

REVIEW

Estrogen receptors in breast and bone: from virtue of remodeling to vileness of metastasis

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Bone metastasis is a prominent cause of morbidity and mortality in cancer. High rates of bone colonization in breast cancer, especially in the subtype expressing estrogen receptors (ERs), suggest tissue-specific proclivities for metastatic tumor formation. The mechanisms behind this subtype-specific organ-tropism remains largely elusive. Interestingly, as the major driver of ER+ breast cancer, ERs also have important roles in bone development and homeostasis. Thus, any agents targeting ER will also inevitably affect the microenvironment, which involves the osteoblasts and osteoclasts. Yet, how such microenvironmental effects are integrated with direct therapeutic responses of cancer cells remain poorly understood. Recent findings on ER mutations, especially their enrichment in bone metastasis, raised even more provocative questions on the role of ER in cancer–bone interaction. In this review, we evaluate the importance of ERs in bone metastasis and discuss new avenues of investigation for bone metastasis treatment based on current knowledge.

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INTRODUCTION

Steroidal and non-steroidal hormones regulate bone formation. Bone supports muscles and shapes vertebrates. Bone rigidity and strength are mostly derived from phosphate and calcium, which are the most enriched minerals during ossification. Multiple factors are involved in bone formation including hormones.^{1,2} They are particularly important for bone development and remodeling in both male and female. For instance, parathyroid hormone (PTH) is critical for bone remodeling and calcium-level adjustment.³ Deficiencies in glucocorticoid, progesterone, androgen as well as estrogen often translate into severe pathological bone conditions.⁴ Sexual dimorphism usually affects hormonal responses between genders. Higher estrogen levels in females accounts for more assessable functions of estrogen receptors (ERs) in bone development and remodeling. Similarly, androgen receptors tend to contribute more to bone formation in males.^{5,6} Reduced availability of estrogen negatively impacts bone mass and strength, which leads to higher risks of bone fractures in postmenopausal women.^{7,8} To palliate osteoporosis, hormone replacement therapies consisting of estrogen alone or in combination with progesterone have been successfully used despite some side effects associated with higher risks of breast cancer development.⁹ Overall, a balanced hormonal production is *sine qua non* to maintaining healthy bones in both males and females.

Osteoprotective role of ERs

Estrogen treatment is well known to protect against bone loss. Most of this effect is mediated by ERs (ER α and ER β), which are highly expressed in osteoblast and osteoclast lineages, suggesting protective functions of ERs in bone.² Osteoblast-specific

ER α -knockout mouse models showed loss of bone mass as well as strength.¹⁰ ER α is more expressed in cortical bone, which suggests predominant roles of the receptor in bone formation. This may also explain bone alterations observed in ER α -knockout mice.¹⁰ ER β is highly expressed in trabecular bone cells, but very few studies have investigated the function of ER β in bone development, remodeling and metastasis.¹¹ Understanding the primary roles of ER α and ER β in bone can open new opportunities to better target postmenopausal and cancer-induced bone loss.

Bone stromal cells construct and remodel the skeleton

The skeleton is a dynamic structure, which is constantly remodeled by several bone cell lineages. Osteoclasts are large multinucleated bone macrophages deriving from monocytes.¹² They degrade bone matrix by creating acidic environments and secreting enzymes such as collagenases.¹² These gaps are often refilled by mature mesenchymal cells called osteoblasts, which secrete bone matrixes to prevent bone loss.¹³ When osteoblasts stay embedded in the bone matrix, they differentiate into osteocytes that interconnect with each other. They are believed to have mechanosensory functions, which allow them to control bone remodeling.¹⁴ Hence, maintaining a good balance between osteoclast and osteoblast activity is primordial for healthy bone formation. Although, significant progress has been made to better understand the function of bone stromal cells during bone development and remodeling, their role in breast cancer bone metastasis still remains unclear. The finer appreciation of how bone stromal cells influence cancer dissemination may allow better prevention and treatment of bone metastasis.

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Luminal breast cancers highly metastasize to the bone

Metastasis is the primary cause of cancer-related death. Cancer cells can migrate in group (collective migration) or individually (single-cell migration) to invade the stroma.^{15,16} Single-cell cancer metastasis has been more scrutinized. It involves multiple steps including epithelial to mesenchymal transition (EMT), which allows invasion of blood vessels and lymphatic system (intravasation).^{3,17} Circulating tumor cells can then migrate to other organs, extravasate and seek for hospitable environments with higher chances of survival.^{18–20} Disseminated tumor cells can stay latent for years before forming secondary tumors.¹⁷ Despite significant improvement in understanding the mechanism of cell dormancy, more research is needed before we can efficiently target latent cells.

Several types of cancer can metastasize to skeletal bone.²¹ Intriguingly, bone is the preferred metastatic niche for a few cancers including breast cancer.^{3,17,22} It is still unclear why such selectivity is seen for bone. It is plausible that bone offers a better microenvironment for tumor survival.^{23,24} However, this answer may not be sufficient as breast cancer subtypes display different trends of migration to bone.²⁵ Clinical evidence shows that luminal breast cancers have a higher selectivity for bone metastasis when compared with other breast cancer subtypes,²⁵ such as hormone receptor-negative breast cancers, which tend to metastasize to visceral organs.²⁶ Thus, factors intrinsic to breast tumor subtypes could determine their bone metastatic potential.

With the majority of breast cancer being luminal subtype, ER α has been the predominant targeted nuclear receptor for breast cancer treatment. To inhibit cancer progression, selective ER modulators, including tamoxifen have been commonly used. Although selective ER modulators antagonize ER α function in breast, they may also impede its activity in other tissues such as bone. The resulting bone loss observed in patients undergoing adjuvant therapy, strongly implies the need for functional ERs in bone. More importantly, such hormonal therapy may indirectly affect ER α -positive cancer progression via altering activities of osteoblasts and osteoclasts. However, this microenvironmental effect remains to be investigated.

In this review, we summarize advancements in bone development and remodeling in connection with bone metastasis, and highlight the role of ERs in both of these processes. Although the primary breast cancer targets, ERs, are significantly expressed in bone, their roles in bone metastasis is still largely equivocal. An integrative understanding of ERs in both bone and cancer cells will be a strong asset toward developing new approaches to prevent and cure metastasis, while maintaining the health and strength of skeletal bone.

