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Review

The estrogen receptor: two or more molecules, multiple variants, diverse localizations, signaling and functions. Are we undergoing a paradigm-shift as regards their significance in breast cancer?

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INTRODUCTION

Estrogen receptors (ERs) belong to the NR3 class of nuclear receptors.[1] This class includes, in addition to ERs, progesterone, androgen, glucocorticoid and mineralocorticoid receptors which mediate endocrine actions of steroids, as well as the orphan receptors ERRα-γ. Classically, NR3 receptors act as nuclear transcription factors. However, in recent years, the discovery of receptor isoforms, together with the identification of alternative steroid-initiated mode of action and signaling pathways, has enlarged our view on estrogen action and furnished novel clues and regulatory elements regarding their role in cancer. Nuclear-transcriptional actions of estrogen have been extensively reviewed vis-à-vis their actions in endocrine-related cancer (see for example Ref 2, for a recent review, and references herein). In the present review, we focus on alternative modes of action of estrogen and discuss some elements suggesting possible novel uses of estrogen agonists and antagonists, with a special emphasis on breast cancer, a primary target of estrogen.

Key words: Cancer, Estrogen receptor, Nuclear-extranuclear actions, Pathology, Receptor isoforms, Signaling

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1. ER SEQUENCE AND PLURALITY

1.1. ERs exist in multiple isoforms

Two different forms of the estrogen receptor have been described, ERα and ERβ. They are coded by two distinct genes, located in chromosomes 6 and 14, and produce two proteins with 595 and 530 aminoacids, respectively.[3-5] The internal structure of the ER genes contains 8 exons (Figure 1). Like all members of the nuclear receptor family, ERs present an internal structure comprising 6 evolutionary conserved domains named A-F: at the N-terminal, domains A/B contain the transcription activation function 1 (AF1), a ligand-independent transcription activator part of the receptor; the highly conserved domain C is its DNA-binding domain; domain D is the hinge region, responsible for the recruitment, binding and function of a number of receptor co-modulators, while domains E and F contain the ligand binding

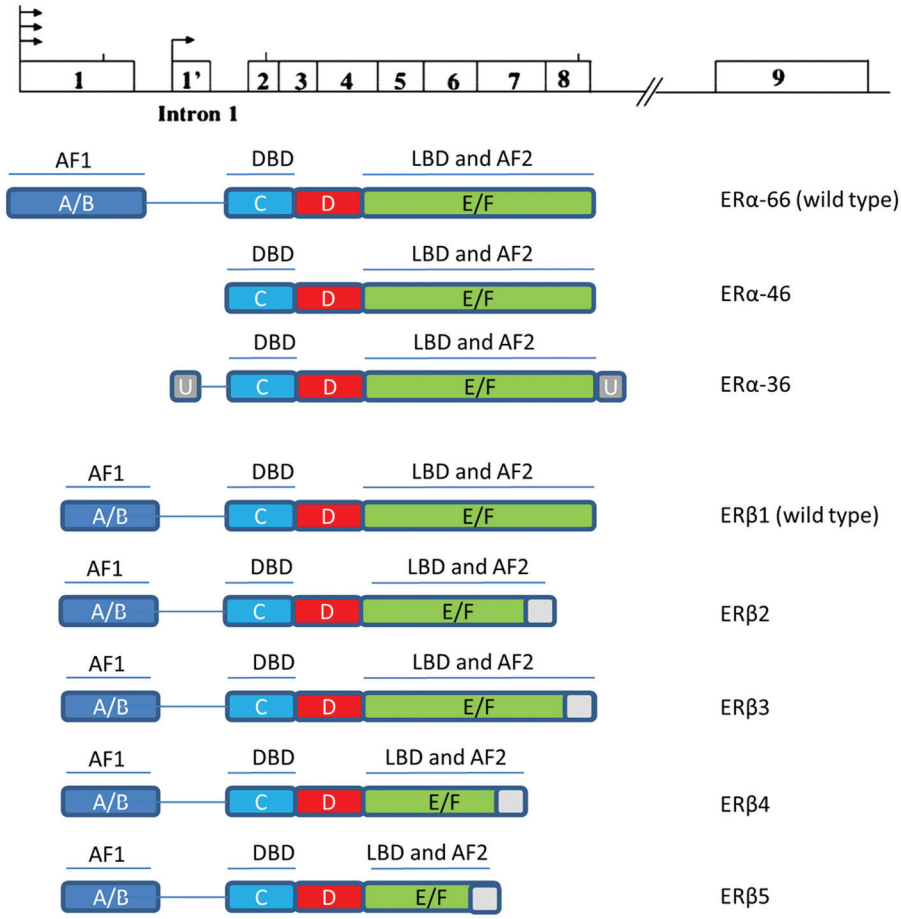

Figure 1. Schematic representation of the different domains of the estrogen receptors. Figure plotted from data presented in references 8 and 2. Only exons are shown in the Figure, with the exception of (1) the intron between A/B and C domains, in which an alternative transcription initiation site has been reported; (2) intron 8, as exon 9 is included only in the sequence of ERα36. Grey boxes represent the alternative specific aminoacid sequences for each isoform; in the sequence of the ERα36, this represents the transcription of a novel exon 9 (shown in A), while in the ERβ isoform it represents sequence differences in exon 8. See text for further details.

domain, together with the transcriptional activation function 2 (AF2), a ligand-dependent activator of transcription (Figure 1).[6,7]

Several splice variants have been described for both receptor types (Figure 1). A shorter ERα isoform, lacking exon 1 and consequently the AF1 (ERα46), has been identified.[9] Moreover, an alternative transcription initiation site, within intron 1 of ERα, may generate another isoform of this receptor, lacking, in addition to AF1, a part of AF2 and containing a unique C-terminal aminoacid sequence (exon 9, ERα36).[8,10] Finally, a number of ERα variants, lacking the 5'UTR of the receptor, have also been found in cancer cell lines.[11] Of note, all these isoforms may heterodimerize with the full-length ERα and thereby repress AF1-mediated activity.[8,9] ERα46 has been found to exert estrogen-related vascular effects,[12-15] while ERα36 has been demonstrated to mediate a wide spectrum of estrogen extra nuclear actions.[16] In addition, several splice variants of ERβ have been identified, with specific differences in exon 8 (Figure 1),[17,18] differentially modulating estrogen signaling.[19-21]

1.2. ER action

Our understanding of the mode of action of ERs has significantly expanded in the last decade. Summing up the available knowledge, two main ER effects can be distinguished (Figure 2):

