling cascades to mediate downstream effects, and also responds to endogenous ligands including tryptophan metabolites. This review comprehensively describes the reported roles of the AhR in skeletal cell biology, focusing on mesenchymal stem cells, osteoblasts, and osteoclasts, and discusses how AhR exhibits sexually dimorphic effects in bone. The molecular mechanisms mediating AhR's downstream effects are highlighted to emphasize the potential importance of targeting this signaling cascade in skeletal disorders.

Introduction

Bone is a critical and specialized organ in vertebrates (Su et al. 2019) that plays key roles in movement, internal organ protection, hematopoiesis, and storage of minerals. The function of bone is maintained through highly controlled mechanisms that regulate the activity of bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). Bone modeling describes the process of bone growth achieved through the independent action of osteoblasts and osteoclasts (Langdahl et al. 2016). This process begins during fetal development and is a major contributor to both peak bone mass and overall bone shape (Siddiqui & Partridge 2016). Bone also undergoes constant remodeling activity where osteoblasts and osteoclasts work together to renew, repair, and adapt the tissue as needed in response to various stimuli and signals such as hormones, cytokines, growth factors, and biomechanical forces (Siddiqui & Partridge 2016, Eisa et al. 2020). Skeletal diseases can reflect the effects of inadequate balance between bone formation and resorption activity. In osteoporosis, osteoclast-mediated bone resorption becomes unbalanced with osteoblast-mediated
bone formation, leading to a net decrease in bone mass and a corresponding increase in risk of bone fracture.

Aging is often associated with bone loss and increased skeletal fragility that adversely impact patients’ quality of life (Kim et al. 2019). While many causative factors for osteoporosis exist, one proposed contributor to bone loss with age is activation of a specialized nuclear hormone receptor called the aryl hydrocarbon receptor (AhR) (Refaey et al. 2017, Eisa et al. 2020). This receptor acts as a transcription factor that responds to environmental pollutants and has been implicated in regulatory mechanisms influencing the immune system, liver homeostasis, and metabolic diseases as well as bone (Wright et al. 2017, Eisa et al. 2020, Kondrikov et al. 2020). AhR functions through canonical and non-canonical pathways to regulate these biological processes. Canonical signaling involves the binding of AhR to a nucleotide sequence (5′-GCGGTG-3′) called the xenobiotic response element (XRE), also referred to as dioxin response elements, via molecular mechanisms that have been studied and reviewed extensively in several previous papers (McMillan & Bradfield 2007, Beischlag et al. 2008). However, there are relatively fewer studies describing the process of non-canonical AhR signaling (Jackson et al. 2015, Wright et al. 2017). The latter pathway has been described after failing to observe a readily identifiable XRE sequence in genes responsive to AhR activation (Huang & Elferink 2012, Wright et al. 2017). In fact, AhR has been shown to form complexes with other proteins and interact with a variety of response elements, exhibiting a plethora of effects (Huang & Elferink 2012, Wilson et al. 2013, Jackson et al. 2014, 2015, Wright et al. 2017).

Tryptophan metabolites such as kynurenine are well-known endogenous ligands to the AhR (Lanis et al. 2017). Since these compounds are polar in nature, they gain access to the intracellular compartment through a transporter known as solute carrier transporter 7a5 (SLC7A5), also referred to as large amino acid transporter 1 (Napolitano et al. 2015, Scalise et al. 2018, Sinclair et al. 2018). Once inside the cell, these ligands can exhibit their biological effects either through canonical or non-canonical AhR signaling cascades. Because AhR mediates diverse endogenous functions, its effect on the skeletal system has not been fully described. Therefore, understanding how AhR could affect the balance between bone formation and bone resorption activity paves the way for new therapeutics in the field. The purpose of this review is to explore the effects of signaling through AhR in mechanisms of bone cell activity.

Bone cells

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can differentiate into bone-forming osteoblasts (Stein & Lian 1993). MSC undergoes an initial proliferation phase where genes involved in cell cycle and cell growth (e.g. c-Myc and c-Jun) are highly expressed (Stein & Lian 1993). This phase is accompanied by the initial stages of extracellular matrix production through the expression of proteins including type I collagen, transforming growth factor beta (TGFB), and fibronectin (Stein & Lian 1993). During the early stages of differentiation, these cells begin to express genes associated with an osteoblastic bone cell phenotype including runt-related transcription factor 2 (Runx2) (Stein & Lian 1993, Komori et al. 1997, Korkalainen et al. 2009). The expression of type I collagen persists during differentiation, although extracellular matrix composition changes considerably as the cells mature (Assis-Ribas et al. 2018). Furthermore, during the differentiation phase, proteins related to osteoblast phenotype including alkaline phosphatase, osteocalcin, and osteopontin are synthesized and released in part to promote matrix mineralization (Stein & Lian 1993), providing bone with its unique biochemical properties (Murshed 2018).

