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Biological Role of Estrogen and Estrogen Receptors

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KEYPOINTS

- There are two different estrogen receptor (ER) subtypes, ERα and ERβ, that mediate the biological effects of estrogens and antiestrogens. Different ligands induce different ER conformations.
- Different mechanisms of target gene regulation affect the agonist–antagonist profile of a ligand. Selective Estrogen Receptor Modulators (SERMs) have a tissue- and gene-specific mixed agonist–antagonist effect.
- Both ERα and ERβ are expressed in human breast cancer. Measurement of both ERα and ERβ is suggested for selection of appropriate breast cancer therapy.
- Both ERα and ERβ are important for normal ovarian follicular development and female fertility.
- ERβ-selective agonists may protect from abnormal prostate growth and may be the therapy of choice for urge incontinence.
- Available data suggest that ERα plays an important role in bone maturation and homeostasis in both men and women, but that ERβ also has a specific role in bone physiology in women.
- The estrogen receptors α and β are expressed in vascular endothelial cells, smooth muscle cells, and in myocardial cells. Beneficial effects of estrogens on cardiovascular function and reactivity stem from direct effects on cells in the vascular system but also from effects on liver and circulating monocytes–macrophages.
- Estrogens are linked to a variety of functions in the central nervous system (CNS): learning, memory, awareness, fine motor skills, temperature regulation, mood, reproductive functions, and depression. The predominant expression and localization of ERβ in rat neocortex, hippocampus, and nuclei of the basal forebrain suggests an important role for ERβ in learning and memory.
- Estrogen and inhibins produced by the ovaries are important feedback regulators of the hypothalamo-pituitary axis and the serum levels of LH and FSH. ERα seems to be more involved in the LH, FSH feedback loop than ERβ.
- ERα and ERβ subtype-selective SERMs may better provide the benefits of estrogen replacement therapy (third generation HRT) than currently used treatments.

EXTENDED SUMMARY

Introduction: Nuclear receptors such as the estrogen receptor (ER) are ligand-dependent transcription factors. There are two different ER subtypes, ERα and ERβ, that mediate the biological effects of estrogens and antiestrogens. ERβ exists in multiple isoforms. Different ligands induce different ER conformations, and there is a dramatic difference in the topology of the ER surface between agonist- and antagonist-bound re-

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ceptor. Coactivators and corepressors interact with ligand-bound ERα and -β and play, together with the receptor, an important role in the regulation of ER target-gene expression. Different modes or mechanisms of target gene regulation affect the agonist/antagonist profile of a ligand. Selective Estrogen Receptor Modulators (SERMs) have a tissue- and gene-specific mixed agonist/antagonist effect. Alternative indirect activation pathways, other than binding of natural or synthetic small organic hormones or drugs, can also modulate the ER activity. Estrogens have also very rapid effects, so-called nongenomic effects. Nonreceptor-dependent antioxidant effects by estrogens have been reported, protecting from neurodegenerative disorders or atherogenesis.

Breast Tissue: There is no pubertal breast development in aromatase-deficient women due to lack of or too-low levels of circulating estrogens. Estrogen therapy of aromatase deficient female patients led to normal pre- and postpubertal breast development. ERα has been shown to be necessary for mouse mammary gland development. ERβ is abundantly expressed in rat breast. Both ERα and ERβ are present in human breast cancer. Measurement of both ERα and ERβ is suggested for the selection of appropriate breast cancer therapy.

Urogenital Tract: ERβ is widely expressed in bladder, urethra, testis, prostate, and ovary in the mouse. The absence of ERα results in infertility in both male and female mice. The absence of ERβ results in partial infertility in female mice but no impaired fertility in male mice. ERβ-deficient mice display hyperplasia, dysplasia, and PIN-lesions of the prostate. Deficiency of aromatase in human females led to ambiguous genitalia and polycystic ovaries. Estrogen replacement therapy of aromatase-deficient female patients led to the resolution of the ovarian cysts and menarche. Male patients with estrogen deficiency or estrogen insensitivity are reported with macroorchidism or oligozoospermia. ERβ-selective agonists may protect from abnormal prostate growth and may be the therapy of choice for urge incontinence.

Bone: Estrogens have an important role in maintaining a balanced bone metabolism. Estrogens protect postmenopausal women from bone loss and the development of osteoporosis. Estrogens may play an important role for the maintenance of bone mass also in aging men. Estrogens are important for the pubertal growth spurt and epiphyseal closure in girls as well as in boys. There are likely both direct and indirect (systemic) effects of estrogens on bone metabolism and homeostasis. Both ERα and ERβ are expressed in the bone-forming osteoblasts. Estrogen insensitivity in a male patient caused by ERα deficiency led to osteopenia and continuous longitudinal growth due to unfused epiphyses. Male and female patients with aromatase deficiency have increased bone turnover, delayed bone maturation, low BMD, and tall stature due to unfused epiphyses. Estrogen replacement therapy of both female and male aromatase-deficient patients resulted in growth spurt, closure of the epiphyses, and increased bone mineral density. Lack of ERβ expression in the female ERβ -/- mice led to a masculinized bone phenotype of the long bones but no effect on the bone phenotype in male mice. Available data suggest that ERα plays an important role in bone in both men and women, but that ERβ perhaps has a role in bone physiology only in women.

The Cardiovascular System: A number of gender-related cardiovascular differences have been reported, (1) lower risk for young women than for young men to develop atherosclerosis and cardiovascular disease, (2) higher prevalence of left ventricular hypertrophy in men than in women, (3) significantly greater intimal thickening after vascular injury in men than in women, and (4)

the rapid vascular response to estrogen in women but not in men. The estrogen receptors α and β are expressed in vascular endothelial cells, smooth muscle cells, and in myocardial cells. Both ERα and ERβ can mediate the vascular injury response to estrogens, suppressing smooth muscle cell proliferation and intimal thickening. Estrogens have both genomic and nongenomic effects on vascular tissue. Part of the beneficial effects of estrogen on cardiovascular function and reactivity comes from liver-specific effects of estrogens on the serum lipid/cholesterol profile. ERα most likely mediates the liver-specific effects of estrogens. Also, monocytes/macrophages are potential targets for the beneficial effects of estrogens on the cardiovascular system and the development of atherosclerosis.

Central Nervous System and the Hypothalamo-Pituitary Axis: Estrogens are reported to influence a variety of functions in the central nervous system (CNS) such as learning, memory, awareness, fine motor skills, temperature regulation, mood, and reproductive functions. Estrogens are also linked to symptoms of depression and treatment of depressive illness. Different brain structures and neurotransmitter systems are involved in the different effects of estrogens. The predominant expression and localization of ERβ in rat neocortex, hippocampus, and nuclei of the basal forebrain suggests an important role for ERβ in learning and memory. Estrogen, through effects on the hypothalamo-pituitary axis (HPA), modulates the expression and secretion of hormones such as LH, FSH, growth hormone (GH), and prolactin (PRL), from the anterior pituitary gland. Female and male patients with aromatase deficiency have elevated levels of LH and FSH, and elevated circulating levels of androgens. Substitution with conjugated estrogens in both male and female aromatase-deficient patients resulted in normalization of gonadotropin and testosterone levels. Clinical data on an ERα -/- male patient also showed increased circulating LH and FSH levels despite high estrogen levels. In ERα -/- mice the serum LH but not the FSH levels were elevated despite 10-fold higher circulating levels of estrogen. Available data indicate that estrogens rather than testosterone (both men and women) together with inhibins are the major regulators of serum gondotropin levels, and that ERα seems to be more involved in this process than ERβ.