ERs ARE NECESSARY FOR BONE DEVELOPMENT AND REMODELING

Estrogen and ERs in bone development

Steroid hormones such as estrogen are necessary for normal development of bone. Two main receptors, identified as ER α and ER β , mediate the effects of estrogen. ERs are structurally highly similar, but they appear to have diverse functions.^{7,10,27} *In vivo* ER knockout mouse models clearly demonstrated that ERs were required for development and maintenance of reproductive organs.²⁸ Considering the importance for estrogen for bone formation, it is plausible that ERs affect bone formation via their effects on ovaries, which are the primary sources of estrogen production in females. Along with reproductive organ alterations, many ER knockout mouse models exhibited bone alterations.^{29,30} ER α and ER β had opposite effects on longitudinal bone growth. ER β knockout (ER β KO) mice had longer bone compared with ER α knockout (ER α KO) mice. Interestingly, double-knockout mice had an intermediate bone size, suggesting inhibitory roles of ER β on

ER α -induced bone growth. In his study, Lindberg did not find significant differences in trabecular bone mineral density (BMD) between ER α KO and ER β KO mice.³⁰ However, other studies found that ER β KO mice had a higher trabecular BMD when compared with ER α KO mice.^{29,31} Similarly, 12 months old ER β KO mice had higher BMD than wild-type mice in both cortical and trabecular bone.²⁹ Overall, these results indicate important functions for ER β in trabecular bone formation and suggest differential activities between ER α and ER β in bone tissues (Figure 1a).

Mechanical loading also increases trabecular BMD in ER β KO but not in ER α KO, suggesting that ER β may inhibit BMD in cancellous bone.^{32,33} Inversely, bone stiffness was increased in female ER β KO mice as a consequence of enhanced periosteal formation under strain. This observation contrasts with ER α KO mice. *In vitro* studies found that osteoblasts derived from ER β KO periosteal bone can divide in response to mechanical strain, but not ER α KO osteoblasts. Despite possible redundancies between ER α and ER β in bone, ER knockout mouse models have helped identify opposite functions between the two ER isoforms.^{32,34–36} The predominance of either receptor during bone formation may be a determinant for the outcome.

ERs are expressed in bone stromal cells

ER α and ER β are well expressed in bone tissues.³⁷ Interestingly, Bord et al.³³ reported a differential expression of ERs in osteoblast and osteoclast lineage cells according to bone histological studies. Although ER α was highly expressed in cortical bone, ER β was predominant in trabecular bone, suggesting that they may have different functions in these tissues (Figure 1a). A change in ER expression was also observed during osteoclast and osteoblast maturation processes (Figures 1b and c). ER α detection was almost limited to pre-osteoclast and pre-osteoblast lineages as low expression was observed in mature cells. These results indicate that ER α may be involved in cell differentiation, but not required for the activity of mature osteoblasts and osteoclasts. In contrast to ER α , ER β expression remained consistent throughout osteoblast differentiation, which strengthens the hypothesis that ERs have different roles during osteogenesis.

Aging also affects ER expression in bone (Figures 1b and c). Using human callus-derived biopsies, Batra et al.³⁸ found that most bone stromal cells, including proliferative chondrocytes, were expressing both ER isoforms. No gender difference was observed between patients under age 40. However, in women close to menopause, both ER α and ER β expression levels were considerably decreased in osteocytes. In osteoblasts and mesenchymal cells, while ER α remained constant, ER β expression was lower. In men above age 40, ER β expression rate was reduced only in mesenchymal cells.³⁸ It is known that bone fracture repair is more challenging and requires more time in older individuals. This corroborates with age-related decrease of ER expression, suggesting possible involvement of ERs in bone repair processes. Yet, the mechanism of ER activity in bone metastasis remains to be determined.

MECHANISMS INVOLVED IN BONE FORMATION

Osteoclast lineage

Osteoclasts are bone macrophages responsible for bone resorption.³⁹ Their activity is often increased under hypocalcemia to rescue blood calcium level. Several factors regulate osteoclastogenesis.^{12,40,41} Macrophage-colony stimulating factor induces expression of a receptor activator of nuclear factor kappa-B (RANK). RANK ligand (RANKL) and osteoprotegerin competitively bind RANK to promote and oppose osteoclastogenesis, respectively.^{42–45} These ligands are mostly secreted by osteoblasts but may also derive from osteocytes and T cells.¹³ RANKL autocrine secretion by tumors has also been proposed, but it is