Osteoclasts, which resorb bone, are multinucleated cells that arise from hematopoietic stem cells (Boyle et al. 2003). These cells solubilize the organic component of bone matrix by secreting proteases such as cathepsin K, matrix metalloproteinases, and tartrate resistant acid phosphatase (TRAP) (Boyle et al. 2003). They also solubilize the mineral component of bone matrix through the release of acidic hydrogen ions in the form of hydrochloric acid (Roodman 1999). Osteoclast differentiation is initiated by two fundamental molecules: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) (Boyle et al. 2003). M-CSF, which is produced by a variety of cells including osteoblasts and stromal cells (Mun et al. 2020), drives preosteoclast survival, proliferation, and surface expression of RANK (Korkalainen et al. 2009). The binding of RANKL to RANK triggers a series of signaling cascades that results in the fusion of pre-osteoclasts to form polykaryons, which then mature to give rise to functional, bone-resorbing osteoclasts (Boyle et al. 2003). Osteoblast-lineage cells can also secrete osteoprotegerin which serves as a decoy receptor for RANKL, thus blocking RANKL–RANK signaling and suppresses osteoclastogenesis (Boyle et al. 2003).

The coupled action of osteoblasts and osteoclasts is key to bone homeostasis. In addition to being
regulated by endocrine factors, exogenous factors such as environmental chemicals and pollutants influence bone remodeling balance (Iqbal et al. 2013). For instance, cigarette smoke is known to impair bone formation and increase osteoclastic bone resorption (Iqbal et al. 2013). In fact, long-term smoking has been associated with elevated risk of osteoporosis and bone fractures (Ward & Klesges 2001, Al-Bashaireh et al. 2018). Smoke toxicants contain molecules like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin) that adversely affect the bone (Iqbal et al. 2013). Interestingly, TCDD mediates its effect at least in part through activating the AhR which in turn drives the expression of genes involved in bone resorption (Iqbal et al. 2013). The role of AhR in bone health has been investigated by several research groups (Herlin et al. 2013, Tong et al. 2017, Eisa et al. 2020), but its exact action remains elusive due to the diversity of cell type specific effects.

**AhR signaling and regulation**

AhR is a ligand-activated transcription factor involved in the response to environmental pollutants and chemicals such as aromatic hydrocarbons (Swedenborg & Pongratz 2010). In addition, it plays a role in a variety of biological processes like drug metabolism (Ramadoss et al. 2005), immune regulation (Stevens et al. 2009), and cardiovascular activity (Zhu et al. 2019). In the absence of a ligand, AhR is present in the cytoplasm in a chaperone complex consisting of heat shock protein 90 (HSP90), P23, aryl hydrocarbon receptor-associated 9 (ARA9), and c-SRC (Carambia & Schuran 2021). This complex ensures that AhR is properly folded and maintained in the correct three-dimensional confirmation that allows ligand interaction (Stevens et al. 2009). Upon agonist binding to AhR, a conformational change is induced which exposes the nuclear localization signal and allows the AhR-chaperone complex to translocate to the nucleus (Stevens et al. 2009). In the nucleus, the chaperone complex disassembles and AhR forms a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT) to generate an activated transcription factor (Stevens et al. 2009). Several co-activators can be recruited to the new complex facilitating its binding to an XRE sequence (Stevens et al. 2009). This binding triggers the expression of AhR target genes such as cytochrome P450 family 1 subfamily A member 1 (CYP1A1) and subfamily B member 1 (CYP1B1) (Zhu et al. 2019). The process described above is referred to as canonical AhR signaling (Fig. 1).

Another pathway for AhR-mediated effects is through non-canonical signaling, where AhR forms a complex with other molecules allowing it to bind to genes lacking XRE sequences (Wright et al. 2017) (Fig. 1). A non-consensus xenobiotic response element (NC-XRE) was first described by Huang and Elferink in 2012 (Huang & Elferink 2012), consisting of a 5'-GGGA-3' tetranucleotide repeat. Subsequent studies demonstrated that this site utilizes Kruppel-like factor 6 (KLF6) as a binding partner rather than ARNT (Huang & Elferink 2012, Wilson et al. 2013, Jackson et al. 2015) and that target genes regulated through this mechanism include PAI-1 (Huang & Elferink 2012) and p21<sup>cip1</sup> (Jackson et al. 2014).

The AhR-ARNT dimer can directly interact with estrogen receptor (ESR) to create a transcriptionally active complex that binds the estrogen response element (Ohtake et al. 2003, Jackson et al. 2015). AhR can also regulate the...
cell cycle after complexing with S phase proteins including E2F, p300, and retinoblastoma (Puga et al. 2000, Marlowe et al. 2004). Accumulating evidence also demonstrates that AhR directly interacts with RelB to regulate NFKB target genes (Vogel et al. 2007). In addition to the genomic effects induced by AhR, it can act through non-genomic manner to control biological processes (Carambia & Schuran 2021). For example, ligand binding triggers the dissociation of the chaperone complex and releases c-SRC, allowing c-SRC to act as a protein kinase that phosphorylates downstream proteins (Carambia & Schuran 2021). One effect of this c-SRC activity is increased focal adhesion kinase activation that can lead to increased cellular adhesion and decreased migration (Larigot et al. 2018). Ligand binding to AhR can also increase intracellular calcium, which when coupled with its c-SRC activation ability promotes cyclooxygenase 2 and arachidonic acid production leading to inflammation (Larigot et al. 2018). In addition, AhR-ligand-binding can promote the degradation of indolamine-2,3-dioxygenase 1 (IDO1) by generating E3 ubiquitin ligase complex (Pallotta et al. 2014). IDO1 is responsible for the conversion of tryptophan to kynurenine and has been implicated in inflammatory processes (Pallotta et al. 2014).