Hormone Replacement: Traditional, Alternatives, and Future Perspectives: The most common regimens in use to treat symptoms of the menopause and postmenopausal health risks are 17β-estradiol, esterified estrogens or conjugated equine estrogens in combination with a progestin, for example, medroxyprogesterone acetate (MPA). The awareness of undesired effects and serious health risks (breast cancer, endometrial cancer, and venous thromboembolism) with existing hormone replacement therapy (HRT) (first generation HRT) calls for alternatives with improved safety profile. Alternative regimens for women who do not wish to take today's first generation HRT exist. Non-ER-subtype-selective SERMs (second generation HRT) display tissue-selective estrogen agonism. Although the most frequent and serious health risk of first generation HRT are set aside by these SERMs, they still suffer from low efficacy compared with first generation HRT, and they aggravate hot flushes. The existence of two ER subtypes, ERα and ERβ, gives the opportunity to develop ER subtype-selective ligands that will most likely better provide the benefits of estrogen replacement therapy, with an improved therapeutic profile (third generation HRT).

I. INTRODUCTION

Around 1960 Jensen and colleagues came to the conclusion that the biological

effects of estrogen had to be mediated by a receptor protein.[1] Since then, two estrogen receptor (ER) subtypes have been cloned, ERα[2] and ERβ.[3] The discovery of rat ERβ was rapidly followed by the cloning of ERβ from other species[4-6] and the identification of several ERβ isoforms with: (1) extended N-termini,[7,8] (2) a variant with an 18 amino acid residue insertion into the ligand-binding domain, with altered ligand-binding characteristics,[9,10] and (3) C-terminal splice variants unable to bind ligand or activate reporter gene transcription.[11,12] Also for ERα various alternatively spliced forms have been described.[13,14] The biological and physiological significance of different isoforms of ERα and ERβ is unknown and remains to be investigated further. Whether there is still another ER subtype, for example, ERγ, to be found remains an open question. However, the vascular protective effect of estrogen in the absence of ERα and ERβ[15] and the fact that ERα and ERβ double knockout mice (DERKO) survive to adulthood[16] may suggest that yet another unidentified estrogen receptor exists.

ERα and ERβ are similar in their architecture to the other members of the steroid–thyroid hormone superfamily of nuclear receptors[17,18] in that they are composed of independent but interacting functional domains: the N-terminal A/B domain, the least conserved among nuclear receptors, enables the receptor to interact with members of the transcriptional apparatus; C domain, devoted to binding to DNA, contains two zinc-binding motifs and a dimerization interface that mediates cooperativity in DNA binding; D domain, also referred to as a "hinge region", necessary to give the receptor some degree of flexibility between the DNA and the ligand binding domains, binds heat shock protein hsp 90 and probably harbors the sequence representing the nuclear localization signal; E/F multifunctional domain recognizes and binds ligand and is involved in receptor dimerization and interaction with transcription factors and cofactors.[19] The gene modulatory effect of a receptor following binding of a ligand depends on the conformational change of the receptor induced by the ligand and the subsequent events, including the release of inhibitory proteins (heat shock proteins), receptor dimerization, receptor:DNA interaction, recruitment of and interaction with co-activators and other transcription factors, and the formation of a preinitiation complex.[20]

There are in particular two regions in the ERα that participate in transcriptional activation of target genes by forming protein:protein contacts with other transcription factors or coactivators, the ligand-independent N-terminal activation function-1 (AF-1) and the C-terminal ligand-dependent activation function-2 (AF-2).[17,20-23] Synthetic ligands with mixed agonist–antagonist activity, so-called SERMs (selective estrogen receptor modulators), such as tamoxifen and raloxifene, display a low but significant partial estrogen agonist activity via ERα on an estrogen-responsive-element (ERE).[24] In contrast, these mixed agonists–antagonists display pure antagonism via ERβ on an ERE site. It has been shown that the partial agonism of the SERM tamoxifen by ERα is mediated by a slightly different part of the ERα AF1 region than required for estradiol (E2) signaling.[25] The pure antagonism of tamoxifen by ERβ was recently explained by the lack in hERβ of this particular function of hERα AF1.[26] Thus, differences in the amino-terminal regions of ERα and ERβ most likely explain the differences in their response to mixed agonists–antagonists such as tamoxifen and raloxifene on an ERE site.

The ER ligand binding domain, similar to other nuclear receptors, is made up of 12 α-helices, named H1-H12. Helix 12 (H12) together with amino acid residues in helices

H3, H4, and H5 constitute the AF-2 coactivator recruitment and interaction surface.[20,27] The resolution of 3D structures of ERα in complex with agonists and antagonists has given a molecular mechanism for agonism and antagonism, respectively.[28–30] When the ER ligand binding domain (LBD) is complexed with estradiol or diethylstilbestrol (DES), H12 is positioned over the ligand-binding pocket, generating the AF-2 surface that promotes interaction with coactivators[29] and transcriptional activation. In contrast, in the ERα- or ERβ-LBD raloxifene complexes[28,30] or in the ERα-LBD 4-OH-tamoxifen complex,[29] H12 was instead positioned in the hydrophobic cleft formed by H3, H4, and H5, foiling the coactivator interaction surface. It is evident that different ligands induce different receptor conformations,[31,32] and that different conformations of the receptor affect the efficiency by which coactivators and corepressors interact with the liganded receptor[29] and consequently the agonist–antagonist profile of ligands.[33]

During recent years there has been a strong focus on the cloning and characterization of nuclear receptor coactivators, corepressors, and their associated histone acetyl transferases (HATs) or deacetylases, respectively.[20] Integrator molecules like CBP/p300 and coactivator and corepressor proteins form protein:protein complexes with liganded nuclear receptors, bringing HATs or deacetylases in juxtaposition to chromatin. These events play a key role in the transcriptional regulation of target genes by liganded nuclear receptors and determine the final outcome on target gene expression.