1. Direct transcriptional effects

Classically, liganded ERs dimerize and translocate

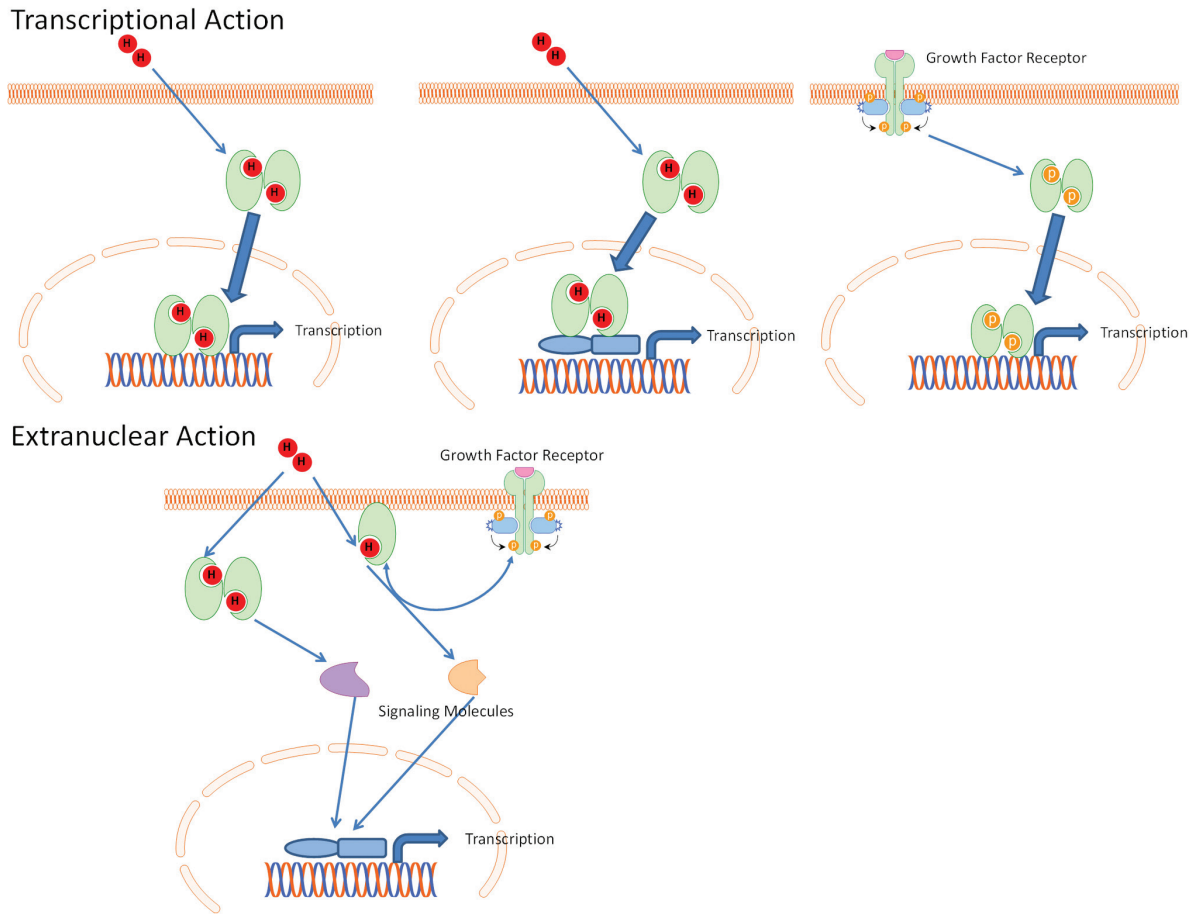

Figure 2. Cellular mode of action of estrogens. See text for details.

to the nucleus, acting as transcription factors per se, binding to specific promoter estrogen response elements (ERE) or through protein-protein interactions with other transcription factors (tethered actions). Through this latter transcriptional activation/repression, ERs can therefore influence the expression of genes lacking EREs.[22-25] In addition, unliganded ERs may be phosphorylated by activated kinases (for example, through growth factor receptor activation[26]), then may dimerize, bind DNA and regulate gene transcription, contributing to the hormone-independence of certain tumors.[27,28]

2. Extranuclear action

Data accumulated during the last two decades suggest the existence of a specific extranuclear estrogen binding component (membrane-bound and/or cytoplasmic-related), which, upon activation, may trigger a number of signaling events (ion mobilization, kinases activation) and ultimately lead to transcriptional events different from those induced by the classical or tethered ER action. This element might be either a classical ER,[29-32] ERα isoforms[10,12,15,33,34] or a novel, non-identified distinct receptor.[35-37]

In the following sections, we will focus on the extranuclear actions of ERs, since accumulating evidence demonstrates their presence in breast cancer (especially in ERα negative tumors) and point to the existence of an alternative novel mode of estrogen action in cancer that could be therapeutically exploited.

2. NATURE OF EXTRANUCLEAR ERs

2.1. Native ER isoforms; the role of receptor palmitoylation

An element which greatly contributed to the elucidation of ER extranuclear and membrane-initiated

actions of ERs is the receptor palmitoylation and anchorage to the plasma membrane.[39,40] Palmitoylation is a regulated, universal, reversible, post-translational modification, mandatory for the regulation of trafficking, membrane localization and activity of many cellular proteins.[41,42] A global cell palmitoylation profile identifies two types of protein palmitoylation: stably lipoylated and proteins sustaining a very rapid and dynamic palmitoylation.[43] Palmitoylation increases protein hydrophobicity and membrane association.[44] ERα as well as ERβ are subjected to rapid palmitoylation/de-palmitoylation at cysteine residues 447 and 339,[45,46] mediated by two palmitoyl-acyl-transferases (PAT).[47] Of particular interest, a similar palmitoylation motif exists in all NR3 family of nuclear receptors,[48] indicating palmitoylation as a common post-transcriptional modification of estrogen, androgen, glucocorticoid and mineralocorticoid receptors. Palmitoylation targets the receptor to the plasma membrane where it exists within caveolar rafts and interacts with specific membrane proteins, including caveolin-1. E_2 association induces both ERα and ERβ de-palmitoylation and differential interaction with specialized proteins. Specifically, the ERα-caveolin-1 complex is dissociated and impairs receptor association with adaptors and/or signaling proteins such as the modulator of the non-genomic activity of estrogen receptor (MNAR) and c-Src.[49] On the other hand, this is not the case for ERβ, which shows an increased association with caveolin-1, occurring along with other cytoplasmic kinases (i.e. p38 kinase) in colon cancer, upon estrogen stimulation and de-palmitoylation.[50] Therefore, palmitoylation/de-palmitoylation is necessary for the induction of some estrogen-evoked extranuclear actions,[51] including the balance between proliferation/apoptosis or proliferation/differentiation.[52-54]

2.2. GPR30

At the beginning of the last decade, a novel membrane bound G-protein-coupled receptor (GPR30) was identified as a novel estrogen-binding protein.[55] GPR30 (or GPER-1, as it was recently renamed, reviewed in Ref 56) has a structure totally different from the classical ERs. It is an integral membrane protein and a classical G-protein-coupled molecule which is present in various cell membrane elements (including the plasma membrane and the endoplasmic reticulum). Through activation of adenylate cyclase, GPR30 triggers the release of EGF membrane-tethered molecules.[57] Although it was introduced as the prototype of membrane ERs, the localization of this protein among cell membrane components is a matter of controversy: older publications suggest that this receptor is an integral membrane element,[57-59] a finding debated by others and attributed to methodological problems;[60] more recent publications provide hints as to the localization of this protein within the endoplasmic reticulum, or on the Golgi apparatus, subjected to a trans-Golgi turnover.[61-63] The research on this receptor has been enriched by the introduction of specific agonists and antagonists (G1 and G15-G36, respectively). It was found that GPR30 may mediate a number of estrogen actions in ERα-positive or negative tissues (reviewed in Ref 61).