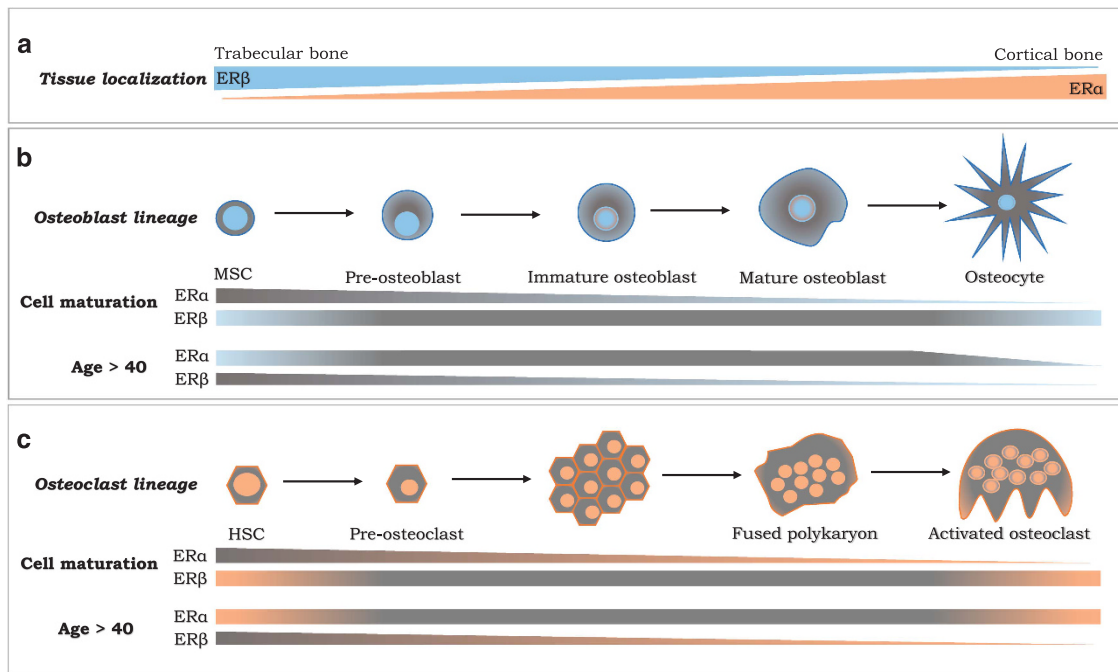


Figure 1. Differential expression of ERs in bone stromal cells. **(a)** Tissue-specific comparison of ER α (orange) and ER β (blue) expression in trabecular bone versus cortical bone. **(b)** Shows osteoblast lineage (top), with high detection of ER α in mesenchymal stem cells (MSC) and pre-osteoclasts, but with decreased expression in mature osteoblasts and osteocytes. ER β expression is maintained throughout the maturation cycle (middle). Disparate expression of ERs in females aged 40 or older (age > 40) (bottom) showing expression of ER α in all cells except osteocytes and decreased levels of ER β during osteoblast differentiation. **(c)** Osteoclast lineage (top) with ER α and ER β expression during cell maturation (middle) and based on aging.

still uncertain whether these ligands will be enough to activate osteolytic lesions.³ Activated RANK promotes nuclear factor kappa-B, as well as p38 mitogen-activated protein kinase signaling.^{46,47} Downstream effectors such as nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 mediate transcription of tartrate-resistant acid phosphatase and cathepsin K.^{48,49} These enzymes are crucial for bone resorption by osteoclasts. Others factors control osteoclast differentiation, which is based on fusion of multiple monocytes into mature osteoclasts. This differentiation process involves dendritic cell-specific transmembrane protein, a key transmembrane protein required for multinucleated osteoclast formation.⁵⁰ Dendritic cell-specific transmembrane protein-depleted monocytes do not differentiate into mature osteoclasts, which often leads to osteopetrosis. In addition to osteoclast differentiation factors, estrogen was found to affect osteoclast survival through ER α . In fact, ER α -knockout mice had less apoptotic osteoclasts than control mice when estrogen was supplemented. The authors proposed that ER α was transcriptionally upregulating the pro-apoptotic factor Fas ligands in osteoclasts.³¹ Although the function of ER β was not specifically addressed, this finding gave a better insight into osteoprotective roles of ERs in bones.

Osteoblast lineage

Osteoblasts have three different fates. They either undergo apoptosis, become lining cells or embed themselves in bone matrix. Osteoblast-specific ER α -depleted mice develop abnormal bone mass and strength.⁵¹ This observation stipulates that ER α osteoprotective effect may be mediated by its role in osteoblasts. Osteoblast maturation from mesenchymal stem cells is regulated by multiple factors some of which are Runt-related transcription factor 2, activating transcription factor 4, Osterix, Twist, activator protein 1 and specificity protein 3.^{52,53}

Several of these factors are involved in ER signaling. In fact, ERs often bind to activator protein 1 and Sp1 sites to regulate gene transcription in an ERE-independent manner.⁵⁴ ER β , in particular, was shown to regulate many genes without estrogen requirement through interaction with other transcription factors including nuclear factor kappa-B, Sp1, activator protein 1 and p53.⁵⁵ Although the mechanism of ER transcriptional activity is not fully understood, it is possible that ERs regulate bone matrix formation by affecting key factors involved in osteoblast differentiation and maturation.

In vitro studies showed that ER activation could enhance osteoprogenitor and RANKL expression in estrogen-treated osteoblasts.⁵⁶ More, the use of ER antagonist ICI-182,780 was able to abolish osteoprogenitor/RANKL production, indicating that ERs mediate their transcription.⁵⁶ Further, the inhibitory effect of Twist in osteoblast differentiation negatively correlates with ERs function in bone matrix production.⁵⁷ In fact, ERs are known to regulate Twist-dependent metastasis in cancer, as well as many other factors including Snail and Zeb1/2. These factors are highly involved in bone development, but also in EMT.⁵⁸ Hence, it is possible that ERs effect on some of these EMT factors induces osteoblast differentiation, which promotes bone formation.

Literally, skeletal bone formation by osteoblasts involves a multi-step procedure. Type 1 collagen is secreted along with other proteins including osteopontin (OPN) and osteocalcin to form the organic matrix called osteoid.⁵⁹ Calcium-phosphate-hydroxide salt (hydroxyapatite matrix) is then added to osteoid allowing bone mineralization. Although this process of bone deposition may be highly influenced by ERs, the mechanisms involved have not been defined.

Osteocyte lineage

The majority of bone stromal cells are osteocytes, which derive from embedded osteoblasts in the bone matrix.⁶⁰ These are long-lasting cells interacting with each other via cytoplasmic

extensions. However, little is understood about osteocyte differentiation and maturation into neuron-like networks. So far, we know that matrix metalloproteinase 14, E11 antigen, dentin matrix protein 1, transforming growth factor (TGF)-beta inducible factor, osteoblast/osteocyte factor 45, Klotho and lysophosphatidic acid, are required for dendritic and canaliculi formation.^{61–66} In addition, oxygen has a protective role in bone formation. Under mechanical strain, hypoxia-inducible factors 1 α was strongly stabilized and found to inhibit anabolic signals in bone.⁶⁷

Mechanical loading and unloading on bone also affect gene transcription in osteocytes, suggesting the hypothesis that osteocytes function as mechanosensors and may regulate bone remodeling.^{68–70} These results may also explain why physical activities maintain stronger and healthier bones.^{71,72} With aging, decreased physical activity often promotes osteocytic senescence causing osteoporosis.⁷³ Reduced osteocyte activity may be one of the drivers of osteopenia (bone loss) observed in spaceflight members. Despite all the evidence involving bone stromal cells in bone development and remodeling, we still do not know much about their mechanism of action. Dextrin matrix protein 1 regulates fibroblast growth factor 23, which is involved in phosphate metabolism in mature osteoblasts.^{61,62} This is supported by clinical evidence showing autosomal recessive hypophosphatemia in patients with Dextrin matrix protein 1 loss-of-function mutations.⁶² Osteocytes can also inhibit Wnt signaling by inducing sclerostin that can bind the Wnt co-receptor LRP5/6, thereby opposing bone formation.⁷⁴ Similarly, osteocytes can activate osteoclast formation through RANKL. We can stipulate from the above studies that osteocytes may serve as messengers between bone stromal cells to protect bone integrity. However, the mechanisms involved in osteocyte activity remain to be clarified.