AhR can be activated by a wide range of endogenous and exogenous ligands (Denison & Nagy 2003), and it is important to note that the downstream signaling mediated by AhR upon binding these molecules is both ligand- and cell-type dependent (Safe et al. 2020). Smoke toxicants such as TCDD and benzo [α] pyrene (BaP) are common AhR agonists that have been reported to influence the skeletal and immune systems (Table 1) (Iqbal et al. 2013). However, to our knowledge, less is known about the skeletal effects of non-classical AhR agonists such as thiabendazole (Seidel et al. 2001, Iqbal et al. 2013). Furthermore, proton pump inhibitors like omeprazole have been described as AhR agonists in breast cancer cells, but their influence on bone also occurs through non-AhR-mediated effects such as blocking acid secretion and hindering calcium absorption (Hyun et al. 2010, Jin et al. 2014).

AhR activity can be downregulated by negative feedback and proteasomal degradation (Davarinos & Pollenz 1999, Mimura et al. 1999). When the AhR/ARNT heterodimer binds an XRE, it induces the expression of AhR repressor (AHRR), which is a polypeptide structurally similar to AhR but carries a potent transcriptional repressor domain (Mimura et al. 1999). AHRR can dimerize with ARNT and reduce AhR transcriptional activity by competing for XRE binding (Mimura et al. 1999). Similarly, CYP1A1 and CYP1A2 are directly activated by AhR to promote the degradation of AhR ligands and limit the activity of this pathway (Carambia & Schuran 2021). Another mechanism to reduce AhR activity is through proteasomal degradation of the ligand-activated AhR after being exported from the nuclear compartment (Davarinos & Pollenz 1999). In addition to the processes described above, AhR-mediated signaling activity can also be epigenetically regulated via mechanisms including changes in DNA methylation,

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<th>Compound</th>
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<td><strong>Agonists</strong></td>
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<td>Kynurenine</td>
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<td>BAY 2416964</td>
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<td>CH-223191</td>
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<td>GNF351</td>
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Growing evidence indicates that AhR signaling interacts with critical cellular pathways including vascular endothelial growth factor (Takeuchi et al. 2009), WNT (Arinze et al. 2022), fibroblast growth factor (FGF) (Girer et al. 2016), TGFβ (Gramatzki et al. 2009), toll-like receptor (Kado et al. 2017), the CXCL12/CXCR4 axis (Elmansi et al. 2020), and ESR (Vert & Chory 2011) signaling, among others. While a detailed summary of each of these mechanisms is beyond the scope of this review, clearly these pathways are all well-established regulators of myriad MSC, chondrocyte, osteoblast, and osteoclast functions as reviewed elsewhere (Plotkin & Bruzzaniti 2019, Hallett et al. 2021, Yahara et al. 2022), and accordingly AhR crosstalk with these pathways may drive downstream cellular responses. However, the relative impact of signaling directly mediated by AhR as compared to its modulation of other pathways remains to be elucidated in discrete skeletal cell populations.

**Role of AhR in MSCs**

MSCs constitute a heterogenous population of cells that can differentiate into several mesodermal cell lineages such as chondrocytes, adipocytes, and osteoblasts (Abney & Galipeau 2021). The AhR is strongly expressed in MSCs (Huang et al. 2022), and many independent reports indicate that AhR-mediated signaling inhibits MSC proliferation (Zhou et al. 2017, Jia et al. 2021, Huang et al. 2022). Human bone marrow MSC proliferation was inhibited by AhR ligand kynurenine and stimulated by AhR inhibitor SR1 in a concentration and time-dependent manner (Jia et al. 2021); proliferation was also stimulated through siRNA-mediated knockdown of AhR expression, which protected against the inhibitory effects of kynurenine (Jia et al. 2021). The cigarette smoke toxicant BaP decreased the proliferation and self-renewal of rat MSCs, comparable to the reduced proliferation rate seen in MSCs isolated from human smokers as compared to non-smokers (Zhou et al. 2017). However, some disagreement exists, as the AhR ligand FICZ did not affect rat bone marrow MSC proliferation (Huang et al. 2022). Other cellular processes relating to cell survival are also affected by AhR signaling in MSC, for instance, kynurenine disrupted autophagy and promoted senescence in murine bone marrow stromal cell (BMSC) in an AhR-dependent manner (Kondrikov et al. 2020).

MSCs play a role in a variety of biological processes including inflammation (Abney & Galipeau 2021), and the AhR is implicated in the anti-inflammatory effect of MSCs (de Almeida et al. 2017, Abney & Galipeau 2021). Upon tissue injury, MSCs migrate to the affected site to modulate inflammatory response (Ou-Yang et al. 2011). Xu et al. demonstrated that AhR knockout mice display heightened lung inflammation following allergen exposure due to impaired migratory potential of MSCs (Xu et al. 2015). Mechanistically, after MSCs arrive to the site of injury, they induce a switch in macrophage phenotype from the proinflammatory M1 to the anti-inflammatory M2 (Cui et al. 2020) and pharmacological inhibition of AhR by CH223191 reduced the polarization of macrophages from M1 to M2 phenotype and exacerbated inflammation (Cui et al. 2020). Moreover, AhR signaling affected the ability of MSCs to produce soluble cytokines (Jensen et al. 2003). This was revealed when an AhR-expressing bone marrow stromal cell line (BMS2) was treated with the AhR agonists 7,12-dimethylibenz [a]anthracene (DMBA) and TCDD, both of which reduced NFκB levels (Jensen et al. 2003). Since the interleukin-6 (IL6) promoter features NFκB binding sites, the reduction of NFκB reduced the expression of IL6, and lipopolysaccharide-induced increases in IL6 levels were blunted in the presence of DMBA and TCDD (Jensen et al. 2003). In addition, the AhR antagonists DMF and CH-223191 inhibited kynurenine-induced downregulation of gene and protein levels of the inflammatory and multifunctional cytokine CXCL12 in human and murine BMSCs (Elmansi et al. 2020). Collectively, these effects indicate that AhR stimulation directly affects the inflammatory responses of MSCs.