GPR30 activity has been reported in different systems, including organs of reproduction (breast, endometrium, ovary and testis), the thyroid gland, the central nervous system (reviewed in Ref 56, and references herein) and the cardiovascular system.[64] However, a number of authors have questioned these results,[60] while others have suggested that GPR30 might be a co-modulator of ERα rather than a pure estrogen receptor.[65] In support of this hypothesis, it has been suggested that GPR30 actions may be triggered through the ERα isoform ERα36.[16] Interestingly, in a recent transcriptomic analysis in breast cancer cells,[66] we have reported that over 95% of early modified genes, inhibited by the GPR30 antagonist G15, were equally inhibited by ICI 182780, a pure ER antagonist, implying either a cooperation of GPR30 with classical or alternatively spliced estrogen receptors or a direct dependence of GPR30 receptor on ER-regulated effects. Moreover, in the same study we reported a small number of genes, related to apoptosis, metabolism, immune functions and different signaling pathways as being GPR30 specific, a result supporting previous publications on GPR30 functions (critically discussed in Ref 66, and references herein).

2.3. Novel receptor molecules

In addition to the above cited molecules, there is evidence of the existence of (an)other membrane-located site(s), binding estrogen. The evidence is based on the following data. (i) pharmacological data

indicate that some of rapid E_2 effects are not inhibited by ER antagonists.[37,67] Indeed, ER-antagonists have been proposed as agonists of GPR30[56] or ERα36.[10] However, they do not have any effect on some E_2-mediated ion movements and signaling cascades, suggesting additional interactions with other molecules. (ii) The use of plasma membrane impermeable E_2 analogs (E_2-covalently linked with protein macromolecules, such as BSA, or with polysacharide dendrimers[68]) triggers rapid actions and induces transcriptional effects different from those attributed to nuclear-acting ERα.[35,69] (iii) Finally, a small number of reports[67,70] point to the existence of an unknown protein, identified through affinity chromatography and E_2-binding, from membranes of E_2-interacting cells, which is partially recognized by ERα antibodies. Unfortunately, until now this molecule(s) has not been conclusively identified. These data suggest that probably, in addition to the identified receptor forms, additional molecules may mediate some of the actions of E_2, acting preferentially at the plasma membrane. Corroborating this conclusion is the fact that in breast cancer xenografts, additional molecules, interacting with ER-antibodies, are identified, suggesting a more complex regulation of ERs during cancer evolution (see below).[71]

3. ER SIGNALING

3.1. Nuclear signaling: the role of co-regulators and receptor shuttling

The main localization of ERs (ERα and ERβ) is within the nucleus.[72,73] However, their nuclear distribution changes, following ligand binding, as a consequence of their role as transcriptional modifiers: in a ligand-free state, the receptor is fairly homogeneously distributed within the nuclear matrix; however, after E_2-interaction, ERs adopt a dotted pattern, typical of an interaction with specific nuclear elements.[74,75] In addition, a constant shuttling of the receptor between the cytoplasm and the nucleus occurs, while the binding of agonists and antagonists modify this highly dynamic process.[76] The nuclear import of ERs is regulated by their binding to specific proteins, facilitating their transport, through a sequence of basic aminoacids (PKKKRK, or, in the case of ERs, KRSKK), named nuclear localization signals (NLS, critically discussed in Refs 74, 77). ERs contain at least three such sequences, ensuring their binding to carrier proteins and nuclear import. In contrast, ERs do not contain the prototype leucin-rich nuclear export signal (NES),[78] but have sequences with a limited homology to them.[79,80] Furthermore, ERs may interact with other proteins, containing NESs, and be transported to the cytoplasm as protein-protein complexes (discussed in Ref 81). In consequence, the ERs' cellular localization is a dynamic process, involving continuous cycles of nucleo-cytoplasmic shuttling.[82] In addition, after ER-DNA interaction and/or phosphorylation, the receptor molecules become polyubiquitinated, dissociate from DNA and transiently accumulate in the nuclear matrix to be subsequently degraded by the action of the 26S proteasome.[80,83-85]

In the nucleus, ligand- or phosphorylated-activated ERs bind to specific DNA sequences, named estrogen response elements (ERE), to modify the transcription of estrogen-responsive genes, or they interact with other transcription factors through protein-protein interactions, to regulate genes lacking EREs.[86] This binding is enhanced or attenuated by the interaction of ER with co-regulator proteins (co-activators or co-repressors, respectively).[87-90] Co-activator binding and activity are relatively well characterized for the AF2 region of ER. It is of note that these co-regulator proteins contain the characteristic motif LxxLL[73,91] and bind and activate liganded (or phosphorylated) ERs, by modifying their conformation. It is important to mention that a platform (P_{295}-T_{311}) between the hinge (D) and the ligand binding domain (E/F) of the ERα, well-conserved among all NR3 class receptors, actively participates in co-regulator binding and activity modification of the receptor.[92] In contrast, the AF1, ligand-independent, co-regulator binding region is less well studied. It has been proposed that different domains of this region (aminoacids 1-62, 80-113 and 118-149) represent the active platform of this function, while an interaction of AF1 and AF2 may also occur.[26,93] In recent years, there has been an intense research effort with a view to identifing, modifing or *de novo* synthesizing ER co-regulator peptides with a therapeutic profile,[94] especially in the light of the increasing emergence of resistance of (breast) cancer patients to endocrine therapy. Such peptides are called PERMs, for "peptidomimetic

estrogen receptor modulators",[95] or CBIs, for "co-activator binding inhibitors".[86]

3.2. Extranuclear signaling

Even in the earliest studies (mid-50's) on steroid actions, it was observed that steroids can also act sometimes too fast, an effect that could not be attributed to the classical, time-consuming, genomic mode of action.[96,97] The key publication, however, which introduced the concept of non-genomic, rapid, extranuclear estrogen effects was that of Szego and Davis.[98] The authors reported an acute, early (30 seconds) increase of uterine cAMP in rats treated with physiological concentrations of E_2. This work was followed, during the 70's and 80's, by the publication of several reports implying the existence of a rapid steroid action that is initiated at the plasma membrane level.[99-115]

Extranuclear estrogen receptor signaling has been critically reviewed in Ref 38,116,117. Their action can be categorized as follows:

1. Ion movement through the plasma membrane

Soon after the extranuclear/membrane actions of estrogen had been determined, a direct activation of Ca^{2+} flux into cells was reported.[118] This effect was further confirmed by different groups, including ours,[119] in a number of cell lines (reviewed in Ref 120-122), a result recently identified as being involved in E_2-dependent cardiac function.[123] In addition, a specific effect of E_2 on K^+-channels has been found, both in the CNS[124,125] and other tissues, including renal tubules and the gut.[126-130] These rapid activities were recently reviewed in Ref 131. This effect may also explain gender differences in epithelial water and ion movements in health and during water and ion-related disease states.