ERS IN BREAST CANCER

Factors affecting ER status of breast cancer

More than 80% of breast cancers are positive for hormone receptor.⁷⁵ Epidemiological studies have identified factors specifically associated with these breast cancer types, including female socioeconomic status, age, age at menarche, gravidity-parity, menopause, body habitus, exposure to exogenous hormones, BRCA mutation and breastfeeding.^{76,77} In general, inverse associations between these factors and negative hormone receptor (ER-PR-) breast cancers are stronger than direct associations with positive hormone receptor subtypes. Higher socioeconomic status has been linked with a higher overall incidence of breast cancer, particularly those expressing ER and/or PR.^{77,78} In regards to race, the highest prevalence of ER- breast cancer (to include the triple negative) has been demonstrated for African-American women, followed by Hispanics and Caucasians.^{77,79,80} Younger women more frequently develop ER- breast cancer.^{75,81–83} Female body habitus assessed by body mass index was positively associated with the risk of developing ER+PR+ breast cancer; this relationship was significantly stronger for overweight and obese compared with normal-weight women.^{77,84} Early age at menarche and late age at menopause have been shown to be independently associated with an increased incidence of ER+ and/or PR+ breast cancer subtypes.^{77,85,86} Nulliparity has been shown to increase the risk for hormone receptor-negative breast cancer.⁸⁷ Parous women with more advanced age at first full-term pregnancy showed significantly higher prevalence of ER+/PR+ subtypes compared with their younger counterparts.⁸⁸ Furthermore, having more children was associated with a lower risk of developing breast cancer expressing hormone receptors. The use of hormonal contraceptives (estrogen-progestin) was linked with a greater incidence of ER+ breast cancer; a similar but weaker association

was observed for menopausal women using hormonal replacement therapy.^{89,90} Women who are BRCA1 gene mutation carriers are at higher risk for developing ER-PR- breast cancer subtypes, whereas those with a BRCA2 gene mutation are more likely to have hormone receptor positive subtypes.^{91,92}

Among the factors affecting hormonal receptor status of breast cancer, lactation may deserve special consideration because of its potential implications to skeletal metastasis of breast cancer. Many studies have reported an inverse relationship between breastfeeding and the overall incidence of breast cancer, particularly with ER-PR- breast cancer.⁹³ A recent meta-analysis of 27 distinct breast cancer studies corroborated a protective effect of breastfeeding against hormone receptor-negative breast cancers.⁹⁴ Ever breastfeeding versus never breastfeeding in parous women was associated with a 10% risk reduction of developing ER-PR- breast cancers when adjusted for age, body mass index, number of full-term pregnancies and family history.⁹⁴ This risk reduction was even twice as strong for the triple-negative breast cancers. Furthermore, the length of breastfeeding demonstrated a dose-response inverse relationship with the incidence of hormone receptor-negative breast cancers.⁹⁴ Women who breastfed for a combined duration of 2 years or more in their lifetime have a significant reduction of developing ER-PR- breast cancers, particularly before menopause. Despite a natural link of lactation with parity, there is an independent 4% reduction in breast cancer risk for every 12 months of breastfeeding, in addition to a 7% reduction for each full-term pregnancy.⁹⁵

The mechanisms by which lactation affects ER-PR- breast cancer subtypes remain unclear. Altered exposure to endogenous hormones, such as low estrogen/progesterone and higher androgen levels, during the process can suppress ER expression, thereby selectively promoting ER- cancer cell induction and/or proliferation.⁹⁶ Nonhormonal mechanisms, including changes in the immune system, alterations in cellular communication, adhesion and apoptosis may also have a role. Furthermore, irreversible changes in the breasts that take place upon lactation and the effects of breast milk within the ducts may provide protective effects against cancer through the cellular and molecular maturation and/or involution of the breast tissue.⁹⁷

Mammary gland and bone homeostasis share common factors

Many factors involved in mammary gland formation and EMT also associate with bone homeostasis and remodeling. For instance, RANK/RANKL signaling, which promotes osteoclast differentiation in bone, has been found to have a major role in lobuloalveolar development during pregnancy.⁹⁸ RANK-knockout mice develop highly apoptotic mammary epithelia associated with R-spondin-mediated inactivation of Wnt signaling, which prevents lobuloalveologenesis.⁹⁸ Mice overexpressing RANK had increased epithelial cell proliferation, less apoptosis and impaired differentiation of lobuloalveolar structures.⁹⁹ Importantly, transient increased expression of RANKL during pregnancy promotes mouse mammary stem cell proliferation, which correlates with higher pregnancy-related breast cancer incidence.¹⁰⁰ RANKL depletion can also inhibit tumor formation and reduce bone metastasis in mice.¹⁰¹ Further, in BRCA1-deficient mouse model, RANKL inhibition with denosumab considerably reduces tumor growth.¹⁰² Beside RANK/RANKL, vitamin D receptor, which protects from osteoporosis, was shown to attenuate mammary gland formation and may contribute to post-weaning mammary gland regression.^{103–105} Tumor-suppressive functions of vitamin D have also been proposed in breast cancer.¹⁰⁶

Other main factors involved in bone formation are also essential for mammary development. Indeed, the expression of a key regulator of osteogenesis, Runt-related transcription factor 2, in developing mammary epithelial cells was found to induce OPN during lactation.^{107,108} Mammary-specific OPN-depleted mice had

lactation deficiencies because of lower alveolar structures suggesting a determinant role of OPN in olveogenesis.¹⁰⁹ Further, OPN expression was drastically increased in spontaneous mammary tumors in c-MYC transgenic mice and OPN expression was found to associate with metastasis.¹¹⁰ Another factor known as Calcitonin, is involved in calcium blood calcium regulation and inhibits osteoclast activity in bone.¹¹¹ Basically, Calcitonin opposes PTH activity. Therefore, in response to hypercalcemia, thyroid cells release calcitonin to inhibit bone resorption. Intriguingly, breast paracrine production of calcitonin increases during pregnancy but quickly drops before parturition, which may imply a local protective role of calcitonin against calcification.¹¹² Further, clinical data reveal decreased calcitonin expression in metastatic breast cancer.¹¹³ These observations suggest commonality of factors involved in bone and breast development.

