

Estrogen receptors in health and disease

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Summary

Estrogen signalling is a balance between two closely related transcription factors (TFs), the estrogen receptors (ER α and ER β), both of which bind to similar DNA element called estrogen response element (ERE). ERs do not function by themselves but require a number of co-regulatory proteins (SRC1, A1, NCoR) whose cell-specific expression elucidates some of the divergent cellular actions of estrogen. A considerable body of evidence has shown that over-expression of ER α leads to uncontrolled cell growth and proliferation resulting in carcinomas of breast, ovary, uterus and prostate while ER β down-regulation causes colon cancer. ERs are well-known regulators of several aspects of metabolism, including glucose and lipid metabolism, whereas impaired estrogen signalling is associated with the development of metabolic bone disorders as in post-menopausal women. We review new evidences depicting the importance of ER in understanding the normal physiological functions and how the disruption of typical estrogen signalling leads to the development and progression of various forms of endocrine cancers and metabolic disorders including post-menopausal osteoporosis, diabetes and obesity. Re-examination of available therapies will enable us to therapeutically address fundamental issues towards the design of pharmacologic molecules so as to target crucial metabolic cascades and genes.

Key words: Estrogen receptor, Cancer, Osteoporosis, Diabetes, Obesity, SERMs.

Introduction

Estrogen is a steroid hormone that regulates several vital physiological processes such as cell differentiation, sexual development, bone remodelling, glucose and lipid metabolism, and energy homeostasis. The three naturally occurring forms of estrogen are estrone, 17 β -estradiol (estrogen), and estriol. Estrone is predominantly present during menopause state, estrogen during reproductive years and estriol during pregnancy. Estrogen is present in all vertebrates, mainly produced by ovaries, and during pregnancy by placenta. It is also produced in smaller amounts by liver, adrenal glands and breast tissues. Fat cells are the secondary source of estrogen (Nelson, 2001).

Estrogen actions are mediated by genomic and non-genomic mechanisms. In classical genomic mechanism 17 β -estradiol exerts its effect through estrogen receptors (Rogers, 2009), which belong to the family of nuclear hormone receptors (NHR) (Mangelsdorf, 1995; Couse, 1999). Two major forms of ERs are ER-alpha (ER α) and ER-beta (ER β) which act as ligand-inducible transcription factors. The non-genomic effects exerted by estrogen are through ER membrane form. The newly described membrane forms of ERs are G protein-coupled

estrogen receptor (GPR30), and non-classical membrane estrogen receptor (ncmER) (Foryst-Ludwig, 2010). GPR30 is involved in some of estrogen-related diseases like cancer. The exact role of GPR30 and ncmER requires much detailed understanding. Both the forms, ER α and ER β , are expressed in bone cells, adipose tissue, skeletal muscles, liver, pancreas, and the central nervous system. The expression pattern of ER α and ER β differs between sexes, species and specific tissues (Foryst-Ludwig, 2010).

Receptors, when activated by estrogen, form dimers and, since the two forms are co-expressed in many cell types, the receptors may form ER α ($\alpha\alpha$) or ER β ($\beta\beta$) homodimers or ER $\alpha\beta$ ($\alpha\beta$) heterodimers (Mangelsdorf, 1995). ER α and ER β have divergent functions which is shown by ER α (ER α -/-), ER β (ER β -/-) and double knockout mice (Mueller, 2001; Windahl, 2001). The phenotypic alterations due to these knockouts include incompletely differentiated epithelium in prostate, ovary, mammary gland and salivary gland. ER α -/- male and female mice develop obesity, insulin resistance, and complete infertility, whereas ER β -/- mice develop polycystic ovary-like syndrome. Imbalance of ER α /ER β leads to metabolic syndrome (Imamov, 2005; Barros, 2011).

Effects of estrogen on reproductive system

Estrogen is involved in normal uterine function, ovulation and secondary sexual character development. Mice lacking ER α show defects in folliculogenesis, which results in formation of haemorrhagic cysts and infertility. Loss of ER β leads to poor ovulation but supports normal implantation and gestation. Mice lacking both ER α and ER β show phenotype similar to ER α -null mice. Thus, estrogen action in uterus is mainly mediated by ER α (Prossnitz, 2008).

Effects of estrogen on non-reproductive system

Various metabolic effects of estrogen are observed in adipose tissue, skeletal muscles, bones, liver, pancreas, and the central nervous system (Fig. 1).

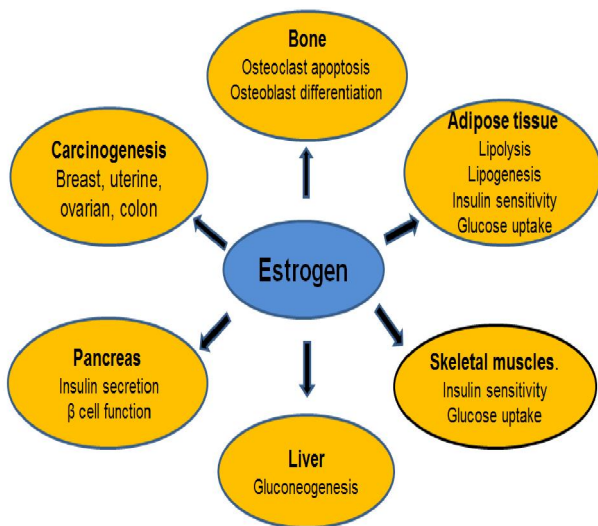


Fig. 1. Metabolic effects of estrogen on pancreas, bone, skeletal muscle, adipose tissue, liver and carcinogenesis.