The presence of different ER subtypes and isoforms, different coactivators and corepressors, and our increasing knowledge of mechanisms by which ERα and ERβ, respectively, can modulate target gene expression, have significantly added to our understanding of the physiology and pharmacology of estrogens and antiestrogens, and have given a plausible explanation why antiestrogens and SERMs sometimes behave more like estrogen agonists than estrogen antagonists. Initially it was believed that the ER affected the transcription of estrogen-sensitive genes only by direct binding of the ligand-activated receptor to EREs on DNA. Today we have learned that ERα and ERβ can modulate the expression of genes also in an indirect manner, either by blocking the ability of a transcription factor to bind to its response element on DNA,[34] thereby inhibiting gene expression, or by stimulating gene expression by indirect binding of ER to DNA response elements through protein:protein interaction with other transcripton factors.[35–38] The different mechanisms by which ERα and ERβ modulate gene expression have changed our view on the pharmacology of estrogens and antiestrogens. The terms agonism and antagonism should be used with care. Natural or synthetic hormones that we today categorize as estrogen agonists and estrogen antagonists should perhaps not, in general terms, be categorized in this way. The reason for this caution is exemplified by the transcriptional effect of ERα and ERβ via an AP1 site in the presence of estrogens and antiestrogens.[39] With ERα, typical agonists such as estradiol and diethylstilbestrol but also the antagonist tamoxifen function as equally efficacious agonists in the AP1 pathway, the antagonist raloxifene being only a partial agonist. In contrast, with ERβ, estradiol acted as an antagonist inhibiting the agonistic activity of the two antagonists tamoxifen and raloxifene.[39]

In addition to the classic activation of the ERs by natural or synthetic hormones, alternative, indirect activation pathways of the estrogen receptors (at least α) in the

absence of ligand has been described[40–43] as a consequence of the activation of membrane receptors like those for IGF-I,[44] EGF,[45] and TGF and dopamine.[46] The exact mechanism involved in this process is still debated. Several studies utilizing specific inhibitors of trandsduction signals like ras, protein kinase A, and C (PKA and PKC) have clearly shown that the full activity of these molecules is essential for unliganded ER activation. Furthermore, point mutation studies have shown that two serines located in the N-terminal A/B domain are required for this process.[42] These findings indicate that phosphorylation of ER or of molecules interacting with ER is involved in transcriptional activation of unliganded estrogen receptor. More studies are necessary, particularly to better define whether this mechanism is conserved among different cell types. In fact, studies carried out in various cell systems favor the hypothesis of differential mechanisms depending on the system taken into consideration.[47,48] The current hypothesis on the physiological significance of these alternative pathways implies that they may be of relevance in those phases of embryo development in which neither estradiol nor its metabolites are available (e.g., during the maturation of the reproductive and the nervous systems).[47] These mechanisms might also be of pharmacological interest, for example, in the treatment of neoplastic forms that express the ER but that have lost the responsiveness to treatment with ER antagonists (e.g., certain type of mammary carcinomas).

Another emerging and potentially important pathway is constituted by the very rapid so-called nongenomic effects of ligands to nuclear receptors.[49] In endothelial cells, estrogen-ER complex-mediated membrane effects led to sequential activation of ras, raf, mitogen-activated protein kinase kinase (MEK), and, subsequently, activation of mitogen-activated protein kinase (MAPK).[50] It is proposed that this may lead to the activation of endothelial nitric oxide synthase (eNOS) and the stimulated release of nitric oxide (NO). In neurons membrane effects by estrogen lead to the stimulation of src, ras, MEK, and MAPK, resulting in neuroprotection, and in the bone-specific osteoblasts the membrane effects of estrogen may be involved in control of apoptosis, cell proliferation, and differentiation.[50]

An important aspect of the physiology and pharmacology of estrogens that does not require the presence of the receptor protein is their described antioxidant effects, suggested to offer protection from neurodegenerative disorders caused by oxidative stress, as in Alzheimer's disease, or atherogenesis due to excess uptake of oxidized low density lipoproteins (LDL) in the vascular wall.[51,52] Components of conjugated equine estrogens have also been tested for their antioxidant effects.[51] Equilin and derivatives thereof were reported to be better antioxidants than estradiol at inhibiting peroxidation of fatty acids and cholesterol in LDL particles. Other agents reported to exert neuroprotective or antiatherogenic effects caused by oxidative stress are phenolic compounds, vitamin E, insulin-like growth factor-1, and mifepristone (RU486).[51–55] In the context of antioxidant effects, it is appropriate to mention also that antiestrogens (trans-hydroxytamoxifen, tamoxifen, and ICI 182,780) but not E2 have been shown to activate the transcription of the quinone reductase gene and to increase NAD(P)H:quinone oxidoreductase enzyme activity via an electrophilic/antioxidant response element (EpRE/ARE).[56] Furthermore, E2 inhibited the agonistic effect of the antiestrogens, and ERβ was shown to be more efficacious than ERα in stimulating EpRE/ARE-containing reporter gene expression.[35] These findings suggest that antiestrogens are also potent antioxidants

and stimulators of phase 2 detoxification enzyme genes, protecting cells from damage by radicals and other toxic byproducts of metabolic oxidation.

ERα gene polymorphisms may also provide important information about the physiology of estrogen action. Different ERα polymorphic forms have been linked to increased litter size,[57] breast cancer susceptibility,[58] bone mineral density and osteoporosis,[59] hypertension,[60] spontaneous abortion,[61] and body height.[62]

II. BREAST TISSUE

The importance of estrogens in the development of female breast tissue is well documented. Female aromatase-deficient patients, unable to convert C_{19} steroids (e.g., testosterone) to estrogens, showed no sign of breast development at the onset of puberty.[63] However, the administration of estrogen to the two described female patients led to normal pre- and postpubertal breast development.

ERα knock-out (ERKO or ERα-/-) female mice have lost their capacity to develop mammary gland tissue beyond the embryonic and fetal stages despite elevated levels of circulating estrogens (17β-estradiol). This impairment of breast development has been attributed to the lack of both direct and indirect (regulation of growth factor, e.g., EGF, and progesterone receptor [PR] expression) stimulatory effects of estradiol on breast epithelial and stromal tissues, due to missing ERα expression.[64] Applying tissue recombinant experiments, making a series of wild-type and ERα-/- breast stromal and epithelial tissue combinations, it was concluded that ERα expression in the stromal cell layer was essential for growth stimulation of the ductal epithelium in the mammary gland in mice.[65] Furthermore, prolactin (PRL) also expressed and secreted from the anterior pituitary plays a crucial role in mammary gland physiology. In ERKO female mice the expression of PRL from the anterior pituitary, and the circulating levels of PRL in serum, are decreased by 20- and 5-fold, respectively. Therefore, it is conceivable that the impaired mammary tissue development in ERKO female mice is also due to decreased levels of PRL, in part due to lack of ERα expression in ERKO pituitary.[57]

More than 70% of primary breast cancers in women are ER (should be read ERα) positive and show estrogen-dependent growth that undergoes regression when deprived of supporting hormones. Patients whose breast tumors lack significant amounts of ERα rarely respond to endocrine ablation or treatment with antiestrogens, whereas most patients with ER-containing cancers benefit from such treatment.

Immunochemical determination of ER in tumor biopsies has become a routine clinical procedure on which the choice of therapy is based. However, the currently available immunochemical procedures for ER measurements are based on ERα-specific antibodies that do not detect ERβ protein (unpublished observations).