2. Membrane-related kinases and interaction with growth factor receptors

The activation of a number of kinases, implicated in membrane signaling, was an early observation in the study of E_2-related extranuclear actions. They include cyclic nucleotide (cAMP, cGMP) generating enzymes[132-144] and subsequently activated protein kinases,[145-155] lipid kinases (such as PI3K),[14,144,145,156-168] Src kinases[119,145,158,159,163,169-176] and their interaction (direct as suggested for GPR30, or indirect through Src[144]) with growth factor receptors. The most extensively studied interaction is that with epidermal growth factor receptor (EGFR),[57,58,156,177-185] in view of its prominent role in cancer cell biology and its place as a therapeutic target. Indeed, an E_2-membrane binding component seems to interact either physically[57,58] or through Src kinases, transactivating the EGFR and inducing specific signaling cascades. Interestingly, in a whole transcriptome analysis that we have performed in breast cancer cells (Notas et al, Mol. Oncol. in press, 2013), we report that early (3 hours) E_2 treatment of cells induces, through ERα, a direct increase in the transcription of EGFR and human epidermal growth factor receptor 3 (HER3), while human epidermal growth factor receptor (HER4) mRNA is decreased and human epidermal growth factor receptor 2 (HER2) levels remain unaltered, providing the first evidence of a direct modulation of EGFR by estrogen.

3. Modification of intracellular signaling cascades

The activation of membrane-related kinases triggers or modifies a number of intracellular signaling cascades. Estrogens have been implicated in the modulation of all major intracellular signaling cascades: MAP kinases,[10,26,27,35,54,145,146,156,158,167,176] Jnk-cJUN,[10,24,35,38,53,63,145,172,181,185] JAK-STAT,[28,35,39,81,119,142,162,186] cAMP signaling, leading to CREB transcription initiation[119,145,187] and finally PI3K/Akt signaling.[14,35,40,119,145,157,162,167,169-172] This latter cascade may subsequently lead to transcription and/or to cytoskeletal modification. Indeed, E_2 is a major modulator of actin cytoskeleton, in a variety of cell types,[170,171,188-199] leading to effects as different as axonal growth, endothelial remodeling or cell migration.

4. Transcriptional actions

As stated above, the final outcome of signaling pathways triggering by estrogen is the activation of a number of nuclear transcription factors. Interestingly, the differential transcriptional signature of membrane-acting (E_2-BSA) or permeable estrogen (E_2) leads to the modification of different genes.[35,66,170] Classical ER-mediated signals lead to the activation of genes and transcription factors regulating cell proliferation, migration and inhibition of apoptosis (Notas et al, Mol. Oncol. in press, 2013). In contrast,

ERα36-mediated signals modulate immune functions, while membrane-initiated signals lead to actin modification, modification of intracellular signaling cascades and negative regulation of immune functions. In this respect, a clear distinction of genomic and non-genomic initiated transcriptional actions of estrogen is described for the first time.

4. POTENTIAL ROLE OF EXTRANUCLEAR ERs IN BREAST CANCER

Breast cancer, being the most common neoplasm affecting the female population worldwide, has been one of the main targets for the elucidation of the extranuclear effects of estrogen. Below, we will sum up work conducted on this tissue, categorizing it as *in vitro* and animal experiments, and work with patients' samples.

4.1. Cell biology

Membrane localization of estrogen binding components has been reported in ER-positive and negative breast cancer cell lines, using fluorescently labeled membrane impermeable estrogens (E_2-BSA-FITC)[106,109,113,145,170,194,200] or dendrimers.[68] In addition, in a number of interesting publications, by means of specific antibodies, a membrane association of ERs has also been identified.[12,13,15,30,39,40,46,48,62,67,68,70,106,109,179,197] Interestingly, membrane estrogen receptors (mER) have been associated with lipid rafts,[30,67,179,182,193,197] cellular anchoring molecules,[198,199] growth factor receptors,[58,62,63,163,193] or related to receptor pamitoylation.[39,40,48,51] In addition, native ERs (ERα),[39,40,48,51,201] or truncated forms of the receptor have been implicated.[8,10,12,13,16,33] All signaling cascades, described in the previous paragraph, have been reported in breast cancer cells. Activation of mER in breast cancer cells leads to cell survival, by activation of anti-apoptotic and suppression of pro-apoptotic molecules,[119,145] positioning this receptor localization as a pro-survival mechanism in breast cancer cells. In this respect, mER might be regarded as a negative prognostic factor in breast cancer (see the following paragraph).

In the field of cross-talk with growth factors affecting cell survival, we herein report the interaction of membrane ERs with the erythropoietin (EPO) system. As presented in Figure 3, E_2-BSA acts in an additive manner with EPO to protect breast cancer cells from serum starvation-induced apoptosis. This is mediated in an ER- independent manner, as the effect is not reverted by the addition of the pure anti-estrogen ICI182780 (ICI) and relies on Akt intracellular signaling, as the addition of the PI3K/Akt inhibitor wortmannin reverts this anti-apoptotic effect. In addition to this antiapoptotic effect, membrane-acting estrogen also decreases the transcription of the EPOR, a result observed 6 hours after E_2-BSA stimulation, which returns to basal levels thereafter, perhaps as a cellular response to overcome the pro-apoptotic stimuli of serum deprivation. This effect was further explored by using erythropoietin receptor (EPOR) promoter constructs (which do not contain EREs), containing its proximal and distal parts. We show that this membrane-initiated estrogen action is exerted on the distal part of the EPOR promoter, although a more detailed analysis is still needed to further elucidate this interaction. These data provide, for the first time, evidence of an interaction of mER with the EPO/EPOR system. Interestingly, previously published data indicate that inverse modifications were induced by membrane-acting androgen: testosterone-BSA-induced apoptosis is reverted by EPO, an effect also mediated by Akt signaling.[202] Androgen, in that case, acts on EPOR transcription through the classical (proximal) promoter.