A connection between lactation and skeletal metastasis of breast cancer has not been addressed in the literature. As breastfeeding protects from breast cancer overall, particularly from ER-PR- subtypes, proportionally more ER+ cancer types are represented by this group. Furthermore, as ER+ breast cancer subtypes tend to metastasize to bone more frequently compared with the ER- counterparts, which commonly target the visceral organs,²⁶ lactation may indirectly implicate skeletal breast cancer dissemination. Lactation is stimulated by prolactin, a hormone released from the anterior pituitary gland already during pregnancy. This contribute to a rapid bone turnover in lactating women because of increased osteoblastic and osteoclastic activities.^{114,115} During breastfeeding, nerves within the nipples stimulated by suckling connect with the central neural system and suppress the gonadotropin-releasing hormone in the hypothalamus, which culminates in a decline of circulating estradiol. This estrogen deficiency results in postpartum amenorrhea,¹¹⁶ and contributes to bone loss similar to menopause. Interestingly, the profound bone loss during lactation cannot solely be attributed to low estrogens.^{117,118} Lactating breasts secrete PTH-related protein (PTHrP) into systemic circulation,¹¹⁹ and PTHrP blood levels correlate with the bone resorption markers and lactation-induced total bone loss.¹²⁰ Low estrogen levels appear to synergize the PTHrP-related bone loss during lactation. Actually, lactation is the only nonmalignant state in which PTHrP is present in the circulation.¹²¹ However, these phenomena are followed by fast recoveries upon weaning, indicating potential crosstalk between breast and bone. This observation associates with high mobilization of calcium for milk production knowing that nursing mothers secrete 300–400 mg of calcium into milk daily.¹¹⁸ The physiologic levels of PTHrP during lactation are controlled the calcium-sensing receptor, the master regulator of systemic calcium metabolism. Activation of the calcium-sensing receptor in lactating breasts downregulates PTHrP, and increases calcium transport into milk.^{120,122} Interestingly, breast cancer utilizes exactly these mechanisms to invade, colonize and destroy bone, but the expression of calcium-sensing receptor in breast cancer cells upregulates PTHrP production.¹²³ Perhaps these molecular connections between mammary gland functions and bone homeostasis are part of the mechanisms underlying bone tropisms of breast cancer, especially the ER+ subtypes.¹²⁴ It can be speculated that mammary epithelial cells may preferentially survive and proliferate in a foreign environment with similar molecular and ionic properties.

ER α promotes tumor growth

ER α is the primary target for breast cancer treatment. Approximately 75% of breast cancers are ER α positive.^{88,125} Upon estrogen binding, ERs dimerize and translocate to the nucleus where they regulate transcription of target genes in a ERE dependent or independent manner.^{126–128} ER α induces cell

growth by activating multiple growth factors and inhibiting tumor suppressors.^{127,129} Hormonal therapy inhibits ER α activity and can significantly enhance survival of patients.^{115,130} Unfortunately, resistance often occurs, leading to cancer recurrence. Recurrent tumors develop new properties, which makes them more resistant to therapeutic treatments.¹³¹ After years of controversy, we now know that the *ESR1* gene can acquire mutations that confer further resistance in breast cancer tumors.^{132–134}

The *ESR1* mutations found in metastatic breast cancer patients appear to cluster in a mutational 'hot-spot' surrounding residues 536–538 in the hormone binding domain, resulting in constitutive activation of the receptor. The most frequent *ESR1* 'hot-spot' mutations in metastatic breast cancer to date are the E380Q, Y537N, Y537S and D538G somatic alterations. These mutations appear to be selected by aromatase inhibitor treatment,¹³⁵ and in some genetic backgrounds also confer resistance to the antiestrogen tamoxifen.¹³⁶ However, clinical evidence suggests that some of the *ESR1* mutations are sensitive to fulvestrant, and/or everolimus, or palbociclib treatment, thus these agents are useful in metastatic mutation-positive patients.^{137,138} Recent pre-clinical studies suggest that the newer orally available selective ER degrader drugs, such as AZD9496 and GDC-0810, are also very effective in reducing tumor growth of *ESR1* mutant models.^{139,140} Thus, patients with *ESR1* mutations can be treated with our currently approved, and highly efficacious targeted therapeutic regimens for ER+ breast cancer.

Using next-generation sequencing, approximately 20% of metastatic patients have acquired *ESR1* mutations, whereas the frequency of these mutations in primary invasive breast cancers is low.¹⁴¹ It is now apparent that clinical monitoring of *ESR1* mutations in circulating cell-free DNA in ER+ cancer patients is a feasible and very sensitive method to detect acquired mutations, especially in metastatic breast cancer.¹⁴² Using cell-free DNA coupled with sensitive digital drop PCR methods, the frequency of *ESR1* mutations in metastatic patients is now estimated to be between 30 and 50% in breast cancer.¹⁴³ Importantly, *ESR1* circulating mutations in the blood are independent risk factors for poor outcome after aromatase inhibitor treatment failure.¹⁴⁴ Circulating *ESR1* mutations are also frequently detectable before clinical progression is observed, thus cell-free DNA *ESR1* mutant assays may prove useful for earlier interventional studies and change of treatment decisions in metastatic patients. Further studies are warranted to determine if these liquid biopsy assays for *ESR1* mutations will also prove to be useful predictive factors to guide the treatment of patients with metastatic breast cancer. Another important clinical question is whether these mutations have a role in metastatic behavior, and whether they may directly influence tumor progression in addition to conferring hormone resistance.¹⁴⁵ Hopefully, ongoing pre-clinical studies will provide the answers to this critical question.