ERβ mRNA and protein, together with ERα mRNA and protein, have been detected in human breast cancer biopsies and in human breast cancer cell lines.[66] With the use of receptor-specific antibodies, both ERα and ERβ were found to be expressed in the normal rat mammary gland but the presence and cellular distribution of the two receptors was distinct.[67] Furthermore, while the level and number of cells expressing ERβ was more or less constant during prepubertal and pubertal stages and throughout pregnancy, lactation and postlactation, the level and percentage of ERα-containing cells varied dramatically. The possible role of ERβ in normal breast tissue development and

physiology, or in breast cancer development and/or therapy, is, however, as yet unknown.[68]

III. UROGENITAL TRACT

ERα and ERβ are both expressed in uterus, ovary, testes, and prostate, but with different cellular localization. In ovary ERα is mainly expressed in thecal cells and in prostate mainly in the stroma compartment. ERβ, in its turn, was found to be expressed mainly in glandular epithelium of the uterus, in ovary primarily in the granulosa cells, and mainly the epithelium of the testes and prostate.

Aromatase deficiency in female patients led to excess circulating androgens in the fetus and at puberty, resulting in virilization and ambisexual development. The two aromatase-deficient female patients described[63] were reported with ambiguous genitalia at birth, a phenotype that was further pronounced at pubertal age, and with polycystic ovaries, characterized by a disproportionate number of atretic follicles and dense fibrotic subcortical stroma. The elevated serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in these patients, as a result of perturbed estrogen-dependent negative feedback on gonadotropin production, were suspected to be the cause of the polycystic ovaries. Estrogen replacement in these affected female patients led to normalized gonadotropin and androgen levels, resolution of the ovarian cysts, and menarche.[63] Like female aromatase-deficient patients, aromatase knock-out (ArKO) female mice had low serum estrogen levels and high testosterone and gonadotropin levels.[69] Female ArKO mice also displayed genital anomalies with underdeveloped external genitalia and uteri, and the ovaries contained numerous follicles that appeared arrested before ovulation. The stroma of the ovaries was found to be hyperplastic with structures that appeared to be atretic follicles. No corpora lutea were present.[69]

Male patients with either defective estrogen production[63] and the male patient with estrogen insensitivity caused by a nonsense mutation in the ERα gene[70] are reported to have macroorchidism or oligozoospermia and/or decreased sperm viability or motility. The fertility of the aromatase-deficient or estrogen-resistant male patients is not known. Male ArKO mice were initially fertile but developed progressive infertility[71] due to arrested spermatogenesis. These findings suggest that estrogen has direct effect on male germ cell development and fertility.

Deletion of ERα gene in mice results in infertility in both females and males. ERα-/- female mice show complete infertility with hypoplastic, estrogen-resistant uteri, and hyperemic ovaries with no ovulatory capacity.[64] Similar to the aromatase-deficient female patients ERKO female mice also have elevated testosterone and LH levels. Treatment of these mice with a gonadotropin releasing hormone (GnRH) antagonist reduced the serum levels of LH and reverted or prevented the cystic ovarian phenotype,[72] in agreement with the disappearance of polycystic ovaries in aromatase-deficient female patients following estrogen substitution and subsequent normalization of serum gonadotropin levels. To challenge the ovulatory deficiency of ERKO female mice, immature mice were treated with exogenous gonadotropins. Although the ovulatory capacity was reduced compared with age-matched wild-type mice, the collected oocytes were fully competent to undergo successful *in vitro* fertilization,[72] suggesting that ERα is not critical for follicle maturation and ovulation.

Also, ERβ knock-out mice (BERKO or ERβ-/-) have been generated. Female BERKOs of these animals showed reproductive defects as well (20% of normal fertility),[64] while males showed normal fertility. The LH and estrogen levels in BERKO females are comparable to the wild type, but their fertility is compromised due to reduced ovarian efficiency. Superovulation of BERKO female mice exhibited several mature but unruptured follicles. The number of corpora lutea was considerably smaller than in wild-type mice, suggesting an attenuated response to the ovulatory hormone surge in the absence of ERβ.

Female mice unable to express either ERα or ERβ, double ER knock-out mice (DERKO), exhibited normal reproductive tract organ development but were, as expected, infertile.[16] Similar to ERKO female mice, the DERKO females showed uterine hypoplasia but no polycystic ovaries. The ovaries of prepubertal DERKO females displayed precocious maturation evidenced by multiple large antral follicles not observed in control wild-type females. The very high serum levels of LH in these animals explain the prepubertal precocious ovarian phenotype of the DERKOs.[16] The ovarian phenotype of the adult DERKO female was distinct from the ERKO and BERKO female phenotypes, most notably by the presence of structures resembling seminiferous tubules of the testis. The sex reversal of the adult DERKO ovary phenotype was judged to be caused by a redifferentiation of ovarian components rather than by a developmental phenomenon.[16] In summary, based on the ovarian phenotype in DERKO females it was concluded that both ERα and ERβ are required for the maintenance of germ and somatic cells in the postnatal mouse ovary.

Male ERKO mice are infertile with atrophy of the testes and seminiferous tubule dysmorphogenesis resulting in decreased spermatogenesis and inactive sperm.[64] Recently, a more detailed study of the cause of the infertility of male ERKO mice provided biological evidence that ERα plays an important role in the reabsorption of luminal fluid from the efferent ductules during the transit of spermatozoa from the testis to the head of the epididymis.[64] Concentration of sperm is claimed to improve their survival and maturation during epididymal storage. Administration of high levels of estrogen to men is known to cause infertility. Thus, another possible explanation that may contribute to the nonreproductive phenotype of ERKO male mice is the relatively high levels of estrogens in ERKO mice and the presence of ERβ in seminiferous epithelium, spermatids, and spermatocytes, causing infertility by a direct action on the testes. In contrast to male ERKO mice, male BERKO mice are fertile,[64] suggesting a different role for ERβ compared with ERα in the male reproductive system. As expected, DERKO male mice are infertile, with an 80% reduction in the number of sperm produced in the testis.[16]

Estrogens are claimed to be effective in the treatment of urge incontinence in postmenopausal women. Recently, it has been shown that ERβ is highly expressed in the inner epithelial cell layer of the rat bladder and urethra.[73] These results may explain the beneficial effect of estrogens in urinary incontinence and suggest that female patients with urinary incontinence might benefit from ERβ-selective agonist therapy.