The well-regulated expression of transcription factors is critical for MSCs differentiation into different cell lineage including osteoblasts (which will be described in the next section) and adipocytes, and disruptions in the expression of transcription factors can therefore compromise MSCs differentiation capacity. With regards to adipocyte commitment of MSCs, the AhR ligand BaP was administered to human bone marrow-derived MSCs and suppressed adipogenic differentiation (Podechard et al. 2009) by reducing mRNA expression of CCAAT/ enhancer-binding protein beta (CEBPB) and peroxisome proliferator-activated receptor γ (PPARG), key transcription factors for adipogenesis (Podechard et al. 2009). BaP also repressed expression of adipocyte markers like fatty acid-binding protein-4 (FABP4), whereas the AhR antagonist α-napthflavone successfully counteracted BaP’s inhibitory effects on adipogenesis (Podechard et al. 2009). A similar study reported that treating canine MSCs with BaP inhibited adipocyte differentiation by activating AhR and downregulating PPARG (Rathore & Cekanova 2015). Intriguingly, in vivo administration of the AhR ligand...
TCDD also decreased bone marrow adiposity (Fader et al. 2018).

**Role of AhR in osteoblasts**

Several studies suggest that activation of AhR by environmental toxicants like TCDD and BaP impedes the differentiation of MSCs into osteoblasts (Fig. 2) (Korkalainen et al. 2009, Tong et al. 2017, Zhou et al. 2017, Watson et al. 2019). For instance, TCDD-treated MSCs showed a dose-dependent decrease in mRNA levels of osteoblastic markers such as Runx2, osteocalcin, and alkaline phosphatase, mediated through inhibiting β-catenin expression (Tong et al. 2017). Zhou et al. verified that BaP inhibited alkaline phosphatase and matrix mineralization of MSCs and showed that *in vitro* treatment with BaP inhibited TGFβ1/SMAD4 and TGFβ/ERK/AKT signaling pathways (Zhou et al. 2017). Korkalainen et al. treated mice and rats with TCDD and isolated MSCs from bone marrow of tibia and femur, monitoring the progress of osteoblastic differentiation in these cells (Korkalainen et al. 2009). Their quantitative real time-PCR results showed reduced mRNA levels of Runx2, osteocalcin, and alkaline phosphatase, suggesting impaired osteoblastic differentiation (Korkalainen et al. 2009). To directly test the role of the AhR in these mechanisms, the authors repeated several experiments in AhR-knockout mice and found that the impaired osteoblastic differentiation attributed to TCDD was rescued in the knockout strain (Korkalainen et al. 2009). In another study, skeletal changes including decreased cortical thickness and elevated cortical bone porosity were evident after 10 weeks of TCDD treatment (Herlin et al. 2013). Bone matrix was harder after TCDD treatment, suggesting increased matrix brittleness in TCDD treated mice comparable to what is seen in older bone with impaired osteogenesis (Herlin et al. 2013). A suppressive role of AhR activation on osteoblast differentiation was also evident in human-derived MSCs (Watson et al. 2019), where TCDD treatment suppressed alkaline phosphatase activity, matrix mineralization, and expression of the osteoblastic differentiation in these cells (Korkalainen et al. 2009). Their quantitative real time-PCR results showed reduced mRNA levels of Runx2, osteocalcin, and alkaline phosphatase, suggesting impaired osteoblastic differentiation (Korkalainen et al. 2009). To directly test the role of the AhR in these mechanisms, the authors repeated several experiments in AhR-knockout mice and found that the impaired osteoblastic differentiation attributed to TCDD was rescued in the knockout strain (Korkalainen et al. 2009). In another study, skeletal changes including decreased cortical thickness and elevated cortical bone porosity were evident after 10 weeks of TCDD treatment (Herlin et al. 2013). Bone matrix was harder after TCDD treatment, suggesting increased matrix brittleness in TCDD treated mice comparable to what is seen in older bone with impaired osteogenesis (Herlin et al. 2013). A suppressive role of AhR activation on osteoblast differentiation was also evident in human-derived MSCs (Watson et al. 2019), where TCDD treatment suppressed alkaline phosphatase activity, matrix mineralization, and expression of the osteoblastic differentiation in these cells (Korkalainen et al. 2009). Their quantitative real time-PCR results showed reduced mRNA levels of Runx2, osteocalcin, and alkaline phosphatase, suggesting impaired osteoblastic differentiation (Korkalainen et al. 2009). To directly test the role of the AhR in these mechanisms, the authors repeated several experiments in AhR-knockout mice and found that the impaired osteoblastic differentiation attributed to TCDD was rescued in the knockout strain (Korkalainen et al. 2009). In another study, skeletal changes including decreased cortical thickness and elevated cortical bone porosity were evident after 10 weeks of TCDD treatment (Herlin et al. 2013). Bone matrix was harder after TCDD treatment, suggesting increased matrix brittleness in TCDD treated mice comparable to what is seen in older bone with impaired osteogenesis (Herlin et al. 2013). A suppressive role of AhR activation on osteoblast differentiation was also evident in human-derived MSCs (Watson et al. 2019), where TCDD treatment suppressed alkaline phosphatase activity, matrix mineralization, and expression of the
transcription factor DLX5 and the osteogenic markers osteopontin and integrin-binding sialoprotein (Watson et al. 2019). Fibroblast growth factor 9 (FGF9) and FGF18 expression, known to inhibit MSC differentiation into osteoblasts, were upregulated by TCDD treatment (Watson et al. 2019). Blocking AhR with GNF351 attenuated TCDD-induced effects on matrix mineralization and rescued expression of genes related to extracellular matrix, osteogenic regulation, and maintenance of multipotency (Watson et al. 2019).