Recently, accumulation of *ESR1* mutations upon aromatase treatment has been observed especially in bone metastatic tumors, suggesting a selective role of bone microenvironment for such mutations.^{135,146,147} Alas, there is not enough evidence to support the role of ER mutations in bone.

ERs regulate EMT

EMT-driving factors are often critical regulators in developmental biology and pathological conditions. As such, TGF β , hypoxia, Notch, Wnt, bone morphogenetic protein (BMP), matrix metalloproteinases, platelet-derived growth factor, PTHrP, vascular endothelial growth factor, epithelial growth factor receptor, interleukins (IL-6, IL-8, IL-11 and IL-1), cathepsin K, and α v β 3 integrin, which are involved in EMT and mesenchymal to epithelial transition processes in breast cancer are also found in the bone microenvironment. EMT-regulated genes, such as E-cadherin, N-cadherin, Zeb1/2, vimentin, mir200, snail, slug and twist1, are

crucial for bone metastasis.^{58,148–150} Interestingly, ERs regulate most of these factors. ER α was shown to affect E-cadherin expression in cells such as MCF7 where knocking down the receptor induced loss of E-cadherin expression.^{151,152} However, this property can be lost in advance stages of breast cancer.¹⁵³ Intriguingly, the ER β isoform displays strong regulatory effects on EMT/mesenchymal to epithelial transition factors and metastasis in breast cancer. ER β expression was enough to induce E-cadherin expression in basal-like ER α -negative cells, suggesting anti-metastatic functions of the receptor in breast cancer.^{154,155} Despite significant progress, the role of ERs in bone metastasis is still uncertain.

Pre-metastatic niche in bone

A new concept of pre-metastatic bone niche has recently emerged. Primary tumors may instigate the formation of distant pre-metastatic lesions by activating bone stromal cells to secrete various chemokines, cytokines and other factors. For instance, C-X-C motif chemokine 12 (CXCL12), insulin-like growth factor, bone morphogenetic protein, tumor necrosis factor alpha, matrix metalloproteinases, TGF β , chemokine (C-C motif) ligand 2, colony stimulating factor 1, semaphorin 3A and vascular endothelial growth factor A were expressed upon development of primary tumor.^{156,157} Further, Lysyl oxidase secreted from hypoxic primary tumors was found to accumulate at pre-metastatic sites of distant organs and promote the recruitment of bone marrow-derived cells.¹⁵⁸ CD11b+ myeloid cells were found to mediate this effect through secretion of metalloproteinases-2.¹⁵⁸ The extracellular matrix proteoglycan versican activates macrophages through the Toll-like receptor complexes (TLR2 and TLR6) to maintain pre-metastatic inflamed micro-environments.¹⁵⁹ Interestingly, tumor-derived exosomes can educate bone marrow progenitors located at distant metastatic niches through the tyrosine receptor kinase MET.¹⁶⁰ Further, mir-122 secretion from primary tumors inhibits glucose metabolism of distant-niche cells, thereby increasing nutrient availability at these pre-metastatic niches.¹⁶¹ The survival promoting effect of this observation implies critical roles of glucose metabolism for disseminated tumor survival, and it may be of great interest to elicit how glucose influences cell awakening from dormancy, especially in the context of bone metastasis. The vascular endothelial growth factor receptors 1 is also involved in pre-metastatic niche vascularization, which may allow circulating tumors to reach tissue-specific sites.^{162,163} Although all these factors prepare hospitable niches for future metastatic cells, it is still unclear whether disseminating tumors choose their niche or whether pre-metastatic niches are the one choosing their 'guests'. Perhaps both options matter considering all obstacles tumors have to overcome to reach metastatic sites.

Luminal cancer dormancy in bone

Little is known about dormancy of ER+ cancer cells in the bone,¹⁶⁴ mainly due to the lack of pre-clinical models. Nevertheless, several recent studies have significantly advanced our understanding of dormancy mechanisms in other systems,¹⁶⁵ which may also be applicable to ER+ breast cancer. In particular, it has become increasingly evident that dormant and viable metastatic cells are often found adjacent to blood vessels in the 'peri-vascular niche'.¹⁶⁶ Mechanistically, crosstalk between endothelial cells and cancer cells via thrombospondin-1-mediated signaling may keep cancer cells quiescent. Recently, IL-6 cytokine leukemia inhibitory factor receptor, as well as signal transducer and activator of transcription 3, were found to promote dormancy states in breast cancer cells disseminated in bone.⁶⁷ Immunosurveillance by natural killer cells may help to reinforce the dormancy of peri-vascular cancer cells by eliminating those that re-enter cell cycle.¹⁶⁷ How do cancer cells survive during the prolonged dormancy period? Along with others, we have previously found

that c-SRC is a key factor in ER+ cell colonization of bone. The proto-oncogene tyrosine-protein kinase c-SRC promotes bone metastasis and survival by activating AKT/mammalian target of rapamycin signaling in response to C-X-C motif chemokine 12 binding to CXCR4.¹⁶⁸ It is noticeable that c-SRC also promotes estrogen independence in ER+ cells,⁶² thus linking survival in dormancy to therapeutic resistance. In addition, C-X-C motif chemokine 12 and CXCR4 signaling may also be responsible to retain cancer cells in the peri-vascular niche.¹⁶⁹ Taken together, these findings suggest a signaling network that dictates the dormancy behavior of cancer cells in the bone marrow.