Estrogens have also been linked to prostate disease. In different species it has been shown that estrogens synergize with androgens in inducing glandular hyperplasia and dysplasia and adenocarcinoma in the prostate.[74] Immunohistochemical studies revealed that ERβ is the predominant ER subtype in prostate, located in the epithelial cells along the ductal network of the prostate. ERα has been detected only in the

stromal compartment of the prostate.[73–75] It has been suggested that ERβ is regulated by androgens in the prostate because the abundance of ERβ mRNA was rapidly reduced following castration but restored after testosterone replacement.[76] Exposure of wild-type mice to the estrogen 5α-androstane-3β,17β-diol, a metabolite of dihydrotestosterone, caused a decrease in the level of androgen receptor (AR) in the prostate.[75] In ERβ-/- mice, however, the level of AR is elevated and 5α-androstane-3β, 17β-diol was without effect, suggesting that the AR gene is an ERβ target in the prostate.[75] Exogenous estrogens have a negative effect on epithelial cell differentiation, ductal morphogenesis, and prostate growth,[74] and prostate of adult rats neonatally exposed to estrogens shows hyperplasia, dysplasia, and presence of *in situ* carcinoma. It was hypothesized that ERβ is a marker of epithelial differentiation and that its decline in epithelial cells in neonatally estrogenized rats is a result of altered epithelial cell differentiation.[74,76] ERβ-/- mice display signs of prostatic hyperplasia with aging,[75] suggesting that ERβ may protect against abnormal prostate growth.

IV. BONE: DEVELOPMENT AND HOMEOSTASIS

It is well established that estrogens exert an important influence on bone physiology; clinically this is manifested by the occurrence of osteoporosis in postmenopausal women. There is also compelling evidence that estrogens protect postmenopausal women from bone loss and the development of osteoporosis, maintaining a balance between bone resorption and bone formation.[77,78] The level of estrogens may play a more important role than testosterone for the maintenance of bone mass also in ageing men,[79,80] showing a positive correlation between bone mineral density (BMD) and serum estradiol concentrations rather than testosterone levels.

As in other tissues, there are most likely both direct and indirect effects of estrogens in maintaining a balanced bone metabolism. The likelihood of important direct effects of estrogens on bone is based on the presence of ERα in the bone-forming osteoblasts[81,82] and in the bone-resorbing osteoclasts.[83] ERβ mRNA has been found in primary rat osteoblasts and in rat osteosarcoma cells,[84] and in immortalized human fetal osteoblasts.[85] Evidence for indirect effects of estrogens on bone metabolism stems from studies in mice, rats, and humans,[86–91] suggesting a coupling and cooperativity between growth hormone (GH) and estrogen in bone metabolism. Taken together these studies indicate that estrogen substitution can increase the circulating levels of GH[87] and the levels of GH receptor on osteoblasts,[90] and that there is a mutual dependence of GH and estrogen action on bone growth, mineral density, and maintenance.[86,88,89,91] In addition, estrogens may have indirect effects on osteoclast differentiation, maturation, and activity by inhibition of cytokine expression,[92–95] and via stimulation of osteoprotegerin expression from human osteoblasts.[96] The importance of these interactions for maintenance of bone health, however, still needs to be evaluated.

Despite approximately 10-fold higher levels of circulating estradiol in ERα-/- mice there was a significant decrease in the length and size of femur in the females but only slight decrease in males.[64] In contrast, the decrease in BMD and bone mineral content (BMC) was more pronounced in ERKO males than females.[64] In ERβ knock-out mice (BERKO) the bone phenotype of male mice was unaffected compared with wild-type male mice, while there was a masculinization of the long bones (femur) in the female BERKO mice.[97] Lack of ERβ expression in

the female BERKO mice led to increased length of the femur, thicker cortical bone (increased BMC due to increased periosteal circumference), and increased size of the vertebrae, approaching the corresponding characteristics of wild-type male mice. There was no effect on trabecular architecture or BMD in the male or female BERKO mice. Ovariectomy of female mice leads to loss in trabecular BMD to a similar extent in both BERKO and wild-type animals,[97] suggesting an important role for ERα in the maintenance of trabecular BMD and architecture in mice. A further support for the importance of ERα in bone physiology was obtained from examination of the male estrogen-insensitive patient.[70] Similar to the ERKO mice, he had elevated levels of LH, FSH, and estrogen. Despite the elevated levels of estrogens, he suffered from low BMD and continuous linear growth because of unfused epiphyses, suggesting an important role for ERα also in human bone biology. The decrease in length and size of femur in the female ERKO mice may be indicative of an effect of ERβ in the presence of excessive amounts of estradiol or metabolites thereof. The possible effects of disrupted ERα and ERβ expression in ERKO and BERKO mice, respectively, on GH expression and consequences thereof for the bone phenotype in these animals are not yet known.

Male and female patients with aromatase deficiency[63] have increased bone turnover, delayed bone maturation, low BMD, and tall stature due to unfused epiphyses. They have elevated circulating levels of androgens, FSH, and LH but very low or undetectable levels of estrogen. Estrogen replacement therapy of both female and male aromatase-deficient patients resulted in growth spurt, closure of the epiphyses, and increased bone mineral density,[63] suggesting a very important role for estrogens not only in females but also in males.

The pubertal growth spurt starts earlier in girls than in boys,[98] beginning at midpubertal stage in boys. Furthermore, the average duration of pubertal growth spurt in girls is shorter than in boys, possibly explained by higher levels of estrogen in prepubertal girls than in prepubertal boys, hypothesized to drive a more rapid skeletal maturation and epiphyseal closure in girls than in boys. Using an ultrasensitive assay for determination of serum estrogen levels, the rise and decline in estrogen levels in boys have been assessed in correlation to age, pubertal growth peak velocity, bone maturity, and epiphyseal closure.[99] In this study it was found that there was a close correlation between rise in estrogen level and the rise in the level of testosterone, and that the rise in estrogen level correlated with time of peak growth velocity.[99] Following growth spurt in these boys there was a further increase in estrogen levels that were sustained toward the end of puberty, hypothesized to accelerate epiphyseal fusion.[99]

VI. THE CARDIOVASCULAR SYSTEM

Women's risk to develop cardiovascular disease at an early age is lower than for men. However, the cardiovascular disease risk increases with age also for women, approaching the same incidence rate as for men at the age of 65 and older. Based on observational epidemiological studies, menopausal hormone therapy seems to have a cardiovascular protective effect in postmenopausal women, decreasing the risk to develop atherosclerosis and cardiovascular disease.[100–102] However, the cardio-protective effect of estrogens is debatable, as no such data from clinical trials are available yet. On the other hand, the outcome of the HERS study,[103] which

did not show any overall cardiovascular benefit in postmenopausal women (treated with oral conjugated equine estrogen plus medroxyprogesterone acetate) with established coronary heart disease (CHD), can be explained on the basis of recent findings, according to which medroxyprogesterone antagonizes positive effects of estrogens on the vasculature. The recommendation drawn from HERS was not to initiate estrogen treatment for the purpose of secondary prevention of CHD. However, fewer CHD events were observed over time in the hormone treatment group, in comparison with the placebo group and therefore it was also concluded that women already on menopausal hormone therapy may very well continue the treatment.