Surprisingly, however, at least two studies have shown a net benefit of TCDD treatment on bone (Herlin et al. 2013, Fader et al. 2018), resulting in part from increasing osteoblasts (Fader et al. 2018) (Fig. 2). Likewise, treatment of MC3T3-E1 pre-osteoblasts with the endogenous AhR ligand 6-formylindolo[3,2-b] carbazole (FICZ) accelerated osteoblastic differentiation and expression of osteoblastic genes including alkaline phosphatase, osteocalcin, and type 1 collagen (Yoshikawa et al. 2021). Moreover, in vivo administration of FICZ increased bone formation rate and mineral apposition rate in WT mice (Yoshikawa et al. 2021). Similarly, a recent report by Huang et al. demonstrated that FICZ treatment promoted osteogenic differentiation of rat bone marrow stromal cells (Huang et al. 2022). However, kynurenine (another endogenous AhR ligand) inhibited differentiation of BMSC-derived osteoblasts, reducing mineralized matrix production by these cells, and also decreased dynamic indices of bone mineralization activity (Refaey et al. 2017). These studies highlight the critical point that AhR-mediated signaling can be ligand-dependent, with different ligands capable of producing opposite phenotypic responses both in vivo and in vitro in a cell type-specific manner, as previously reviewed (Safe et al. 2020) (Fig. 2). Many studies have also demonstrated that the effect of AhR can be species dependent (Unkila et al. 1995, Karchner et al. 2006, Xu et al. 2021). One such example is the distinctive sensitivity to TCDD among mammals, fish, toads, and birds (Xu et al. 2021). Among all vertebrates, the most sensitive species to TCDD are fish, while toads and frogs are considered the least sensitive (Peterson et al. 1993, Xu et al. 2021). Intraspecies variability is also observed, with 120-fold difference in the response to TCDD between two fish species: bull trout (Salvelinus confluentus) and zebrafish (Danio rerio) (King-Heiden et al. 2012). Mammals exhibit differential responses to TCDD as well, for example, rats and hamsters demonstrate teratogenic effects upon exposure to this agent, but this impact is undetectable in guinea pigs (Kransler et al. 2007).

Discrepancies in the sensitivity of different species to AhR-mediated signaling could be impacted by differing primary structures of the AhR ligand-binding domain among species, where small amino acid variations in this domain greatly alter the extent of AhR activation (Xu et al. 2021). Supporting this idea is a site directed mutagenesis study demonstrating that TCDD binding affinity was reduced upon replacing Ala375 with Val (Poland et al. 1994). Changes in the primary structure of AhR affect ligand and coregulator interactions, likely contributing to varied downstream responses (Pandini et al. 2007). Many different transcription factors and coregulators can bind to AhR depending on its microenvironment (Gargaro et al. 2021). For instance, AhR can either directly interact with signal transducers and activator of transcription (STAT) to regulate proinflammatory responses, or it can form a complex with STAT which then binds to NFκB, suppressing the transcription of inflammatory mediators (Kimura et al. 2009). It has also been reported that a ligand-bound AhR/ARNT complex interacts with hypo-phosphorylated retinoblastoma protein which inhibits the cell cycle transition from G1 to S phase (Puga et al. 2000). Through this interaction, cell cycle progression and cellular proliferation are slowed down by AhR in humans and rats (Gargaro et al. 2021). Taken together, these findings suggest that different coregulators of AhR present in different species could promote signaling through various AhR pathways to drive the expression of distinct gene sets.

Tissue-targeted genetic models used to directly investigate the role of AhR in osteoblasts are sparsely reported in the literature. AhR-floxed mice crossed with α1(I)-Collagen-Cre were developed to investigate the effects of osteoblast-targeted AhR knockout, but surprisingly these mice presented with a bone phenotype that was comparable to their WT littermates when examined at young ages (Yu et al. 2014). However, it is worth noting that the detrimental effects of the AhR ligand kynurenine on bone mass are attributed to an aging skeletal phenotype (Refaey et al. 2017), raising the possibility that the role of AhR in osteoblasts is age-dependent. It is surprising that, to our knowledge, the role of AhR in osteocytes (which are terminally differentiated osteoblasts) has not yet been directly tested. Given the importance of osteocytes in the regulation of skeletal mechanobiology, mineral homeostasis, and the regulation of bone remodeling activity, such studies are likely warranted to better understand the overall role of AhR in skeletal biology.