4.2. Human breast cancer specimens

In contrast to breast cancer cell lines, data on human breast cancer specimens are very rare. Our group was the first to provide data on patients' specimens.[203] We have reported that mER are present on cell membranes of both ER-positive and negative samples. In contrast, no staining of non-tumoral tissue is found, suggesting that mER expression is a specific feature of breast tumors. Whenever neo-adjuvant chemotherapy was applied prior to surgery, the intensity of mER was increased. It is of note that in the same publication we also reported a negative association of increased EPO and EPOR expression with disease outcome. These results provide a translational outcome of the negative control, exerted by mER on EPOR transcription, shown in the previous paragraph and advance membrane ER(s) as possible therapeutic targets. Nevertheless, it is essential to elucidate the nature of mER and differentiate between possible specific effects of alternative membrane-localized

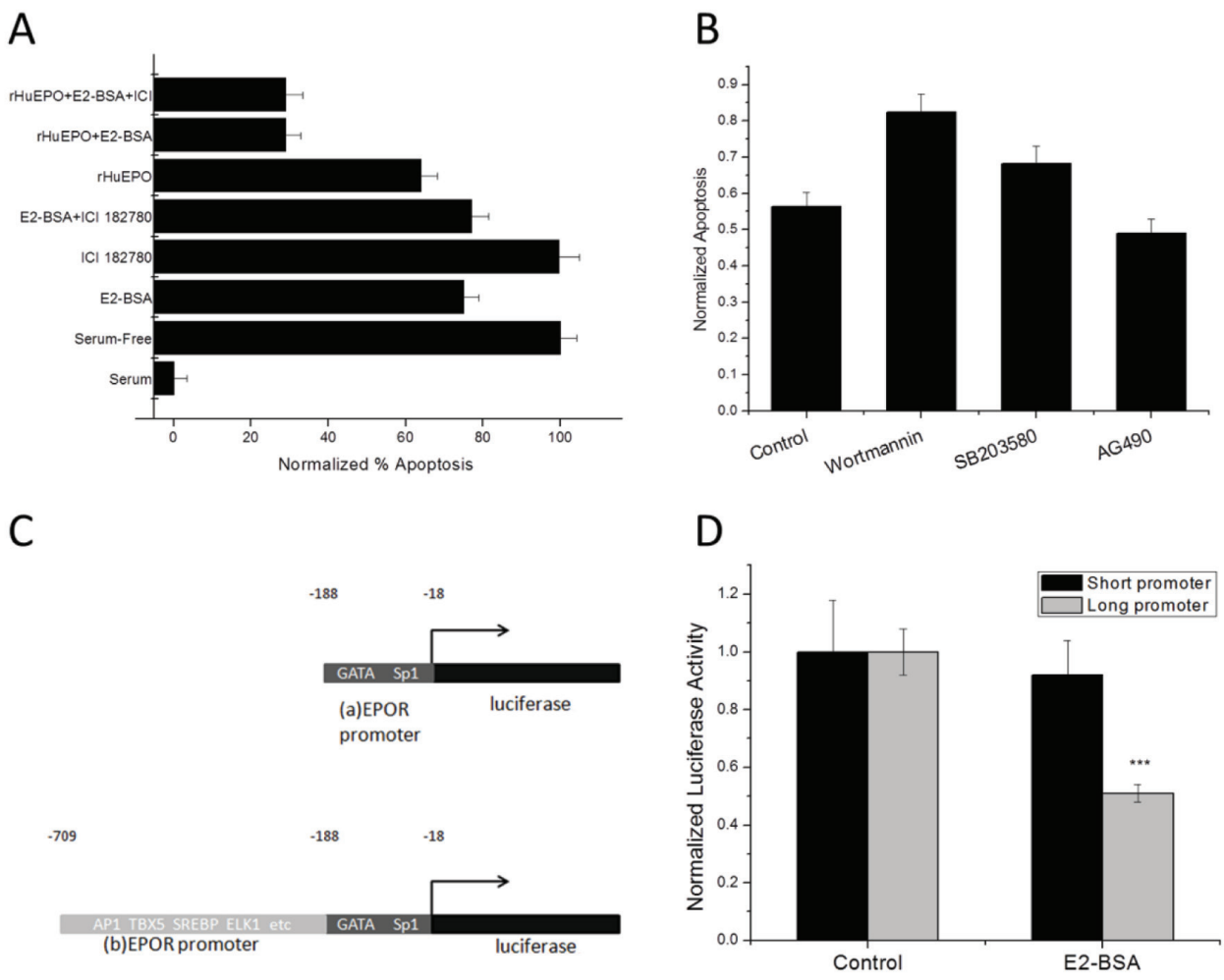

Figure 3. Membrane-acting estrogens interact with EPOR. A. T47D breast cancer cells were incubated with 10^{-7}M E_2-BSA, in the absence or presence of 10^{-6}M ICI182780 (a pure ER antagonist), or 10^{-7}M erythropoietin (Epoetin® alpha), in a culture medium devoid of serum. Apoptosis was assayed after 24h. Control conditions=non-treated cells in the presence of 10% FBS. **B.** Cells were incubated with equimolar concentrations (10^{-7}M) E2-BSA and erythropoietin, in the absence (control) or the presence of wortmannin (PI3K inhibitor), SB203580 (p38 kinase inhibitor) and AG490 (JAK2 inhibitor). Apoptosis was assayed after 24h incubation. **C.** The constructs used for EPOR promoter assay. See Ref 202 for details. **D.** E_2-BSA decreases EPOR transcription, acting on the distal part of the EPOR promoter. All data represent the mean±SEM of three different assays in triplicate.

receptor forms. More recently, we[204] and others[205] reported the expression of ERα36 staining in a series of triple-negative breast tumors. The majority of tumors (~97%, and 83%, respectively, in the two publications) expressed this ERα variant. Interestingly, the membrane association of ERα36 was a good prognostic factor in our series, being associated with an increased survival of patients. Perhaps, therefore, the assay of this isoform or the identification and administration of specific modulators of the membrane localized ERα36 might be beneficial for the outcome. This of course necessitates further verification and testing, while the fact that ERα36 can mediate a biphasic anti-estrogen signaling in ER-negative breast cancer cells, as recently reported,[206] should be taken into account. The above results match those of Lee et al,[207] who also reported the expression of ERα36 in a small series of ER-positive and negative breast tumors. However, in a series of 896 patients,[208] ERα36 expression was associated with a poorer prognosis. Nevertheless, the authors did not specify the relevance of ERα36 localization (membrane and cytosolic) to patients' survival, a feature which was found discriminant in our series.