The estrogen receptors α and β are expressed in vascular endothelial cells,[102] smooth muscle cells,[104] and in myocardial cells.[105] A number of direct effects of estrogen on vascular tissue have been reported:[101,102,16,107] nongenomic vasodilatation as an effect of estrogen on ion-channel function[108] and nitric oxide (NO) synthesis,[109] long-term effects by modulation of, for example, prostaglandin synthase, NO synthase and endothelin gene expression,[105,110] regulation of AT1 receptor density on vascular smooth muscle cells,[111] and inhibition of injury-induced vascular intimal thickening.[112] Furthermore, reduced heart contractility in ovariectomized female rats was normalized following estrogen replacement,[113] an effect, in part, explained by estrogen-mediated changes in expression of contractile proteins.[106,114]

Besides a higher risk for men to develop atherosclerosis and cardiovascular disease at an early age compared with women, there are also other gender-related cardiovascular differences reported. Men have a higher prevalence of left ventricular hypertrophy than women,[106,115,116] hypothesized to be an effect of the difference in the level of circulating estrogens in men compared with women[106] but possibly also to gender-specific differences in estrogen receptor levels and in the induction of endogenous gene expression in cardiac myocytes in response to estrogen.[105,106] In rats, intimal thickening after vascular injury is significantly greater in males than in females,[107] although male level of intimal thickening was obtained in female rats after ovariectomy, an effect that was reversed by estrogen therapy.[112] The primary inhibitory effect of estrogen on intimal thickening was found to be performed by its direct effect on vascular smooth muscle cells, inhibiting their migration and proliferation.[112] Still another gender difference is the rapid response to estrogen after acetylcholine-induced coronary arterial constriction in men and women with coronary artery disease.[117] In the female patients, administration of estrogen reversed the constriction response to acetylcholine, while there was no response to estrogen in male patients. Furthermore, coronary blood flow was significantly enhanced in the presence of estrogen in the female patients but again with no response to estrogen in the men. A plausible explanation for these differences may be that the vascular endothelium in women produces more nitric oxide in response to estrogen than in men.

The specific role of ERα and ERβ in maintaining normal cardiovascular function and in prevention of the development of atherosclerosis and CVD is still largely unknown. However, disruption of the ERα gene, as in ERα knock-out mice (ERKO), showed a reduced production of NO.[64] ERα also seems to be involved in neovascularization, as there was no angiogenic response to estrogen in ERKO mice.[64] An increased number of L-type Ca^{2+} channels were reported in ERKO male mice,[64] suggesting an involvement of estrogen and the ERα in the regulation of cardiac excitabil-

ity. In the man bearing an ERα nonsense mutation[64] there was absence of endothelium-dependent vasodilatation of the carotid arteries following ischaemic cuff occlusion.[118] However, this lack of ischemic response may relate more to atherosclerosis-induced endothelial dysfunction than to a direct consequence of lack of ERα-mediated responses in the vascular endothelium.[107,119] Sublingual estrogen delivery did cause a rapid vasodilatory response in the ERα-deficient man,[118] possibly suggesting a role for ERβ in rapid vascular responses to estrogen. In contrast, there was no negative effect of vascular endothelium-dependent vasodilatation in ERα-/- mice.[64] Estrogen treatment protects against vascular injury, suppressing smooth muscle cell proliferation and intimal thickening, in ERKO mice.[64] The expression of ERβ but not ERα was dramatically increased in wild-type mice after vascular injury.[102] These data suggest an important role for ERβ in vascular injury response and protection. However, a similar estrogen-dependent vascular injury protection was also seen in ERβ knock-out mice (BERKO), suggesting an ERα and ERβ redundancy in vascular injury protection or that an unknown signaling pathway or a still unidentified estrogen receptor is involved.[102] It should be added in this context that the estrogen-resistant man[70] showed intimal thickening of the common carotid arteries[118] despite elevated circulating levels of estrogen.

Part of the beneficial effects of estrogen on cardiovascular function and reactivity comes from liver-specific effects, regulating serum lipid–cholesterol levels.[101] Estrogens increase the level of apolipoprotein A1 in postmenopausal women.[120] Apolipoprotein(a), the major protein component of the atherogenic lipoprotein(a), is downregulated in the liver by estrogen at the mRNA level, resulting in decreased plasma levels of Lp(a).[121] Estrogen also increases the expression of angiotensin in postmenopausal women,[122] regulates the level of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase at the protein level, and increases low-density lipoprotein (LDL) receptors on the surface of liver cells.[101] Regulation of the apoE gene by estrogen has been demonstrated in rat[123] and mouse.[64]

Like in skeletal tissue, growth hormone (GH) may play an important role for estrogen effects also in liver,[91] hypophysectomy blunting the cholesterol-lowering effect of estrogen in ovariectomized rats.[91] It has been shown that liver in female rats has more GH receptors than in male rats,[124] and that liver GH receptor expression positively correlates to estrogen status in male and female rats.[125]

So far, ERα but not ERβ has been shown to be expressed in liver,[126] thus most likely all effects of estrogen reported on liver-specific gene expression are ERα mediated. Further support for the physiological role of ERα and estrogens in the regulation of liver-specific gene expression and lipid–cholesterol homeostasis stems from the analysis of the ERα-deficient[70] and the aromatase-deficient patients[63] and from the ERKO mice,[64] demonstrating glucose intolerance and lipid abnormalities as a consequence of estrogen resistance or estrogen insufficiency.

In addition to liver and cardiovascular cells monocytes–macrophages are also involved in health and disease of the cardiovascular system.[127,128] Several studies, both in vitro and in vivo, have indicated that growth factors and cytokines that mediate the critical processes of inflammation and wound healing also play a central role in vascular disease and during the initiation and progression of atherosclerosis. The cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) have been implicated in the processes of vascular injury and atherogenesis.[129] Both ERα and ERβ are reported to be expressed in monocytes–macrophages.[130,131] Estradiol has been shown to

inhibit LDL oxidation and cholesteryl ester formation and accumulation in macrophages,[132] and to reduce the uptake of acetylated LDL into macrophages, resulting in reduced rate of foam cell formation.[133] Estrogen downregulates the expression of TNFα in human macrophages[134] by a mechanism that involves ERβ but not ERα.[131] These results suggest that monocytes–macrophages are also potential targets for the protective effects of estradiol on the cardiovascular system and the development of atherosclerosis.[128]

VI. CENTRAL NERVOUS SYSTEM AND THE HYPOTHALAMO-PITUITARY AXIS

Estrogens are reported to influence a variety of functions in the central nervous system (CNS) such as learning, memory, awareness, fine motor skills, temperature regulation, mood, and reproductive functions.[135] Estrogens are also linked to symptoms of depression and treatment of depressive illness.