Estrogen helps in maintaining blood pressure by up-regulating or down-regulating various elements of

Renin-Angiotensin-Aldosterone-System (RAAS). Estrogen down-regulates renin, angiotensin-converting enzyme (ACE), and AT-1 receptor whereas up-regulates angiotensinogen. The net effect of estrogen under well-defined experimental condition is suppression of RAAS (Nakamura et al., 2007). Meta-analysis suggests that women using contraceptive medications show overall mild increase in blood pressure while women undergoing estrogen replacement therapy show mild decreases (Fischer, 2002).

Estrogen increases the number of hematopoietic stem cells (HSC) in the vascular niche of bone marrow, but does not affect the number of HSC in endosteal niche. Increase in HSC number is also observed in ER α knockout mice; thus, estrogen-induced increase in HSC number is independent of the hormone's effect on bone (Illing, 2012).

Estrogen affects gluconeogenesis, glycolysis, lipolysis, lipogenesis, glucose uptake, insulin secretion and sensitivity. Anorexigenic and orexigenic signals from the CNS resulting in food intake and energy homeostasis are also governed in part by estrogen. Metabolic syndrome, which is cumulative effect of obesity, dyslipidemia, hypertension, insulin resistance, prothrombotic and proinflammatory states, is also result of aberrant functioning of ERs. In subsequent sections, we will discuss estrogen- and ER-mediated cancer cell proliferation, skeletal development, growth and maintenance along with diabetes and obesity.

Molecular structure of ER

ER α and ER β are encoded by *ESR1* and *ESR2* genes. ER's molecular structure is similar to other NHR, having five domains. Like other NHR domains, A/B is N-terminal transactivation domain (AF-1), C is DNA-binding domain (DBD), D is hinge region, E is C-terminal ligand-binding domain (LBD) and F is second activation domain (AF2) (Fig. 2) (Delaunay, 2000; Barkhem, 2004).

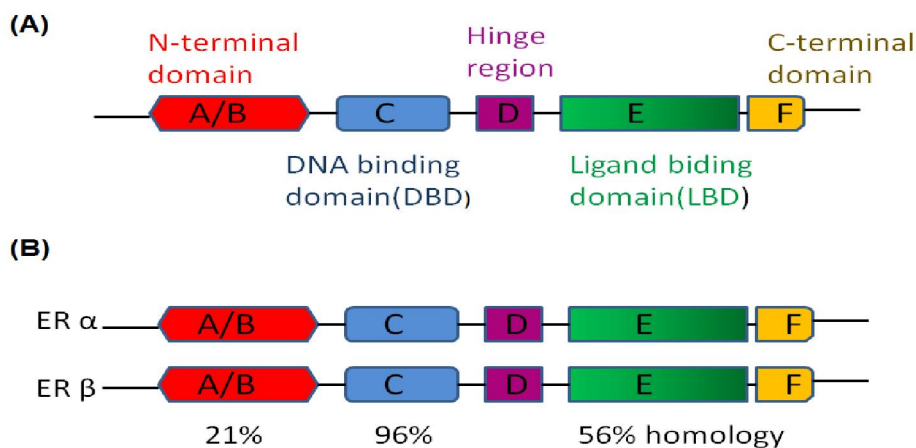


Fig. 2. Structural organisation of (A) estrogen receptor domain, and (B) sequence homology between ER α and ER β .

ER α in cancer

Estrogen, a natural hormone, despite having diverse roles in normal human physiology, was recently declared as a carcinogen by NIH. Several evidences showed that estrogen may cause breast, endometrial and uterine cancers. The carcinogenic effect is primarily mediated by ER signalling. There are several mitogenic genes downstream of ERs which are turned-on by estrogen to induce cell proliferation. During proliferation, any discrepancy in DNA repair system results in accumulation of mutations in genes that regulate cell cycle and proliferation resulting in cancer. Therefore, entities that induce apoptosis, prevent metastasis, and regulate inflammation via modulation of ER signalling are important to stall carcinogenesis.

Breast cancer

Estrogen induces proliferation and supports the metastatic behaviour of breast cancer cells. Breast cancer is classified in two types: ER α -positive and ER α -negative, depending upon the need of estrogen for proliferation. Its mechanism of action involves binding to ER and ultimately leading to a physiological response involving protein-protein interactions and recruitment of co-regulators. After ER activation by estrogen, ER α homodimerizes and undergoes conformational changes so as to recruit co-activators ultimately leading to transcriptional activation (Bocchinfuso, 1997). The continuous activation of ER α by estrogen leads to uncontrolled proliferation of ER α -positive breast cancer cells. Among the two isoforms of ER, ER α is a predictive marker in breast cancer, while the role of ER β is still not clear but recent studies have revealed down-regulation of ER β in cells undergoing rapid proliferation (Campbell-Thompson, 2001), thus suggesting an anti-mitogenic effect of the latter. Studies with ER α KO mice demonstrated that it is essential for tumor development (Bocchinfuso, 1997). Despite the currently available hormonal therapies for ER α -positive breast cancers there is a need of new therapies for ER α -negative breast cancers.

Chemotherapies targeting ER in breast cancer management

SERMs are highly selective in regulating the estrogen signalling in different tissues without affecting the normal signalling. Most commonly used anti-estrogens that block the activation of ER α by endogenous estrogen include tamoxifen, raloxifene and ICI 182,780 (Fabian, 2005). Though non-steroidal anti-estrogens like droloxefene and toremifene are known to have activity

comparable to tamoxifen, the latter is preferred to be used as first line of treatment (Ibrahim, 1998). Toremifene is also reported to be effective against ER α -negative breast cancers. Arzoxifene is a new potent SERM that is highly effective in breast and endometrial cancers.