ERs in early cancer arousal in bone

Some dormant cancer cells are eventually activated and resume aggressive growth to become overt metastases. Our understanding of this process is equally scarce especially in ER+ tumors. It has been observed that proliferating cancer cells often target special structures lining the inner side of bones called endosteal that are enriched in osteoblasts and their precursors, which is designated as the 'osteogenic niche'.^{169,170} The fate of the tumor cell is in part determined by their ability to interact with osteoblasts through cell-cell contact proteins such CXCR4, E-cadherin, annexin II receptor, AXL receptor, IL-6.¹⁷¹ In particular, the heterotypic adhesion junctions between the E-cadherin of cancer cells and N-cadherin of osteogenic cells can activate the downstream mammalian target of rapamycin signaling in cancer cells and trigger proliferation.¹⁷⁰ The interaction between ER α with these pathways¹⁷² suggest a role of ER in metastatic cancer re-activation within the osteogenic niche. However, the direct interaction with osteogenic cells may not be sufficient, and the re-activation may be an integrated result of other cellular components of the niche, and the availability of cytokines and growth factors produced from the niche,¹⁷³ including estrogen. Indeed, Ogba *et al.*¹⁷⁴ recently observed that estrogen could trigger tumor revival from dormancy. In an elegant study, it is demonstrated soluble VCAM1 may be secreted by cancer cells including ER+ ones to recruit activated osteoclasts.¹⁷⁵ These findings demonstrate the involvement of osteoclasts in full activation of dormant cancer cells. It is possible that there are distinct stages of the re-activation process. In an earlier stage, the osteogenic niche drives initial proliferation of cancer cells via direct cell-cell interaction and paracrine mechanisms. This process may take a long period of time until osteoclasts are recruited and foster a faster progression of micrometastases.

ERs in osteolytic vicious cycle

Metastasized breast cancer often drives osteolytic lesions, which are due to increased bone resorption as a result of unbalanced activities between bone stromal cells. This process of bone resorption releases multiple factors such as insulin-like growth factor, fibroblast growth factor and substantial amount of TGF- β , which are often stored in the bone matrix.¹⁷⁶ TGF- β induces secretion of paracrine factors including PTHrP and IL-11, which promote osteoclast maturation. In addition, cancer cells express tumor necrosis factor alpha, chemokine (C-C motif) ligand 2, soluble intercellular adhesion molecule 1, soluble vascular cell adhesion molecule 1, matrix metalloproteinases and Jagged 1, which foster more osteoclastogenesis.¹⁷⁷ This will perpetuate malicious crosstalk between degenerating bones and growing tumors thereby promoting bone loss. Clinical data suggest a strong association between bone loss prevention and decreased bone metastasis in postmenopausal, but not premenopausal women, indicating a role of ERs in bone metastasis.¹⁷⁸ Further, ER α -positive cancer cells had almost fivefold increased bone colonization in ovariectomized mice when compared with control mice.^{179,180} These results suggest a protective role of estrogen in bone, but the role of ERs remains to be clarified (Figure 2).

Clinical implication of ER and current therapeutic options

The majority of luminal cancers display bone metastasis often accompanied with intense bone pain and pathological fractures.¹⁸¹ Bone pain management may require complex treatment especially when neurologic pains are involved.¹⁸² Breast metastatic tumors are known to induce bone resorption as bone fractures considerably increase. Factors involved in breast cancer treatment may also contribute to rapid bone loss in patients. Therapies such as ovariectomy or aromatase inhibitor

treatment including letrozole and exemestane promote bone weakening because of the inhibition of estrogen-dependent ER activity.¹⁸³ Ultimately, these cancers often become resistant to hormonal treatment. Many of the recurrent tumors develop ER-independent survival mechanism or acquire ER mutations, which make the receptor constitutively active.

New therapies are being investigated to reduce side effects of ER inhibition on bones. One solution has been the use of selective estrogen modulators with less deleterious effect on bones. For

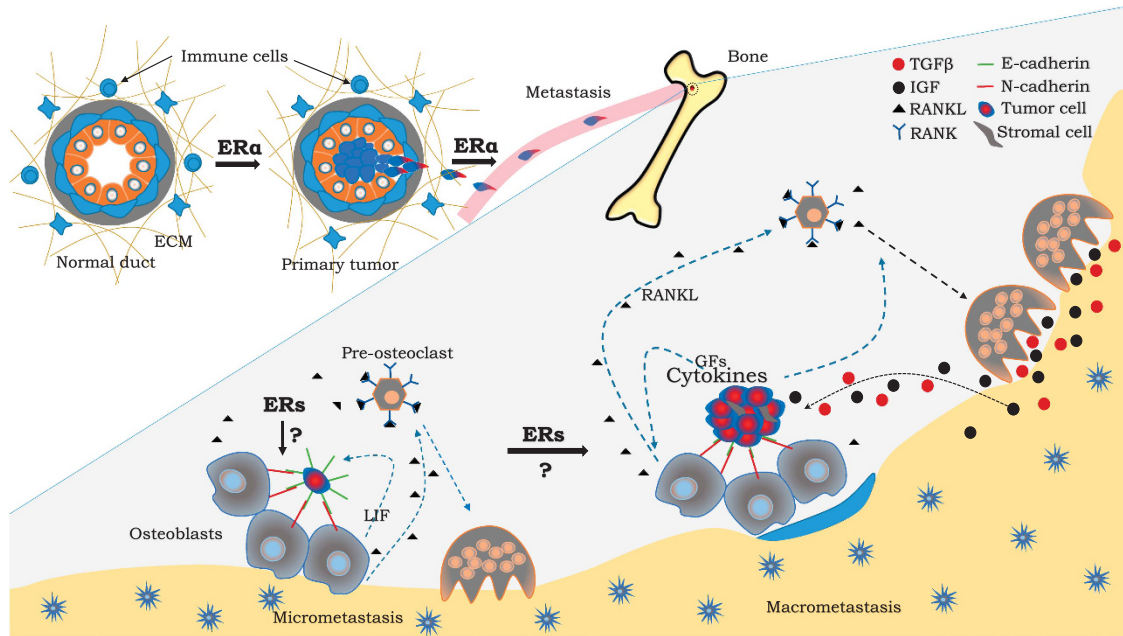


Figure 2. Role of ERs in bone metastasis. ERα is a predominant driver of primary tumor formation from normal mammary glands. ERα regulates several EMT factors to drive metastasis but this seems to require E2. Some of these metastatic effects may be attributed to ER mutations. Circulating tumors often disseminate to bone and form micro-metastatic niches by interacting with osteoblasts. Factors such as IL-6 cytokine leukemia inhibitory factor (LIF) promotes cell dormancy. Several other factors involved in cell-cell adhesion (cadherins and integrins) may be ER regulated. Increased bone macro-metastases following E2 treatment suggests a role of ERs in tumor re-activation and growth.