Different brain structures and neurotransmitter systems are involved in the different effects of estrogens.[135] The serotonin (5-HT) system, with neurons projecting from the dorsal and medial raphe of the midbrain/brainstem raphe nuclei to multiple forebrain areas such as the hypothalamus, hippocampus, and cortex,[135] is involved in the modulation of reproduction, mood, sleep, and cognition. Serotonin levels and activity in CNS are altered by serum estrogen fluctuation in rodents and estrogen substitution of ovariectomized rats positively affects the serotonergic system.[135,136] Estrogen has been reported to increase the expression of tryptophan hydroxylase (TPH), the rate-limiting enzyme for serotonin synthesis,[137] and to suppress the expression of the serotonin reuptake transporter (SERT) in raphe nuclei of ovariectomized monkeys.[138] Estrogen reduced the level of the 5-HT_{1A} autoreceptor subtype in the dorsal raphe nucleus of spayed monkeys[139] and reduced agonist stimulated 5-HT_{1A} receptor inhibition of dorsal raphe neuron firing in rats,[140] suggesting that estrogen may facilitate 5-HT neurotransmission.[139] The level of postsynaptic 5-HT_{1A} receptors, mainly localized in the limbic brain areas and the cerebral cortex, are also affected by estrogen.[141] 5-HT_{2A} receptors, suggested to be involved in the control of hormone and transmitter release, control of sexual activity, regulation of sleep, motor behavior, and psychiatric disorders such as anxiety and depression,[142] are positively regulated by estrogen in the dorsal raphe, olfactory bulb, and cerebral cortex.[143–145] Also in male rats 5-HT_{2A} receptor levels are regulated by estrogen.[146] ERβ mRNA within the dorsal raphe of the rat has been reported,[147] and an ERα immunoreactive protein has been detected in neurons adjacent to serotonergic cells in rat dorsal raphe.[135] An autoradiographic study in ERα-/- mice indicated the abundant presence of ERβ in mouse dorsal raphe,[148] and no ERα protein has been detected in the macaque raphe,[135] suggesting that ERβ may play a more important role than ERα in mediating estrogen effects on the serotonergic system.

The dopaminergic system, involved in motor function, motivation, reward, cognition, and hypothalamic-pituitary control is also affected by estrogen.[149,150] Dopamine levels and turnover fluctuate during the estrous cycle,[151] and the administration of estrogen, following ovariectomy, potentiates the release of dopamine.[152,153] Estrogen also increases dopamine transporter binding sites in the striatum[154] as well as the densities of dopamine receptors D_1 and D_2.[155] In the hypothalamus the dopaminergic tuberoinfundibular neurons inhibit prolactin release from the anterior pituitary by

release of dopamine into the hypophyseal portal system, an effect that is inhibited by estrogen.[156]

The basal forebrain cholinergic neurons project to the cerebral cortex and hippocampus and are implicated in learning and memory.[135] Long-term ovariectomy results in impaired learning due to decline in high-affinity choline uptake and choline acetyl transferase (ChAT) activity in rats.[135] Estrogen substitution following ovariectomy in rats induced ChAT enzyme levels and increased ChAT activity in the basal forebrain and possibly ChAT activity also in projection areas ending in cerebral cortex and hippocampus.[135] It has also been shown that ChAT mRNA levels fluctuate in the basal forebrain cholinergic neurons during the estrous cycle in the rat.[135] The colocalization of ERα with nerve growth factor (NGF) receptors in cholinergic neurons of the rat basal forebrain[135] and the stimulation of estrogen of both NGF receptor mRNA and ChAT mRNA in the rat basal forebrain[135] suggests a possible role for ERα in learning and memory functions. However, the predominant expression and localization of ERβ in rat neocortex, hippocampus, and nuclei of the basal forebrain suggests an important role for ERβ in learning and memory.[147,157] This assumption is supported further by the maintained normal memory and learning function in ERKO mice.[64] In a recent study on the human brain, the predominant presence of ERβ message in the hippocampal formation, entorhinal cortex, and thalamus suggests a putative role of ERβ in cognition, memory, and motor functions.[158]

Additional transmitter systems shown to be influenced by estrogens are, for example, vasopressin and oxytocin,[159,160] somatostatin,[161] galanin,[162] the γ-aminobutyric acid (GABA) system,[163] and the glutamate system.[164,165]

The expression patterns of ERα and ERβ, based on mRNA, autoradiographic, or immunohistochemical studies of rat and mouse brain, indicate a more abundant or distinct presence of the two ER subtypes in certain areas of the brain but also areas where they seem to overlap. ERα seems to be more abundant in the hypothalamus (preoptic, arcuate, periventricular, and ventromedial nucleus) and amygdala (amygdala hippocampal area, medial, and cortical nucleus).[147,166] A high level of ERβ mRNA has been found in the medial preoptic, paraventricular, and supraoptic nucleus of the rat hypothalamus. In the amygdala, ERβ is primarily expressed in the medial amygdala nucleus. Moderate to high ERβ mRNA levels are found in olfactory bulbs, bed nucleus of the stria terminalis, hippocampus, cerebral cortex, cerebellum, midbrain raphe, and basal forebrain.[135,147,148,157,166–168]

The hypothalamo-pituitary axis (HPA) regulates overall endocrine homeostasis in the body. Estrogen, through effects on the HPA, modulates the expression and secretion of several hormones from the anterior pituitary gland, such as LH, FSH, GH, and PRL.[64] Both ERα and ERβ are expressed in the pituitary gland, but ERα predominates,[64] in particular in the gonadotrophs and lactotrophs. Both ER subtypes are expressed also in the preoptic area of the hypothalamus, believed to be involved in regulating the expression of pituitary hormones, but ERβ predominates.[147]

Although serum levels of LH and FSH are directly controlled by hypothalamic gonadotropin-releasing hormone (GnRH), it is the circulating level of estrogen, other sex steroids, and the inhibin glycoproteins that are the most important physiological determinants of serum gonadotropin levels.[64,169,170] There is a strong inverse correlation between the circulating levels of inhibin and FSH. The main source of inhibin (inhibin A and inhibin B) production in females is the ovary, inhibin B being expressed in the

early follicular phase with a peak at the midfollicular phase and inhibin A being expressed by the dominant follicle and the corpus luteum with a peak in late follicular — and in the mid-luteal phase.[169,170] In men inhibin B, proposed to be the main inhibin involved in FSH regulation in men, is primarily produced by the Sertoli cell.[169]

Female and male patients with aromatase deficiency are reported to have elevated levels of LH and FSH, elevated circulating levels of androgens but very low circulating levels of estradiol and estrone.[63] Therapy with conjugated estrogens in both male and female aromatase-deficient patients resulted in normalization of gonadotropin and testosterone levels.[63] Clinical data on the patient with the ERα nonsense mutation[70] also showed increased serum LH and FSH levels despite normal levels of testosterone and high estrogen levels. Transdermal ethinylestradiol therapy of this man did not have any effect on lowering serum LH or FSH. Estradiol substitution of ovariectomized rats prevented the expected increase in LH but only partially blocked the rise in FSH. In ERα-/- mice the circulating LH levels but not FSH are elevated despite 10-fold higher serum levels of estrogen.[64] Taken together these data indicate that estrogen is more important than testosterone (also in men[171–173]) in regulating circulating gonadotropin levels and that ERα plays a major role in mediating the effect of estrogen in this process, although the effect of activin–inhibin feedback regulation of pituitary FSH expression is independent of ERα. Whether ERβ has a role in ovarian activin–inhibin expression and the feedback regulation of gonadotropin expression remains to be investigated.