Aromatase inhibitors prevent formation of estrogen in fat tissue. Commonly used aromatase inhibitors are anastrozole and letrozole. These drugs are effective in both early and late stage breast cancers only in post-menopausal women since estrogen formation from fat tissue is inhibited but not the one produced in the ovaries in young women. Several studies have also suggested use of aromatase inhibitors in adjuvant therapy for breast cancer (Hiscox, 2009)

Since the above-mentioned therapies have certain drawbacks, e.g., tamoxifen is known for development of resistance, and use of aromatase inhibitors are limited to post-menopausal women, there is a need for discovery of new therapeutic targets. Recent studies have shown that dysfunction of ER co-regulators (such as PLP1, AIB1 and SRC1), which are over-expressed in breast cancer or ER extra-nuclear signalling, have potential to enhance metastasis in ER-positive breast cancer cells which are responsible for cancer relapse (O'Dowd, 1998).

It was also shown that over-expression of AIB1 co-activator activates the downstream mTOR signalling cascade leading to mammary tumors and mammary hyperplasia. In consistence with the previous reports it has further been reported that several mTOR inhibitors inhibited the growth of ER-positive AIB1-dependent tumors (Liao, 2002).

ER α -negative primary breast cancers and cell lines showed increased level of SRC, a coregulator of estrogen signalling. It regulates estrogen-mediated ER activation and proteolysis. SRC and ER α levels were reported to be inversely correlated in primary breast cancers (Chu, 2007)

Estrogen signalling may also occur independently of ER via GPR30 which, upon activation with estrogen, leads to rapid non-genomic signalling resulting in intracellular calcium changes and synthesis of phosphatidylinositol (3,4,5)-trisphosphate in the nucleus. It is also known to be responsible for estrogen resistance in breast cancer cells (Ignatov, 2010).

Estrogen-ER α complex also activates key signalling cascades which play an important role in cancer cell proliferation like phosphatidylinositol 3 kinase (PI3K)/Akt, Bcl2 family members, several cytokines and their

receptors, p53, nuclear factor kappa B (NF κ B), and specific caspases (Riggins, 2005). Recent studies have shown that estradiol induces cyclin E processing in breast cancer cells. Cyclin E over-expression is closely related with tumor progression, relapse and resistance to antiestrogens following continuous use by patients. 4-Hydroxytamoxifen (OHT)-mediated cleavage of cyclin E and its migration is via GPR30, as cyclin E cleavage occurred in SK-BR-3 cells that express GPR30 and lack ER α , but not in MDA-MB-231 cells that express neither. GPR30 agonist G-1 enhanced cyclin E proteolysis resulting in cell migration while this effect was attenuated by GPR30 antagonist, G15. Similar observations were made with EGFR inhibitors suggesting involvement of EGFR signalling (Li, 2013).

Despite the use of current treatments available, the mortality rate due to breast cancer is high. One of the major reasons is resistance to chemotherapy, arising as a result of mutations in genes encoding the ER α . Point mutations in ESR1, like A908G or K303R, were originally identified in breast hyperplasia and reported to be hypersensitive to estrogen and, later on, these mutations were identified at a low frequency of 6% in invasive breast tumors (Conway, 2007).

Previously it was known that ER α -positive breast cancers are more resistant to chemotherapy than ER α -negative cancers. On the contrary, ER α activation was reported to increase the chemosensitivity of ER α -positive T47D cells and increased resistance in ER-transfected ER α -negative Bcap37 cells. This phenomenon could not be explained by ER α -mediated Bcl-2 and Bax regulation, rather it was found that ER α increased the chemosensitivity by enhancing the cell growth of T47D ER-positive cells and accounted the resistance to anti-estrogens to the decreased cell growth of ER-negative Bcap37 cells (Jiang, 2012). Additional research is needed to further elucidate these mechanisms and their role in breast cancer and its progression.

Ovarian cancer

ER is over-expressed in approximately two-thirds of the human ovarian cancer. Regardless of first line platinum-based chemotherapy i.e., addition of taxanes (Seetalarom, 1997), cyto-reductive surgery followed by carboplatin and paclitaxel enable most patients to achieve complete clinical response; however, over 85% eventually relapse due to the emergence of multidrug resistance. ER α is localized in ovarian stroma, theca and granulosa cells and epithelium in the reproductive system of normal female and in corpus luteum of post-menopausal women.

In support of this, tumor tissue microarray studies were carried out to identify gene expression. 2% of tumors were ER-positive with relatively low gene copy number except for a few exceptional cases (Pelletier, 2000).

ER α is expressed in 50% of ovarian cancers. Previous studies showed that expression of ER α and ER β varies at different stages of reproductive system development in females. Expression level of ER β was significantly higher in stage I as compared with stages II-IV of this cancer. Higher ER β expression was related with longer disease-free survival while down-regulation of ER β may lead to malignant transformation. Determining the expression of ER isoforms may provide us with improved response to hormonal therapy by modifying the existing SERMs (Bossard, 2012).