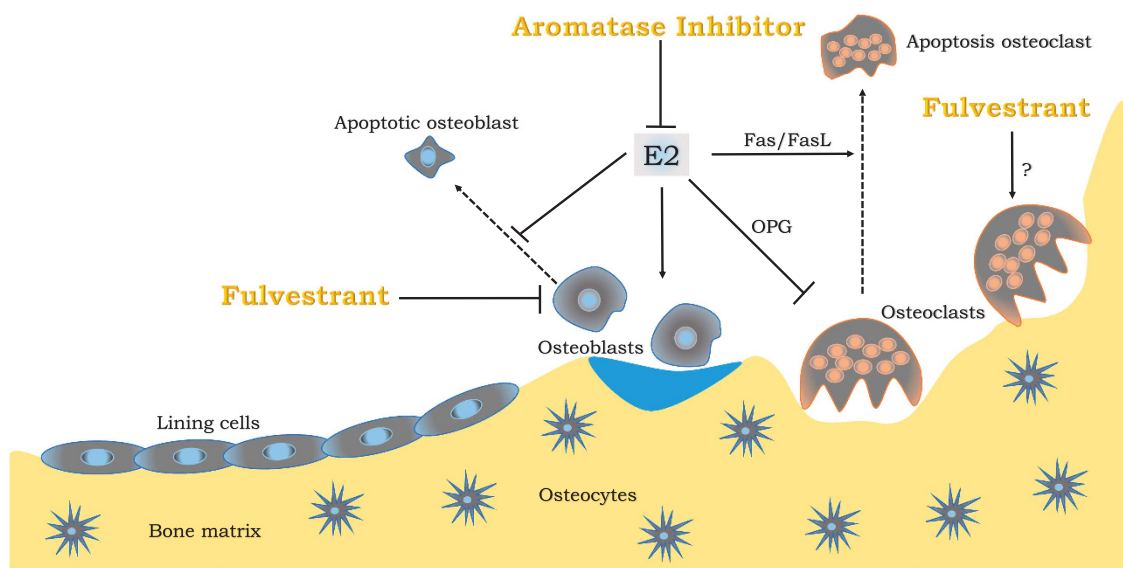


Figure 3. Effect of antiestrogen therapies on bone turnover. Estrogen (E2) promotes bone formation by opposing osteoclastogenesis and enhancing osteoblast activity. Breast cancer therapies affect bone metabolism and may impact bone metastasis. Aromatase inhibitors prevent E2 production, which may leads to more bone loss because of increased osteoclast activity. Fulvestrant alters E2 signaling by inducing degradation of ERs, which leads decreased bone formation.

instance, tamoxifen exhibits some bone protective roles, which contrast with its anti-estrogenic activity in breast. In contrast, the selective ER degrader fulvestrant induces ER degradation in both bone and breast, leading to bone weakening, which may significantly increase the risks of bone metastasis and promote cancer progression. The use of these therapies in clinic makes imminent the need to elicit the mechanisms underlying ER ligand effects in bone with regard to ER isoforms as both ER α and ER β represent potent targets mediating ER signaling in bone (Figure 1). More recently, a new class of selective ER modulator/selective ER degrader hybrid drugs developed to reduce side effects and increase efficacy in breast cancer, showed ER agonist effects in bone.¹⁸⁴ Another way to reduce ER-dependent bone loss has been the association of anti-resorptive drugs such as bisphosphonates to hormonal therapy. Bisphosphonates decrease bone loss by inhibiting osteoclastogenesis and inducing cell death. More anabolic drugs, such as teriparatide, are also used for their potential to rescue bone loss. Although these drugs are well used, the clinical outcome of patients can significantly improve if we get a better understanding of their mode of action.

CONCLUSION AND PERSPECTIVES

The mineralized structure of skeleton makes bone research more challenging. Despite these limitations, tremendous efforts have been made to better understand normal bone biology as well as malignancies in bone. One important family of molecules that we have not fully understood in the bone context are the ERs. Osteoblast and osteoclast lineage cells clearly depend on estrogen to be fully functional. ER knockout mouse models have helped identified some similarities, but mostly divergent functions between ER α and ER β in bone formation. Intriguingly, mammary glands are also highly regulated by ERs and cancer cells derived from breast primary tumors incline to metastasize to bone. Systemic hormone therapies have been implemented to treat ER+ breast cancer before and after they form bone metastasis. Although such therapies are often effective, bone metastasis remains incurable and usually develops resistance. Numerous pre-clinical and clinical studies have been performed to understand endocrine resistant mechanisms. However, only a few of them were done in the context of bone and bone metastases. As a result, the impact of endocrine therapies on osteoclasts and osteoblasts, two cell types that intimately interact with cancer cells during bone colonization, has been largely ignored. Studies are urgently needed to synthesize both microenvironmental and cancer-intrinsic effects of endocrine therapies on bone niche formation, cancer cell survival and metastatic tumor growth (Figure 3).

We are entering a new era of ESR1 mutations and we are just beginning to perceive the contribution of mutant ESR1 to cancer progression and drug resistance. Interestingly, a recent study suggests that bone metastasis patients accumulate ESR1 mutations in their circulating DNAs. Moreover, aromatase inhibitors but not tamoxifen appear to enrich ESR1 mutations.¹³⁵ Understanding the mechanisms underlying these observations will undoubtedly shed light on distinct roles of ERs in bone and cancer cells during bone colonization and development of endocrine resistance. Thanks to significant progress in imaging technologies and new tool developments, we may soon answer some essential questions in bone metastasis. We believe that genetic dissection of microenvironmental and cancerous ERs at different stages of bone metastasis may allow us to better apprehend the molecular mechanisms of ERs in bone colonization, and may open new opportunities for the development of new anti-metastatic therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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