LH and FSH surge is critical to female ovarian cycle and fertility,[64] and it has been demonstrated that elevated estradiol levels in proestrous are required for the preovulatory LH surge from the anterior pituitary, triggered by a discharge of GnRH into the hypophyseal portal system.[174] The anteroventral periventricular nucleus (AVP) of the preoptic region, a sexually dimorphic part of the hypothalamus, is thought to play a critical role in transducing the gonadotropin surge.[64] The AVP is larger in female mice and contains a greater number of dopaminergic neurons than males.[64] Testosterone exposure of neonatal females reduces the number of dopaminergic neurons and precludes an LH surge. The AVP provides direct projections to a subpopulation of GnRH neurons in the preoptic region that are thought to participate in the initiation of the preovulatory LH surge.[64] Also, progesterone and the PR are necessary components of the LH surge.[64,175] Both ERα and ERβ have been shown to trigger PR expression in the preoptic nucleus,[167] suggesting that either of the two ER subtypes or both may participate in triggering the LH surge. Also, other neurotransmitter systems in the brain are suggested to contribute to the induction of the LH surge.[64] In a recent publication,[174] ERα containing histaminergic neurons located in the tuberomammillary complex also were shown to be involved in the positive feedback effect of estrogen in the induction of the LH surge, mechanistically via histaminergic axo-dendritic and axo-somatic appositions onto GnRH neurons and the histamine-1 receptor.

VII. HORMONE REPLACEMENT: TRADITIONAL ALTERNATIVES AND FUTURE PERSPECTIVES

The most common regimens in use to treat symptoms of the menopause and postmenopausal health risks such as osteoporosis and cardiovascular disease are 17β-estradiol, esterified estrogens, or conjugated equine estrogens, each in combination with

a progestin, for example, medroxyprogesterone acetate (MPA), to avoid the increased risk of endometrial or uterine cancer in women with an intact uterus. Another combination used is estrogen with testosterone, claimed to increase libido and decrease depression. However, the awareness of undesired effects (e.g., resumption of monthly bleedings, breast tenderness, and headaches) or health risks (breast cancer, endometrial cancer, venous thromboembolism, ovarian cancer, asthma, and gall bladder disease) with existing hormone replacement therapy (HRT) (first generation HRT) calls for alternatives with improved safety profile. Recent development of nonsteroidal ER ligands with mixed agonist–antagonist activity, the so-called SERMs (selective estrogen receptor modulators, second generation HRT) display a tissue-selective estrogen agonism in, for example, bone and liver, but estrogen antagonism in breast and uterine tissue.[176–178] Additional ER ligands with similar mixed agonist–antagonist activity (SERMs) are in development.[177] Although the increased risk of breast cancer and endometrial cancer is set aside by the SERMs they have not shown the same efficacy as estrogen to prevent, for example, bone fractures of the hip. Furthermore, existing SERMs, increase the incidence of or aggravate hot flushes in postmenopausal women, and the incidence of venous thromboembolism is the same as for first generation MHT. In addition, a serious drawback for this kind of drug became evident as the SERMs Levormeloxifene and Idoxifene had to be withdrawn from further development due to increased incidence of urinary incontinence and uterine prolapse in postmenopausal women. The discovery of a second ER subtype, ERβ, has revitalized the search for improved drugs for MHT that most likely will better provide the benefits of estrogen replacement therapy. Several of the large pharmaceutical companies are engaged in the development of ERα- and ERβ-selective SERMs (third generation HRT), but as yet there is no information as to how far they have come in their development. However, synthetic ER subtype-selective ligands have been reported.[179] The most ERα-selective ligand showed 120-fold higher agonist potency for ERα than for ERβ. Another ER subtype-selective ligand synthesized by the same group showed full ERα agonism but pure ERβ antagonism.

Alternatives for women that do not wish to take HRT are (1) synthetic progestins, megestrol acetate (a synthetic derivative of androgens), tibolone (a synthetic steroid with estrogenic, progestational, and androgenic activity), and clonidine (an α-adrenergic receptor agonist) for alleviation of hot flushes; (2) phytoestrogens such as ginseng, dang gui/dong quai, and remifemine (cimicifuga racemosa) for relief of climacteric symptoms; (3) bisphosphonates, calcitonin, and soy/isoflavones for the prevention of osteoporosis; and (4) statins, antioxidants (e.g., vitamin E), and soy/isoflavone for prevention of cardiovascular disease.[176]

VIII. CONCLUSIONS

Further characterization of the phenotypes of ERα and ERβ knock-out mice will be of continuous importance, not least regarding the effects of ERα and/or ERβ deficiency in aging mice. That ERα/ERβ double knock-out mice are viable needs an explanation: the role of redundant systems to secure viability and functionality, the importance of membrane or nongenomic effects of estrogens, and/or the possible existence of a third ER subtype need to be clarified. Other compelling questions to be answered about the biological role of ERα and ERβ stem from the observation that

both ERα and ERβ are expressed in normal and malignant breast tissue. Phenotypic characterization of the ERβ-/- mice revealed a role for ERβ as an antiproliferative receptor. In several tissues it operates to oppose the effects of ERα (yin-yang principle). Male BERKO mice develop prostate hyperplasia, which becomes malignant with age and aging female BERKO mice develop lymphoma. Furthermore, BERKO mice have severely impaired ovarian function related to the dysregulation of the androgen receptor (AR). Treatment of BERKO females with antiandrogens reversed the phenotype. We have concluded that the major role of ERβ in the ovary is down-regulation of the androgen receptor in maturing follicles. In BERKO ovaries AR remains high, for example, the ovary is in a hyperandrogenic state and is similar to ovaries seen in polycystic ovarian disease in humans. There is a need to identify genes, that are regulated by ERβ but also to understand the regulation of the ERβ gene itself. Furthermore, it will be of importance to identify possible mutations in ERβ and to investigate the role mutated ERβ might play in human diseases.

IX. FUTURE NEEDS

- Understand whether breast tumors arise in cells that already contain one or other of the estrogen receptors.
- Investigate the roles the two estrogen receptors have in the breast (synergistic or opposing).
- Compare BERKO mice susceptibility to develop breast cancer to that of controls or of ERKO mice.
- Determine whether both ERα– and ERβ-containing stromal cells secrete growth factors in response to estrogens.
- Investigate the role in human breast cancers of mutations in ERβ.
- Recognize the role of the splice variants of ERβ in the normal and malignant breast.
- Unravel the mechanism of activation of ER in postmenopausal breast.
- Identify genes that are regulated by ERa and ERb in normal and malignant breast.

More specifically there is a need to:

- Understand why epithelial cells in the prostates of ERβ knockout mice are never in G_0 and identify the stage in the cell cycle in which they are arrested.
- Identify the genes involved in the change from hyperplasia to malignant phenotype in the prostate.
- Characterize the lymphoma, which develops in BERKO females.
- Characterize the role of ERβ in the immune system.
- Investigate the role of ERβ mutations in women with polycystic ovarian syndrome.

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