In a non-published series of breast cancer patients, membrane-associated ERα staining was evidenced in ~20% of cases. Apart from our work, only one recent publication has assayed extranuclear ER localization. In an excellent work, Welsh et al[209] performed a tissue microarray (TMA)-based retrospective analysis of extranuclear ER, in a series of 8 different cohorts of archival breast cancer cases, totaling 3981 different samples. Analysis was performed by automatic methods and verified by pathologists, according to rigorously specified criteria. The authors report that major staining was cytoplasmic and not-membrane associated and that "the overall incidence of cytoplasmic staining only averaged 1.49%, ranging from 0% to 3.2% at best. As many of the cytoplasmic cases were observed in cohorts from outside institutions, we did not have broad access to the original tissue to conduct any follow-up analysis on the individual cases". The discrepancy between the two studies points to the need to establish specific criteria for membrane/extranuclear staining and to train pathologists for such a diagnosis, based on the established standard diagnostic criteria for ER nuclear and extranuclear/membrane positivity.

5. CONCLUDING REMARKS

The data presented here relate to the field of estrogen receptors, with emphasis on their extranuclear localization and action, an area in which considerable progress has been made over the last decade. The identification of receptor isoforms for ERα and β, dotted with specific actions, together with the exploration of extranuclear, rapid actions, have significantly expanded the field and provided novel insights explaining some of estrogen's effects, which have been difficult to be integrated to the genomic action of the hormone. Much work has yet to be done in order to decipher the nature of a putative membrane "ERx" and to determine its effects, as well as the effect of extranuclear ER(s) cellular and molecular actions. In addition, the design, synthesis and evaluation of novel agents modifying the activity of membrane-acting estrogen could provide novel therapeutic approaches for malignancies in which E_2 exerts a specific action.

In the case of breast cancer, estrogen together with progesterone receptors and Her2/neu are nowadays considered as the major predictive and prognostic biomarkers, driving cancer adjuvant therapy. Initial determination of ERs was made by ligand binding assays, in cytoplasmic preparations of tumor specimens. This technique enabled the detection of unliganded ERs and their isoforms, as determined by their ability to bind [^{3}H]-estradiol. However, nuclear fractions of ERs were also evidenced,[210] although liganded ERs could not be detected.[211] Soon after, with the development of specific anti-ER antibodies, ligand binding was routinely replaced by immunohistochemistry, which is the "gold standard" today. The use of antibodies has made possible the identification of both liganded and unliganded receptors,[211] while their nuclear localization is the only one taken into account for diagnostic/therapeutic purposes. However, this method is also controversial: antibodies recognizing different parts of the receptor are on the market (critically discussed in Ref 209) and therefore the results provided are not comparable, especially in view of divergences in the binding capacity and the conformational effect on the receptor induced by anti-estrogen (used in some cases as a neo-adjuvant therapy). In support of this point, a recent publication of our group shows the identification of ERα36 immunoreactive molecules in a series of 52 triple-negative breast cancer cases[204] and also in ERα-negative and positive breast cancer specimens (Figure 4). Furthermore, using a fluorescent non-permeable E_2 analog (E_2-BSA-FITC), we have reported the presence of estrogen membrane binding sites in both ER-positive and negative breast cancer cases, which correlate with patients' survival.[203] Finally, in 20% of breast cancer cases, a membrane immunostaining component could be identified.

So, where do we now stand? Should we integrate the knowledge of extranuclear and membrane-associated ERs into our diagnostic armamentarium? In the authors' opinion, this development is as yet premature; in the absence of specific modulators, membrane ER(s) will not provide added diagnostic or therapeutic clues. However, their presence (either cytoplasmic or especially membrane-bound) might provide additional information on the possible response of patients to hormonal manipulation. In this respect, a "re-education" of pathologists so that they may include the notion of mER within their diagnostic

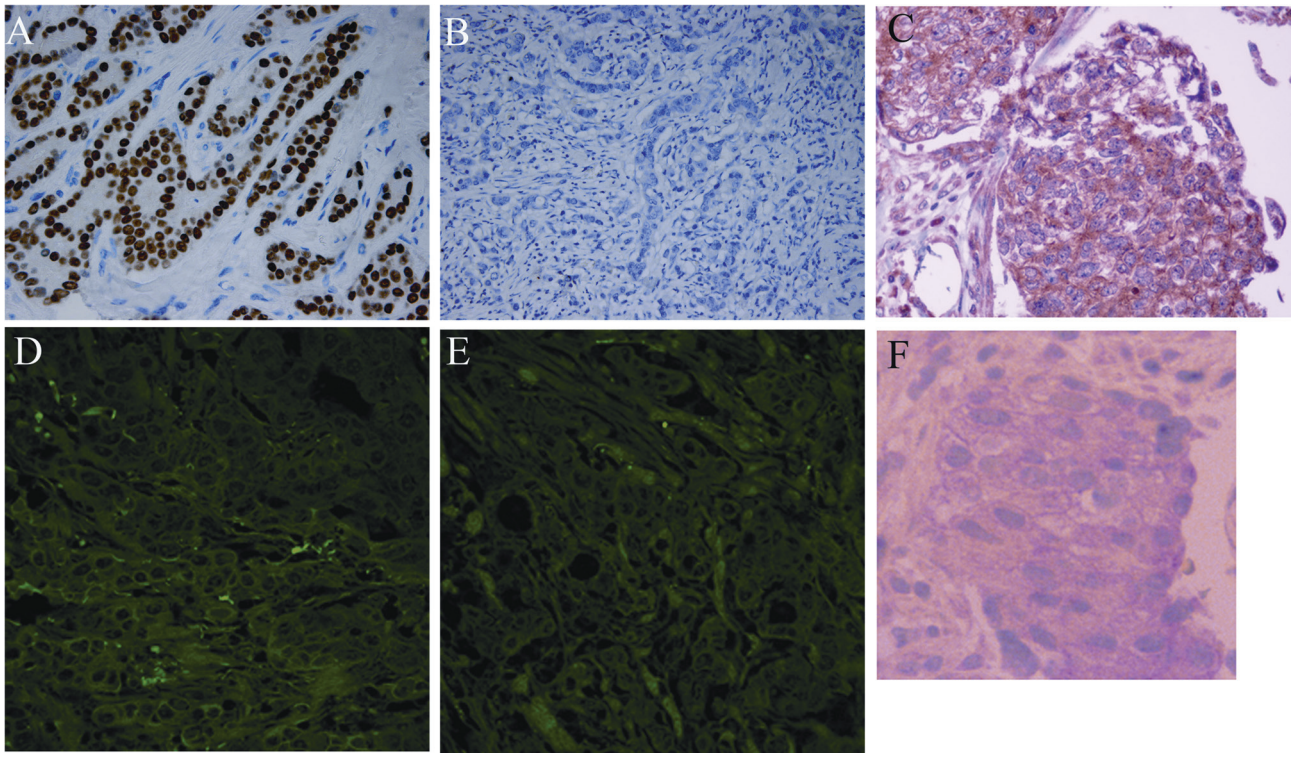

Figure 4. Expression of ERs in breast cancer specimens. **A and B.** Typical cases of ERα-positive and negative immunohistochemical staining of breast tumors. However, as shown in **D and E** these tumors express membrane estrogen receptors, as depicted by fluorescent microscopy after E_2-BSA-FITC binding. In **C**, ERα36 staining is depicted, while in **F** an ER-negative case is presented, in which a dotted membrane immunochemical positivity is shown.

assets seems necessary.