ER α is also reported to be regulated by a pleiotropic hormone leptin, known to promote growth in BG-1 ovarian cancer cells involving ER signalling cascade. The cells over-expressing ER α resulted in increased cell growth upon leptin treatment, whereas no significant difference was observed in ER β -transfected cells. The down-regulation of ER α completely reversed leptin-induced growth of BG-1 cells. Leptin treatment leads to nuclear localization of ER as a result of transcriptional activation and increased expression of ER-regulated gene pS2. Leptin also induces Janus kinase 2/signal transducers and activators of transcription 3 (STAT-3) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. Altogether, leptin involves, at least in part, ER transcriptional activation via the STAT-3 signalling pathways (Choi, 2011). It is known that MAPK regulates nongenomic ER α signalling (Joel, 1998; Clark, 2001) by interacting with ER α in estrogen-independent manner. Earlier studies have identified several kinases in MAPK signalling cascade that phosphorylate residues on ER α leading to transcription of estrogen-dependent target (Atsriku, 2009). On the contrary, a few studies have also shown an inverse relationship between MAPK signalling and ER α genomic activity (Joel, 1998; Clark et al., 2001).

Non-genomic signalling of estrogen is also mediated by GPR30 in various estrogen-dependent cancer cells via epidermal growth factor receptor (EGFR) activation. Since GPR30 binds to most of ER ligands, its role in ER signalling cannot be assessed. Therefore, G-1, specific ligand for GPR30, will offer new insights to further identify the differences in ER family and GPR30 in mediating the complicated mechanisms of estrogen action (Albanito, 2007).

Endometrial cancer

Endometrial cancer is the most common type of gynecological cancer. Based on the dependence on estrogen, endometrial cancer is categorized into two types; type I, which is estrogen-related and occurs in 70-80% of endometrial cancer in peri-menopausal women, and type II, which is not dependent on estrogen for progression and is predominant in older women. Type I endometrial cancer is characterized by endometrial hyperplasia, high serum estrogen and estrogen receptor over-expression while type II is associated with lack of estrogen and progesterone receptors (Lax, 2004).

The prognostic significance of ER α and progesterone receptor in endometrial cancer is controversial. On the other hand, determining the ratios of relative expression of ER isoforms (ER α /ER β 1 and ER α /ER β 2) was thought to be of help in predicting the clinical outcome of endometrial cancer. It was found that ER α /ER β 1 and ER α /ER β 2 are independent prognostic markers for survival. Commonly used treatment for endometrial cancer includes tamoxifen and aromatase inhibitors. Tamoxifen fails to be effective generally due to drug resistance and side-effects caused after prolonged use (Cohen, 2008).

Prostate cancer

Androgens are known to be main contributors in prostate cancer development. In addition to androgen, estrogen, a key player in female reproductive system development, also plays important role in growth and development of male reproductive system and, therefore, any disruption in estrogen signalling may lead to tumor formation in prostate gland. Both the isoforms of estrogen receptor are expressed in normal and malignant prostate cells differentially in stromal and epithelial cells. ER α is known to be present in stromal cells while ER β is expressed in both stromal and epithelial cells (Prins, 2008). Though expression of ER β is higher than ER α , estrogenic effects in prostate cancer are mainly mediated by ER α (Prins, 2001).

Studies demonstrated that transcriptional inactivation of ER α gene by methylation enhances the progression of prostate cancer (Li, 2000). However, some studies also showed that many sequence variations or single nucleotide polymorphisms (SNPs) in ER α gene (ESR1) may be the risk factor for having an important role in prostate cancer progression (Tanaka, 2003). The role of ER α in prostate cancer has been validated through both *in vivo* and *in vitro* studies. Wild type, but not ER α

knockout (α ,ERKO) mice developed prostatic squamous metaplasia (SQM) after treatment of diethylstilbestrol (DES) (Risbridger, 2001). Upregulated aromatase has an influencing role in prostate cancer which increases the intracellular estradiol level and thus leads to aberrant downstream signalling cascade via ER α /GPER activation to induce cell proliferation and metaplastic change in prostate (Albanito, 2007). At present, both SERMs, including raloxifene and aromatase inhibitor, are used in the treatment of prostate cancer (Campbell-Thompson, 2001).

Colon cancer

Development of colon cancer is a multistage process involving transformation from adenomatous stage to carcinoma. With the increasing incidence of colon cancer in aged group of people and advanced stages of disease there is a need of new preventive strategies. It is evident that males are more prone to colon cancer as compared to its frequency in females. Some studies suggested this observation to be due to protective nature of female hormones. ER β , present predominantly in colon, plays protective role against cancer. The mRNA level of ER β was considerably low in colonic tumors as compared to normal mucosa in female patients, whereas in male patients ER β mRNA levels were not different between samples. However, ER β mRNA levels in normal mucosa were similar regardless of age in females. Hypermethylation of CpG islands in the promoter region of ER β alters its expression during colon cancer development from adenomatous stage to carcinoma. In contrast to ER α , decreased expression of ER β promotes tumor progression in the human colon during aging (Toyota, 1999). ER β appears to have anti-cancer role as it induces apoptosis and prevents metastasis.

In colon cancer the advanced Duke stage and loss of differentiation is due to reduced ER β levels (Konstantinopoulos, 2003). The protective role of ER β was confirmed by the *in vivo* studies in mice, which failed to develop colon cancer adenomas (Apc^{Min}) in the presence of ER β agonist whereas ER β deletion resulted in an increase in size and number of adenomas (Giroux, 2008).

Another report revealed that ER β downregulation of IL-6 has important role in inflammatory mechanisms involved in colon cancer. It was also found that ER β is involved in cross-talks between PROX1, a nuclear receptor co-regulator, and the anti-cancer impact of ER β may be a pooled effect of cell cycle regulation, down-

regulation of oncogenes such as MYC, MYB and PROX1, regulation of the anti-inflammatory response, and increased DNA repair capacity (Petrova, 2008). Therefore, enhancing ER β action may prove to be a viable option for prevention and treatment of colon cancer.