**Role of AhR in osteoclasts**

Osteoclasts are derived from hematopoietic stem cells (HSCS), and several reports have found that genetic deletion
of AhR promotes HSC proliferation (Singh et al. 2014, Bennett et al. 2015, Unnisa et al. 2016) (Fig. 2). The literature presents conflicting findings for the role of AhR in osteoclasts (Fig. 2). Some studies show that AhR stimulation drives osteoclastogenesis and increases bone resorption (Iqbal et al. 2013, Eisa et al. 2020), while others demonstrate that AhR inhibits osteoclastogenesis (Voronov et al. 2005, Jia et al. 2019). Kynurenine is an endogenous metabolite that activates AhR signaling pathway (Opitz et al. 2011), and in fact, AhR is the only known receptor for kynurenine thus far, responsible for mediating many of kynurenine’s effects (Eisa et al. 2020). Therefore, kynurenine-centric studies may be informative regarding understanding AhR’s effects in osteoclasts. However, careful interpretation of these studies is critical as kynurenine has been shown to modulate cell bioenergetics (Pierce et al. 2020), and it is not yet known whether this impact is AhR-dependent. A case-control study on elderly patients investigated relationships between bone marrow kynurenine levels, age, and osteoporosis-related phenotypes (Kim et al. 2019). Interestingly, the results revealed that kynurenine accumulates with age and that patients with fragility hip fractures had 39.7% higher bone marrow kynurenine levels than patients without hip fractures (Kim et al. 2019). Relevant to osteoclasts, the researchers also analyzed bone marrow samples for markers of osteoclast activity like TRAP-5b and RANKL, finding elevated plasma level of these biochemical molecules and reduced bone mass density at the total femur with increasing age (Kim et al. 2019). These results suggest that elevated kynurenine levels (and by extension activating the AhR) could contribute to increased bone resorption and higher fragility observed within the elderly population (Kim et al. 2019). The negative influence of kynurenine on bone density and architecture was also noted in mice fed kynurenine or intraperitoneally injected with the agent (Refaey et al. 2017). Markers of osteoclastic activity such as RANKL and pyridinoline cross-links were elevated in the serum of kynurenine treated mice, and histological markers of osteoclastic activity and bone marrow adiposity were also increased in the treated animals (Refaey et al. 2017). These results suggested that kynurenine signaling (likely mediated through the AhR) is involved in promoting osteoclastic resorption and skeletal aging.

On a mechanistic level, Eisa et al. investigated AhR’s role in the molecular pathway for kynurenine’s effect in osteoclasts in vitro, showing that kynurenine acted through the AhR pathway to enhance RANKL-dependent osteoclastogenesis and bone resorption (Eisa et al. 2020). Cotreatment of Raw 264.7 cells with kynurenine and RANKL increased the mRNA and protein levels of c-FOS and NFATC1, which are critical transcription factors that induce osteoclast differentiation (Eisa et al. 2020). The number of TRAP-positive cells was also elevated after kynurenine/RANKL treatment (Eisa et al. 2020). Pharmacological and genetic blockade of AhR pathway attenuated kynurenine’s effect on osteoclasts, which confirmed the involvement of this cascade in osteoclast biology (Eisa et al. 2020). The function of AhR in osteoclasts has been explored in vivo by using systemic and tissue-specific AhR knockout mice (Yu et al. 2014). When AhR was knocked out systemically (body-wide), mice exhibited greater bone mineral density, trabecular bone connectivity density, trabecular bone volume, and trabecular number compared to WT mice (Yu et al. 2014). Both males and females with whole-body AhR knockout had fewer osteoclasts than WT mice with no changes in mineral apposition rate and bone formation rate, suggesting that skeletal effects of body-wide AhR deletion were primarily mediated through reduced bone resorption (Yu et al. 2014). AhR-floxed mice were then crossed with Cathepsin K-Cre mice to drive AhR conditional knockout in cells of the osteoclast lineage (Yu et al. 2014), which showed that osteoclast-targeted knockout mice had higher bone mass with lower bone resorption (Yu et al. 2014).

There is also contrasting evidence in the literature suggesting that AhR signaling inhibits osteoclast activity. For example, at least one report demonstrates that TCDD treatment of juvenile rodents reduced osteoclastic bone resorption (Fader et al. 2018). Through stimulating the AhR pathway, 3-methylcholanthrene (3MC) repressed the differentiation of osteoclasts via a reducing expression of the pro-osteoclastogenic molecule RANKL expression in osteoblast-lineage ST2 progenitor cells (Naruse et al. 2004). These studies suggested that the AhR pathway might reduce osteoclast differentiation indirectly by affecting osteoclast supporting cells rather than osteoclast precursors (Naruse et al. 2004). However, literature support also exists for the idea that AhR-mediated signaling can directly inhibit osteoclasts. For example, the endogenous AhR ligand FICZ dose-dependently inhibited actin ring formation and pit formation by bone marrow macrophage-derived osteoclasts (Yoshikawa et al. 2021). Voronov et al. demonstrated that the AhR agonist BaP also inhibited osteoclast differentiation and bone resorption (Voronov et al. 2005, 2008), attributed to crosstalk between AhR and NFkB pathways (Voronov et al. 2008). NFkB is a transcription factor that resides in the cytoplasm in complex with NFKBIA, RELA, and NFKB1 (Oeckinghaus & Ghosh 2009). When NFkB pathway is stimulated, IKK phosphorylates NFKBIA and targets it for degradation,
allowing NFkB to translocate to the nucleus and drive gene expression (Oeckinghaus & Ghosh 2009). NFkB is a common transcription factor for AhR and RANKL signaling cascades, therefore stimulating AhR creates a competition for NFkB and limits RANKL-induced osteoclastogenesis (Voronov et al. 2008). Although the exact mechanism for the crosstalk between AhR and RANKL is not clearly understood, it was proposed that AhR can complex with p65 and NFKBIA in the cytoplasm (Voronov et al. 2008). Upon AhR stimulation, AhR could act as a ligand-dependent E3 ubiquitin ligase and activate the IKB kinase complex, driving the translocation of NFkB to the nucleus and subsequent gene expression (Voronov et al. 2008). All together, these events reduce the availability of NFkB for RANKL-mediated signaling, limiting osteoclastogenesis (Voronov et al. 2008).