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REFERENCES

1. Germain P, Staels B, Dacquet C, Spedding M, Laudet V, 2006 Overview of nomenclature of nuclear receptors. Pharmacol Rev 58: 685-704.
2. Thomas C, Gustafsson JA, 2011 The different roles of ER subtypes in cancer biology and therapy. Nat Rev Cancer 11: 597-608.
3. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J, 1986 Sequence and expression of human estrogen receptor complementary DNA. Science 231: 1150-1154.
4. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA, 1996 Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93: 5925-5930.
5. Dahlman-Wright K, Cavailles V, Fuqua SA, et al, 2006 International Union of Pharmacology. LXIV. Estrogen receptors. Pharmacol Rev 58: 773-781.
6. Nilsson S, Makela S, Treuter E, et al, 2001 Mechanisms of estrogen action. Physiol Rev 81: 1535-1565.
7. Chang CS, Kokontis J, Liao ST, 1988 Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. Proc Natl Acad Sci USA 85: 7211-7215.
8. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF, 2005 Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. Biochem Biophys Res Commun 336: 1023-1027.
9. Flouriot G, Brand H, Denger S, et al, 2000 Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and

that is able to repress hER-alpha activation function 1. EMBO J 19: 4688-4700.
10. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF, 2006 A variant of estrogen receptor-α, hER-α36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. Proc Natl Acad Sci U S A 103: 9063-9068.
11. Kos M, O'Brien S, Flouriot G, Gannon F, 2000 Tissue-specific expression of multiple mRNA variants of the mouse estrogen receptor alpha gene. FEBS Lett 477: 15-20.
12. Li L, Haynes MP, Bender JR, 2003 Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. Proc Natl Acad Sci USA 100: 4807-4812.
13. Hisamoto K, Bender JR, 2005 Vascular cell signaling by membrane estrogen receptors. Steroids 70: 382-387.
14. Kim KH, Bender JR, 2009 Membrane-initiated actions of estrogen on the endothelium. Mol Cell Endocrinol 308: 3-8.
15. Kim KH, Toomre D, Bender JR, 2011 Splice isoform estrogen receptors as integral transmembrane proteins. Mol Biol Cell 22: 4415-4423.
16. Kang L, Zhang X, Xie Y, et al, 2010 Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic estrogen signaling. Mol Endocrinol 24: 709-721.
17. Fuqua SA, Schiff R, Parra I, et al, 1999 Expression of wild-type estrogen receptor beta and variant isoforms in human breast cancer. Cancer Res 59: 5425-5428.
18. Saji S, Omoto Y, Shimizu C, et al, 2002 Expression of estrogen receptor (ER) (beta)cx protein in ER(alpha)-positive breast cancer: specific correlation with progesterone receptor. Cancer Res 62: 4849-4853.
19. Ramsey TL, Risinger KE, Jernigan SC, Mattingly KA, Klinge CM, 2004 Estrogen receptor beta isoforms exhibit differences in ligand-activated transcriptional activity in an estrogen response element sequence-dependent manner. Endocrinology 145: 149-160.
20. Leung YK, Mak P, Hassan S, Ho SM, 2006 Estrogen receptor (ER)-beta isoforms: a key to understanding ER-beta signaling. Proc Natl Acad Sci U S A 103: 13162-13167.
21. Leung YK, Lee MT, Lam HM, Tarapore P, Ho SM, 2012 Estrogen receptor-beta and breast cancer: translating biology into clinical practice. Steroids 77: 727-737.
22. Saville B, Wormke M, Wang F, et al, 2000 Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. J Biol Chem 275: 5379-5387.
23. Kushner PJ, Agard D, Feng WJ, et al, 2000 Oestrogen receptor function at classical and alternative response elements. Novartis Found Symp 230: 20-6; discussion 7-40.
24. Kushner PJ, Agard DA, Greene GL, et al, 2000 Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74: 311-317.
25. Hall JM, Couse JF, Korach KS, 2001 The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 276: 36869-36872.
26. Kato S, Endoh H, Masuhiro Y, et al, 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270: 1491-1494.
27. Shim WS, Conaway M, Masamura S, et al, 2000 Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology 141: 396-405.
28. Coutts AS, Murphy LC, 1998 Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. Cancer Res 58: 4071-4074.
29. Razandi M, Pedram A, Rosen EM, Levin ER 2004 BRCA1 inhibits membrane estrogen and growth factor receptor signaling to cell proliferation in breast cancer. Mol Cell Biol 24: 5900-5913.
30. Razandi M, Pedram A, Merchenthaler I, Greene GL, Levin ER, 2004 Plasma membrane estrogen receptors exist and functions as dimers. Mol Endocrinol 18: 2854-2865.
31. Hammes SR, Levin ER, 2011 Minireview: Recent advances in extranuclear steroid receptor actions. Endocrinology 152: 4489-4495.
32. Deecher DC, Swiggard P, Frail DE, O'Connor LT, 2003 Characterization of a membrane-associated estrogen receptor in a rat hypothalamic cell line (D12). Endocrine 22: 211-223.
33. Zhang J, Li G, Li Z, et al, 2012 Estrogen-independent effects of ER-α36 in ER-negative breast cancer. Steroids 77: 663-673.
34. Moriarty K, Kim KH, Bender JR, 2006 Minireview: estrogen receptor-mediated rapid signaling. Endocrinology 147: 5557-5563.
35. Kampa M, Notas G, Pelekanou V, et al, 2012 Early membrane initiated transcriptional effects of estrogens in breast cancer cells: First pharmacological evidence for a novel membrane estrogen receptor element (ERx). Steroids 77: 959-967.
36. Doolan CM, Harvey BJ, 2003 A Galphas protein-coupled membrane receptor, distinct from the classical oestrogen receptor, transduces rapid effects of oestradiol on $[Ca^{2+}]$ i in female rat distal colon. Mol Cell Endocrinol 199: 87-103.
37. Condliffe SB, Doolan CM, Harvey BJ, 2001 17beta-oestradiol acutely regulates Cl^- secretion in rat distal colonic epithelium. J Physiol 530: 47-54.
38. Heldring N, Pike A, Andersson S, et al, 2007 Estrogen receptors: how do they signal and what are their targets. Physiol Rev 87: 905-931.
39. Acconcia F, Ascenzi P, Bocedi A, et al, 2005 Palmitoylation-dependent estrogen receptor alpha membrane localization: regulation by 17beta-estradiol. Mol Biol Cell 16: 231-237.