Role of estrogen and ER in bone cells

Bone is a metabolically active organ that is built and rebuilt continuously by two dynamic processes: bone modelling and bone remodelling. These processes require the coordinated actions of bone forming osteoblasts and bone resorbing osteoclasts, present on the bone surface, and the osteocytes, embedded within the bone matrix. A number of systemic hormones and transcription factors directly regulate the proliferation and differentiation of osteoblasts and osteoclasts. Estrogen is a key hormone known to affect the skeletal development, growth and maintenance in humans. High plasma levels of estrogen during puberty are requisite for rapid skeletal growth and epiphyseal closure. After menopause, the levels of serum sex steroids, including estrogens, become reduced. Additionally, a fall in estrogen concentration below 30 pg/mL during menopause corresponds to the period of accelerated bone loss (Marcus, 1998) when bone resorption is more than formation (Rodan, 2000; Teitelbaum, 2007) that eventually results in osteoporosis. Post-menopausal estrogen deficiency in women is believed to be largely responsible for age-related bone loss. Men with genetically impaired estrogen signalling due to defect in either estrogen biosynthesis or function of ER α do develop osteoporosis (Smith, 1994b; Jones, 2006). In the male, although estrogens also play a physiological role, nothing is known about the regulation of estrogen production by extra-gonadal tissues. A direct correlation between estrogen deficiency and reduced bone mineral content is evident by the increased incidence of osteoporosis in women versus men.

Osteoporosis is a systemic skeletal disease characterized by deterioration of bone mass and micro-architecture, with increased incidence of fragility fractures. Bone resorption and formation are tightly coupled and their balance defines bone mass as well as quality. Studies have documented that estrogens act through effects on bone-forming osteoblasts derived from mesenchymal stem cells and on multinucleated, bone-resorbing osteoclasts originating from hematopoietic stem cells to control the number of osteoclasts. Estrogens slow the rate of bone remodelling and help to maintain a balance between bone formation and resorption by regulating the birth rate of osteoclast and osteoblast progenitors in the bone marrow

and exerting a pro-apoptotic effect on osteoclasts and an anti-apoptotic effect on osteoblasts and osteocytes. Post-menopausal osteoporotic bone loss in women can be ameliorated with estrogen replacement and SERMs.

ER α in osteoblasts

The function of estrogens and their receptors in bone homeostasis has been investigated using ovariectomized rodent models and in several mice strains with modified ER α or β genes. Initially, the role of ER α in bone was studied using global ER α KO mice, which have a drastically different skeletal phenotype, e.g., female ER α KO mice had shorter bones compared to littermate controls during growth and maturation, in addition to increased cancellous and cortical bone mineral density in the tibia (Vidal, 1999). However, due to systemic response, high serum estrogen and reduced IGF-1 levels (Lindberg, 2001), both of which are major independent regulators of bone mass, made it difficult to interpret the role of ER α in mice lacking the gene globally. Using ovariectomized and estrogen treated ERKO and DERKO mice, it was shown that ER α regulates the amount of trabecular bone in female mice (Lindberg, 2002; Parikka, 2005) while the regulation of cortical bone was found to be independent of both ER isoforms (Lindberg, 2002). In a clinical study, mutation in the ER α gene showed decreased bone mineral density and resulted in osteopenic bone in both male and female (Smith, 1994a). Moreover, a meta-analysis revealed that polymorphisms in ER α are associated with bone mass in humans (Wang, 2012). Mice expressing an ER α mutation cannot bind DNA directly (ER α /NE RKI) and have impaired bone formation (Syed, 2005, 2007, 2010), and the osteoporotic phenotype of ER α /NERKI mice may involve the suppression of Lef1-mediated Wnt signalling through both the stimulation of secreted Wnt inhibitors and/or disruption of normal β -catenin function (Syed, 2011; Modder, 2012). Further, in U2OS cells, inactivation of classic ER α signalling has been shown to up-regulate negative regulators of Wnt signaling such as dickkopf-related protein 1 and sclerostin (Modder, 2012). Recently, osteoblast-specific ER α KO mice (pOC-ER α KO) generated by Melville et al. (2013) indicated that ER α in osteoblasts is required for appropriate bone mass and strength accruing in cancellous and cortical sites. In another study, the osteocalcin promoter-driven inactivation of ER α in osteoblasts and osteocytes were reported to strongly affect the bone phenotype of female mice by regulating bone formation and maintenance while in male mice the role of ER α is more related to the maintenance of bone and the well-established effects of estrogen on

bone in male mice during rapid skeletal growth are more likely to be indirectly mediated via systemic ER-regulated mechanisms, such as the GH-IGF axis (Maatta, 2013). Pharmacological estrogen administration exerts osteo-anabolic action independent of FSH, and requires DNA-binding of ER α in osteoblasts (Seitz, 2012). The anabolic action of estrogen in mice has been proposed to occur, at least in part, through oxytocin produced by osteoblasts in bone marrow (Colaianni, 2012). The receptor ER α has been proposed to be involved in a number of bone signalling networks, including those activated by estrogen, IGF-1, wnt/ β -catenin, and BMP-2 (Lau, 2006; Armstrong, 2007; Sunters, 2010; Modder, 2012). Another report showed that estrogen replacement following ovariectomy involves a possible coupling mechanism between the vascular endothelium signalling and bone remodelling activity (Prisby, 2012).