**Role of AhR in cartilage and endochondral ossification**

Endochondral bone development begins with a cartilaginous template which osteoblasts and osteoclasts then replace with bone tissue. This process is regulated by various endocrine factors including growth hormone and insulin-like growth factor-1 (Cedervall et al. 2015). The AhR has been reported to play a role in cartilage development and subsequent bone growth. For example, AhR expression was suppressed in cartilage templates, but activation of AhR by the endogenous ligand FICZ positively promoted later processes of endochondral ossification (Huang et al. 2022). Cedervall et al. investigated how AhR acts in growth plate chondrocytes to regulate bone elongation, beginning with the finding that AhR was broadly expressed in the growth plate cartilage of human subjects and more highly expressed in hypertrophic as compared to resting chondrocytes (Cedervall et al. 2015). For mechanistic studies, fetal rat metatarsal bones were cultured and monitored for longitudinal growth after exposure to AhR modulators (Cedervall et al. 2015). TCDD did not affect bone growth at the concentrations tested (1 pM –10 nM), but higher dose of the AhR antagonist, α-naphthoflavone, increased chondrocyte apoptosis and suppressed bone growth (Cedervall et al. 2015). It is worth noting that at lower doses (10 pM and 10 nM), α-naphthoflavone did not affect bone growth, and cytotoxic effects may have occurred through AhR-independent mechanisms (Cedervall et al. 2015). The authors therefore concluded that AhR activation may not directly affect endochondral bone growth and that AhR activation in the rat growth plate may require an unknown co-factor to affect cartilage formation (Cedervall et al. 2015). Yang et al. demonstrated that rabbit chondrocytes were exposed to TCDD at a concentration of 10 nM developed elevated levels of reactive oxygen species and nitric oxide (Yang & Lee 2010). This created cellular stress which eventually triggered chondrocyte apoptosis (Yang & Lee 2010). Inhibiting AhR using 10 μM of α-naphthoflavone attenuated these harmful effects of TCDD (Yang & Lee 2010), raising the possibility that apoptosis of chondrocytes driven by AhR activation could lead to cartilage damage and arthritis development (Yang & Lee 2010). In a parallel study, treating Japanese rice fish (medaka) with TCDD (ppt-ppb concentrations) impaired chondrogenesis and osteogenesis (Dong et al. 2012). The recruitment of MSCs to hypural cartilage anlage was attenuated following TCDD treatment, and chondrocyte proliferation and differentiation were also impaired in a dose-dependent manner (Dong et al. 2012). On a molecular level, the expression of type 2 collagen (a major cartilage extracellular matrix protein) was markedly blunted after TCDD treatment (Dong et al. 2012). This effect was mediated through downregulating the key chondrogenic transcription factor, SRY-box transcription factor 9 (Sox9) (Dong et al. 2012). With regards to subsequent bone formation, TCDD treatment attenuated Osterix expression and reduced perichondral ossification within the fish hypural anlage (Dong et al. 2012). Mechanisms of endochondral ossification are initiated postnatally during fracture repair, and, when administered to rats with tibial fractures, the AhR ligand BaP delayed healing and resulted in a less mineralized callus (Zhou et al. 2017). Together, these results imply that AhR activation may negatively influence cartilage and endochondral bone formation.

In addition to AhR’s role in cartilage during developmental and repair-mediated processes of endochondral ossification, AhR may also play a role in diseases affecting cartilage like osteoarthritis. Expression of AhR was upregulated in human osteoarthritic cartilage as compared to intact controls (Klinger et al. 2013) and was also highly expressed in meniscus and subchondral bone from osteoarthritic patients (Chang et al. 2021). The AhR signaling pathway was reported to be more active in osteoarthritic synovial tissues as compared to those from rheumatoid arthritis (Ogando et al. 2016). As with other cell types described above, the effects of AhR-mediated signaling in chondrocytes with respect to osteoarthritis phenotypes are likely ligand-dependent. Exposure to the exogenous AhR ligand BaP led to the development of osteoarthritis-like lesions, loss of proteoglycans, and reduced expression of aggrecan, type 2 collagen, and Sox9 in the articular cartilage of WT (but not AhR-knockout)
mice (Yoshikawa et al. 2021). Interestingly, however, administration of the endogenous AhR ligand FICZ was protective against the development of mechanically induced osteoarthritis in mice (Yoshikawa et al. 2021). Mechanistically, BaP promoted expression of the active forms of caspase-3 and caspase-9 in ATDC5 chondrocyte progenitor cells, and increased expression of cleaved caspase-3 in the mandibular chondrocytes of WT (but not AhR-knockout) mice, but administration of FICZ reduced metrics of apoptosis (Yoshikawa et al. 2021). The beneficial effects of FICZ described above are consistent with a recent bioRxiv report, where intra-articular delivery of an indoleamine 2,3-dioxygenase and galectin-3 fusion protein (IDO-Gal3), intended to drive local expression of the endogenous AhR ligand kynurenine, improved functional outcomes in a rat surgical model of osteoarthritis (Partain et al. 2021).