ER α in osteoclasts

Estrogen depletion following menopause or ovariectomy is associated with increased resorption and formation leading to high turnover trabecular bone loss through enhanced resorption (Delmas, 2002; Tolar, 2004). The effects of estrogen in restoring bone mass arise mainly from its antiosteoclastic action in a hyper-resorptive state, partly by induction of osteoclast cell death (Faloni, 2007) and by regulation of their differentiation from precursors (Garcia Palacios, 2005). When ER α is ablated from differentiated osteoclast it leads to osteoporosis in females but not males. Also, up-regulation of Fas ligand (FasL) in osteoclast is observed in WT but not in ER α -ablated mice, thereby suggesting that the osteoprotective effect of estrogen in ER α -ablated female mice is mediated by osteoclastic ER α (Nakamura, 2007; Takada, 2010). Osteoclast differentiation is regulated by several autocrine and paracrine mechanisms, and ER α has been reported to inhibit osteoclast function indirectly via osteoblasts by augmenting production of osteoprotegerin (OPG) (Yasuda, 1998; Hofbauer, 1999) and FasL (Takada, 2010), and directly independent of stromal cells by the inhibition of the MAPK/JNK (Shevde, 2000) leading to decreased osteoclast differentiation and induction of osteoclast apoptosis, respectively. However, both *in vivo* and *in vitro* studies, have implicated multiple inflammatory cytokines and growth factors are being involved in bone protective effects of estrogen. These studies have highlighted IL-1 and/or TNF- α (Kimble, 1995, Pacifici, 1989), IL-6 (Girasole, 1992), IL-7 (Weitzmann, 2002), and IL-11 (Shaughnessy, 2002) as potential mediators of osteoclast differentiation. Estrogens attenuate osteoclastogenic

cytokine production from osteoblast progenitors by inhibiting NF- κ B (Manolagas, 1995). Recently, it was shown that selective deletion of ER α from cells of the osteoclast lineage increases osteoclastogenesis, abolishes the effects of estrogens on osteoclast apoptosis, increases bone resorption in female but not male, and causes loss of cancellous bone (Nakamura, 2007; Martin-Millan, 2010). However, ER α deletion from osteoclasts does not affect cortical bone, raising the possibility that effects of estrogens on other cell types may be responsible for their effects on cortical bone (Almeida, 2013). ER α plays essential roles in the accretion and maintenance of bone mass via cell autonomous actions in both osteoblast progenitors and osteoclasts. However, the role of ER α in these two cell types is different in distinct bone compartments: the ER α in osteoblast progenitors promotes bone formation at the periosteal surface of the cortex and prevents resorption at the endocortical surface, whereas in osteoclasts the receptor prevents resorption of cancellous bone (Almeida, 2013).

ER β in bone cells

More profound loss in BMD following ovariectomy in ER α KO mice raises the possibility that other receptors may be important for estrogen action on mouse skeleton. ER β mRNA has been detected in primary osteoblasts as well as in cancellous and cortical bones of rat. Furthermore, ER β has been detected in chondrocytes, osteoclasts, osteoblasts and mesenchymal cells in both men and women (Batra, 2003) suggesting that, apart from ER α , ER β also plays an important role in the mechanism by which estrogen acts on bone. There is also evidence that ER β is more ubiquitously expressed at higher levels than ER α in trabecular bone (Onoe, 1997; Lim, 1999). In contrast, ER α predominates in cortical bone (Bord, 2001).

Several polymorphic studies have been conducted that demonstrate that a CA repeat polymorphism has been associated with BMD in two Asian populations (Ogawa, 2000; Lau, 2002; Geng, 2007) and a North American population (Scariano, 2004). Moreover, genetic variation in this dinucleotide repeat has been linked with incidence of femoral fractures (Honma, 2013). In contrast, blockade of ER β has been suggested as another therapeutic strategy for osteoporotic fracture and non-union fracture (He, 2012).

In vitro experiments have indicated that at low concentrations of estrogen, ER β has the capacity to repress ER α -activated transcription from estrogen response elements. In contrast, at high concentrations of

ligand, ER β does not inhibit ER α action and, moreover, it induces transcription on its own (Hall, 1999, Pettersson, 2000). Thus, at different concentrations of estrogen, ER β might have the capacity to promote different effects, and the growth plate's fusion of ER α KO mice might be mediated through ER β at high serum levels of estrogens.

ER in mesenchymal stem cells (MSCs)

In addition to osteoblasts and osteoclasts, both ER isoforms, α and β , have been detected in MSCs isolated from human and mice (Zhou, 2001; Chen, 2013). ER α is the predominant form expressed in MSCs (Hong, 2004; Wang, 2006). Reports have shown impaired proliferation and osteogenic activity of mesenchymal stem cells after estrogen depletion (Rodriguez, 1999). Estrogen, when given *in vitro*, directly augments the proliferation and differentiation, ER α expression, osteogenic gene expression while inhibits apoptosis and ER β expression in MSCs, suggesting that mouse MSCs are anabolic targets of estrogen action, via ER α activation and ER β function as a repressor in the osteogenic differentiation in MSCs (Zhou, 2001; Hong, 2011). Estrogen is reported to promote proliferation of early osteoblast progenitors, while it inhibited BMP-2-induced osteoblast differentiation of periosteum-derived mesenchymal progenitors (Ogita, 2008). Recently, it has been shown that ER α deletion from MSCs and osteoblast progenitors leads to reduction in cortical bone mass and microarchitecture and is attributed to Wnt/TCF-signaling, independent of estrogen (Almeida, 2013). In addition, there are reports suggesting anti-remodelling effect of estrogen, demonstrating that the effects of the estrogen-activated ER α are opposite to the effects of the unliganded ER α (Di Gregorio, 2001; Almeida, 2010). In support of these findings, it is well documented that estrogens suppress periosteal bone formation and that estrogen deficiency in female rodents and women increases periosteal apposition (Seeman, 2003; Callewaert, 2010).

Selective Estrogen Receptor Modulators (SERMs)

The synthetic, non-steroidal compounds, shown to possess estrogen receptor agonistic or antagonistic selectivity in specific target tissues initiated the development of a new class of drugs known as SERMs, are believed to exert at least their genomic effects mainly via the ERs. Several possible molecular bases for their target tissue selectivity have been suggested. First, by inducing changes in receptor conformations, which contributes to their pharmacological properties in target tissues (Pike, 1999). Second, their differential affinity for

each of the ER α and ER β receptors and the receptor ratio could also explain some of aspects of SERM selectivity (Kuiper, 1997; Gustafsson, 1999). Third, the relative co-activators or co-repressors levels in response to SERMs followed by selective interaction with ER α may be an explanation for their target site specificity (Voegel, 1996). Fourth, in addition to classical ERE, SERMs can interact with target gene promoter possessing the AP-1 (Paech, 1997) or Sp1 binding sites (Mukherjee, 2005). Finally, SERMs are also likely to be able to enhance the signalling potential of ER through intracellular signalling pathways that are induced by extracellular growth factors (Smith, 1998).

Tamoxifen, the first generation SERM, is known to prevent bone loss in female rats and post-menopausal women (Jordan, 1987; Love, 1992). As with estrogens, the final effect on bone is an inhibition of resorption, although the effects are less strong. The main distinctions from estradiol are differences in the binding to ER α and ER β and the fact that ER responsive genes are less strongly induced by SERMs. 4-OH-tamoxifen is the active metabolite of tamoxifen and has a two–three-fold higher binding affinity for ER β than for ER α (Kuiper, 1997).

Alternatively, raloxifene, the second generation SERM, has been demonstrated to have bone protective role (Delmas, 1997; Ettinger, 1999) and in 1998 was approved by the FDA for the treatment and prevention of post-menopausal osteoporosis and vertebral fractures. The molecular mechanism of bone protection by raloxifene is similar to estrogens, and exhibits anti-osteoclastogenic effect directly by reducing osteoclast number (Taranta, 2002) and indirectly by up-regulating FasL or OPG expression in osteoblasts (Viereck, 2003; Michael, 2007) to induce apoptosis in osteoclasts. For the first time under *in vivo* settings, raloxifene treatment in postmenopausal women for 6 months showed a significant increase in OPG levels (Messalli, 2007). In addition to the OPG/RANK/RANKL system, it has been suggested to inhibit production of cytokines, particularly IL-6 and IL-1beta from cultured osteoblasts (Taranta, 2002; Cheung, 2003). Similar to estrogen it also exhibits osteoblast stimulatory effect by increasing mRNA expressions of Cbfa1/Runx2 and alpha2 procollagen type I (Taranta, 2002). Recently, raloxifene has been demonstrated to be a CB2 (Cannabinoid receptor 2) inverse agonist, so it is proposed that a novel mechanism for its anti-osteoporosis activity may involve the inhibition of osteoclast formation in bone via CB2. Both tamoxifen and raloxifene recruit ER α to the FasL enhancer, similar to estrogen (Krum, 2008).

Ospemifene (also known as FC1271a), a metabolite of toremifene, is another SERM used for treatment of osteoporosis. In addition to anti-osteoclastogenic effect, ospemifene is reported to enhance osteoblastic differentiation and the effect was inhibited by ICI182780, suggesting an ER-mediated mechanism (Qu, 1999).

Bazedoxifene, a new SERM in the late-stage clinical trials, is known to reduce bone loss in postmenopausal women (Miller, 2008). The Selective estrogen Menopause And Response to Therapy (SMART) trial tested it in combination with estrogen as a new class of therapeutic agents called tissue-selective estrogen complexes (TSEC). Bazedoxifene/ conjugated estrogen (BZA-10,20 or 50mg/CE-0.625 or 0.45mg) combinations when given daily for 2 years, decreased bone turnover and bone loss in postmenopausal women at increased risk for osteoporosis (Lewiecki, 2007; Lindsay, 2009).

So far, studies carried out using SERMs for the treatment of osteoporosis failed to prove their efficacy to reduce the rate of non-vertebral fractures. In addition, they are contraindicated for patients at risk for deep vein thrombosis and also aggravate hot flushes in some women.

ER α in diabetes

The prevalence of diabetes is more in men than in postmenopausal women and lowest in premenopausal women. Autoimmune diseases strike 10 times more frequently in women than in men (Beeson, 1994) but exception to this is diabetes. Thus, gender dimorphism is seen in β -cell failure (Liu, 2010). These findings indicate that estrogens have protective role against diabetes.

Type I diabetes mellitus (T1DM) is an autoimmune disorder that leads to insufficient level of insulin due to β -cells destruction. On the contrary, Type II diabetes mellitus (T2DM) occurs due to insulin resistance. Estrogen replacement therapy (ERT) in postmenopausal women prevents diabetes suggesting that estrogen improves β -cell function or survival via direct or indirect mechanisms. Estrogens have direct insulinotropic and prosurvival effects in cultured islets. It also protects against oxidative stress and proinflammatory cytokines. Physiological range of estrogen provides insulin sensitivity, whereas hypo- or hyperestrogen level is related to insulin resistance (Livingstone, 2002). Indirect effect includes estrogen-mediated ER association with transcription factors like Fos/Jun which activates transcription of genes containing activator protein-1 response element. AP-1 activated genes help in survival.