Sexually dimorphic effects of AhR in bone

Crosstalk between signaling cascades is a common phenomenon driving biological events (Vert & Chory 2011). Some evidence of sexually dimorphic effects of AhR have been reported in bone, which may be due in part to the interaction between AhR and ESR (Wejheden et al. 2010, Tarnow et al. 2017). Wejheden et al. designed a study to explore the long-term effect of exposing mice to AhR ligands (Wejheden et al. 2010). As their experimental model, they studied 3-month-old transgenic mice expressing constitutively active AhR. Results revealed that continuous AhR stimulation reduced bone formation in transgenic female mice but not in males (Wejheden et al. 2010). Bone dimensions, density, and content were notably altered in female mice, while males were minimally affected (Wejheden et al. 2010). Osteoclastic markers such as carboxy-terminal cross-linked telopeptide of type 1 collagen (CTX-1) and osteoclast volume density were increased by more than 60% and the resorption index of CTX-1/TRAP 5b was increased by 90% in females but not in males (Wejheden et al. 2010). On an mRNA level, female but not male mice demonstrated altered expression of osteoclastic and osteoblastic markers in these studies (Wejheden et al. 2010). Similarly, treatment with the endogenous AhR ligand kynurenine was reported to decrease osteoblasts in young female but not young male mice and likewise impaired cellular energetics in BMSC-derived osteoblasts from females but not males (Pierce et al. 2020). Another report demonstrated that TCDD treatment exhibited gender-dependent effect in C57BL/6J mice (Herlin et al. 2013). For instance, bone biomechanical properties were decreased in females following TCDD treatment, but males were not affected (Herlin et al. 2013). Also, unlike males, TCDD-treated females demonstrated unbalanced bone remodeling activity as indicated by lower PINP/CTX-1 ratio (Herlin et al. 2013). In the same report, bone phenotype of both genders was compared between AhR knockout mice and WT mice, showing that AhR deletion resulted in higher trabecular number and trabecular bone volume fraction in both males and females (Herlin et al. 2013). However, female knockout mice had lower cortical area, cortical thickness, and bone mineral density compared to WT females (Herlin et al. 2013), whereas male knockout mice displayed lower plasticity and harder cortical bone matrix than their WT controls (Herlin et al. 2013). In another report, 8-week-old osteoclast-targeted AhR knockout mice were orchiectomized or ovariectomized then monitored for 4 weeks, showing that the osteoclast-targeted AhR knockout mice were resistant to bone loss induced by gonadectomy (Yu et al. 2014). This implied that sex hormones might influence bone remodeling through AhR.

Multiple mechanisms have been proposed for the crosstalk between AhR and ESR (Tarnow et al. 2017). Firstly, AhR could directly interact with ERα to form a complex which binds to AhR target genes (Matthews & Gustafsson 2006). Alternatively, the complex might bind to ERE and inhibit ESR target gene expression (Jackson et al. 2015). The competition for shared co-factors between the two signaling cascades also influences the outcome of AhR activation (Swedeborg & Pongratz 2010). Moreover, AhR could increase proteasomal degradation of ESR through upregulating CYP expression and estrogen metabolism (Wormke et al. 2003). Estrogen has also been reported to alter kynurenine pathway by inhibiting kynurenine aminotransferase enzymes (Jayawickrama et al. 2017). All together, these mechanisms suggest that AhR stimulation might exert antiestrogenic effects in bone (Wejheden et al. 2010). Since estrogen loss usually increases bone resorption, the crosstalk between AhR and ESR could contribute to the higher sensitivity of female mice to AhR activation (Wejheden et al. 2010).

Conclusions

Taken together, the literature suggests that signaling through AhR likely plays an important role in skeletal homeostasis. Several reports have shown that activating AhR reduces osteoblast differentiation and activity
Moreover, it will be critical to ascertain the contributions of endogenous AhR ligands (like kynurenine and other tryptophan metabolites) to skeletal biology, particularly in the context of aging. Although the literature demonstrates that kynurenine activates AhR, the precise mechanism is not yet understood. For example, it is not clear whether kynurenine and/or other tryptophan metabolites activate canonical, non-canonical, or non-genomic modes of AhR signaling, and the specific signaling molecules and enzymes involved in the effect of kynurenine and other tryptophan metabolites on bone cells are not well defined. It has been recognized that the AhR cascade undergoes important crosstalk with other nuclear hormone receptors such as ESR and the glucocorticoid receptor (Widerak et al. 2006, Monostory et al. 2009, Denison et al. 2011). Among those, the crosstalk between AhR and ESR is the best understood, while other interactions are less studied (Denison et al. 2011). Future studies focused on elucidating such molecular interactions would foster better understanding of the role of AhR in musculoskeletal disorders, ultimately promoting the discovery of new, effective therapeutic skeletal agents.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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