Skeletal muscle is a major site of glucose utilization. Glucose uptake by skeletal muscles is insulin-induced. Phosphorylation cascade switched on by insulin upon binding to its receptor phosphorylates various proteins which include insulin receptor substrate (IRS), phosphoinositide 3-kinases (PI3K), protein kinase B (Akt), and AMP-activated protein kinase (AMPK). The ultimate result is translocation of glucose transporter 4 (GLUT4) to the cell membrane from cytoplasmic vesicles. Estrogen stimulates the phosphorylation of Akt, AMPK, and the Akt substrate TBC1D1/4 in soleus muscle (Rogers, 2009). When estrogen is administered in insulin resistant rat or mouse, it restores insulin sensitivity by increasing IRS-1 content and phosphorylated form of Akt (Rogers, 2009). Stimulatory effect of estrogen on glucose uptake is mainly due to ER α . ER β represses GLUT4 expression because targeted disruption of aromatase (ArKO) in male mice causes decrease in GLUT4 expression in muscle when treated with selective ER β agonist diarylpropionitrile (DPN) (Barros, 2006). This result indicates that activation of ER β may lead to diabetes. In ER α -KO mice tamoxifen, an ER antagonist, improved insulin sensitivity and increased GLUT4 expression, but in ER β KO mice, it does not show any effect (Barros, 2009). On the contrary, in L6 myoblasts propyl pyrazoletriol (PPT), an ER α -selective agonist, increases GLUT4 translocation to the cell membrane (Galluzzo, 2009). Glucose uptake and GLUT4 expression in skeletal muscle is increased in ovariectomized rats after PPT treatment (Gorres, 2011). These data suggest that ERs participate in insulin sensitivity as well as resistance.

ER α in obesity

Imbalance between energy intake and expenditure leads to obesity. Among two types of adipose tissues, white adipose tissue (WAT) and brown adipose tissue (BAT), the latter has abundant uncoupling protein (UCP1) which dissipates energy as heat by uncoupling the respiratory chain from ATP synthesis. Both ER α and ER β are expressed in human subcutaneous and visceral adipose tissues, whereas only ER α mRNA is identified in BAT (Rodriguez-Cuenca, 2007). Estrogen increases central sensitivity to leptin. Leptin is secreted by adipose cells and decreases food intake and enhances food expenditure.

Deficiency of estrogen at menopause is associated with increased adiposity. Truncal obesity, elevated blood lipids, and severe insulin resistance are seen in aromatase-deficient men (Jones, 2006). Body uses carbohydrate as the major source of energy but estrogen promotes use of

lipid as a fuel, and this fuel partitioning reduces adiposity in ovariectomized (OVX) mice and menopausal women undergoing ERT. Besides, estrogen inhibits lipogenesis in adipose tissue, liver and muscles. The hormone enhances fat oxidation in muscles and lipolysis in adipocytes, thus indicating that it is involved in adipocyte turnover, and reduction in adipose mass i.e., adipocyte number and size.

Adipocyte size is direct manifestation of triglyceride storage. Studies in OVX mice treated with estrogen suggest that estrogen effect on adipose mass is mainly in terms of reduction in mean adipocyte size. Chronic estrogen treatment promotes reduced adipose mass independently of reduced energy intake in OVX mice (D'Eon, 2005). The genomic and non-genomic targets of estrogen in lipid metabolism are sterol regulatory element-binding protein 1 (SREBP-1c), peroxisome proliferator-activated receptor- γ (PPAR- γ) and AMPK, respectively. PPAR- γ , which is an adipogenic gene, plays a key role in the regulation of lipid and glucose homeostasis, fatty acid storage and differentiation of adipocytes (Heikkinen, 2007). Down-regulation of PPAR- γ after estrogen treatment prevents obesity. Estrogen also down-regulates expression of genes under the control of SREBP-1c. SREBP-1c is regulated by insulin and is required for glucose metabolism and fatty acid synthesis, resulting in reduced lipogenesis.

AMPK regulates fatty acid synthesis, oxidation and glucose uptake. Estrogen phosphorylates AMPK which results in inactivation of Acetyl Co-A carboxylase (Galluzzo et al., 2009), preventing synthesis of malonyl-CoA and ultimately triglyceride synthesis. Thus, estrogen treatment is associated with reduced adipose mass and adipocyte size and down-regulation of lipogenic genes in adipocytes.

Estrogen increases sympathetic activity and thus promotes catecholamine-stimulated lipolysis. Perilipin is

lipid droplet protein which is key regulator of lipolysis. Estrogen treatment increases perilipin and increased levels enhance responsiveness to catecholamine-stimulated lipolysis, reducing triglyceride accumulation (D'Eon, 2005).

ER α KO mice had larger adipose depots than wild type (Lemieux, 2005). The study of mice knockout for the ER α (ER α KO), ER β (ER β KO) and both (DERKO) suggests that estrogen effect of decrease in adipose tissue deposition is mediated through ER α (Davis, 2013).

Conclusion

Estrogen is a key hormone that is involved in regulation of almost all aspects of cellular and physiological processes, and exerts either positive or negative or direct and/or combinatorial effects by interacting with ER α and/or ER β leading to differential target gene expression. The high abundance and distribution of the receptors in many tissues made it difficult to determine whether a ligand (estrogen, SERMs) will have a particular effect or there is a systemic response (GH or IGF-1) or the effect is ligand-independent (Wnt/TCF or BMP-2) or particular milieu of co-activators/co-repressors mediated regulation or a membrane-bound receptor is involved. Thus, a number of questions regarding molecular mechanism of estrogen involving differential ER α and ER β responses in target tissue need to be answered more extensively by employing stringent set of cell-specific knock out studies before drawing definitive conclusions on the pathogenesis of cancers and metabolic disorders like osteoporosis, diabetes and obesity and for the development of therapies to treat them.

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