40. Marino M, Ascenzi P, Acconcia F, 2006 S-palmitoylation modulates estrogen receptor alpha localization and functions. Steroids 71: 298-303.
41. Smotrys JE, Linder ME, 2004 Palmitoylation of intracellular signaling proteins: regulation and function. Annu Rev Biochem 73: 559-587.
42. Schlesinger MJ, Magee AI, Schmidt MF 1980 Fatty acid acylation of proteins in cultured cells. J Biol Chem 255: 10021-10024.
43. Martin BR, Wang C, Adibekian A, Tully SE, Cravatt BF, 2012 Global profiling of dynamic protein palmitoylation. Nat Methods 9: 84-89.
44. Resh MD, 2006 Palmitoylation of ligands, receptors, and intracellular signaling molecules. Sci STKE 2006: re14.
45. Acconcia F, Bocedi A, Ascenzi P, Marino M, 2003 Does palmitoylation target estrogen receptors to plasma membrane caveolae? IUBMB Life 55: 33-35.
46. Galluzzo P, Ascenzi P, Bulzomi P, Marino M, 2008 The nutritional flavanone naringenin triggers antiestrogenic effects by regulating estrogen receptor alpha-palmitoylation. Endocrinology 149: 2567-2575.
47. Pedram A, Razandi M, Deschenes RJ, Levin ER, 2012 DHHC-7 and -21 are palmitoylacyltransferases for sex steroid receptors. Mol Biol Cell 23: 188-199.
48. Pedram A, Razandi M, Sainson RC, Kim JK, Hughes CC, Levin ER, 2007 A conserved mechanism for steroid receptor translocation to the plasma membrane. J Biol Chem 282: 22278-22288.
49. Song RX, Barnes CJ, Zhang Z, Bao Y, Kumar R, Santen RJ, 2004 The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. Proc Natl Acad Sci U S A 101: 2076-2081.
50. Galluzzo P, Caiazza F, Moreno S, Marino M, 2007 Role of ERbeta palmitoylation in the inhibition of human colon cancer cell proliferation. Endocr Relat Cancer 14: 153-167.
51. Marino M, Ascenzi P, 2006 Steroid hormone rapid signaling: the pivotal role of S-palmitoylation. IUBMB Life 58: 716-719.
52. Hammes SR, Levin ER, 2007 Extranuclear steroid receptors: nature and actions. Endocr Rev 28: 726-741.
53. Acconcia F, Marino M, 2011 The Effects of 17beta-estradiol in Cancer are Mediated by Estrogen Receptor Signaling at the Plasma Membrane. Front Physiol 2: 30.
54. Galluzzo P, Rastelli C, Bulzomi P, Acconcia F, Pallottini V, Marino M, 2009 17beta-Estradiol regulates the first steps of skeletal muscle cell differentiation via ER-alpha-mediated signals. Am J Physiol Cell Physiol 297: C1249-1262.
55. Filardo EJ, Quinn JA, Frackelton AR, Jr, Bland KI, 2002 Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol 16: 70-84.
56. Filardo EJ, Thomas P, 2012 Mini review: G Protein-Coupled Estrogen Receptor-1, GPER-1: Its Mechanism of Action and Role in Female Reproductive Cancer, Renal and Vascular Physiology. Endocrinology 153: 2953-2962.
57. Filardo EJ, Thomas P, 2005 GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. Trends Endocrinol Metab 16: 362-367.
58. Filardo E, Quinn J, Pang Y, et al, 2007 Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology 148: 3236-45
59. Thomas P, Pang Y, Filardo EJ, Dong J 2005 Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146: 624-632.
60. Langer G, Bader B, Meoli L, et al, 2010 A critical review of fundamental controversies in the field of GPR30 research. Steroids 75: 603-610.
61. Prossnitz ER, Barton M, 2011 The G-protein-coupled estrogen receptor GPER in health and disease. Nat Rev Endocrinol 7: 715-726.
62. Cheng SB, Graeber CT, Quinn JA, Filardo EJ, 2011 Retrograde transport of the transmembrane estrogen receptor, G-protein-coupled-receptor-30 (GPR30/GPER) from the plasma membrane towards the nucleus. Steroids 76: 892-896.
63. Cheng SB, Quinn JA, Graeber CT, Filardo EJ, 2011 Down-modulation of the G-protein-coupled estrogen receptor, GPER, from the cell surface occurs via a trans-Golgi-proteasome pathway. J Biol Chem 286: 22441-22455.
64. Nilsson BO, Olde B, Leeb-Lundberg LM, 2011 G protein-coupled oestrogen receptor 1 (GPER1)/GPR30: a new player in cardiovascular and metabolic oestrogenic signalling. Br J Pharmacol 163: 1131-1139.
65. Levin ER, 2009 G protein-coupled receptor 30: estrogen receptor or collaborator? Endocrinology 150: 1563-1565.
66. Notas G, Kampa M, Pelekanou V, Castanas E, 2011 Interplay of estrogen receptors and GPR30 for the regulation of early membrane initiated transcriptional effects: A pharmacological approach. Steroids 77: 943-950.
67. Heberden C, Reine F, Grosse B, et al, 2006 Detection of a raft-located estrogen receptor-like protein distinct from ER alpha. Int J Biochem Cell Biol 38: 376-391.
68. Harrington WR, Kim SH, Funk CC, et al, 2006 Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. Mol Endocrinol 20: 491-502.
69. Madak-Erdogan Z, Kieser KJ, Kim SH, Komm B, Katzenellenbogen JA, Katzenellenbogen BS, 2008 Nuclear and extranuclear pathway inputs in the regulation of global gene expression by estrogen receptors. Mol

Endocrinol 22: 2116-2127.
70. Toran-Allerand CD, Guan X, MacLusky NJ, et al, 2002 ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. J Neurosci 22: 8391-401
71. Piccart MJ, Trivedi S, Maaroufi Y, Debbaudt A, Veenstra S, Leclercq G, 1998 Evolution towards hormone independence of the MXT mouse mammary tumor is associated with a gradual change in its estrogen receptor molecular polymorphism. Cancer Biochem Biophys 16: 169-182.
72. Sheridan PJ, Buchanan JM, Anselmo VC, Martin PM, 1979 Equilibrium: the intracellular distribution of steroid receptors. Nature 282: 579-582.
73. Edwards DP, Martin PM, Horwitz KB, Chamness GC, McGuire WL, 1980 Subcellular compartmentalization of estrogen receptors in human breast cancer cells. Exp Cell Res 127: 197-213.
74. Yamashita S, 1998 Localization and functions of steroid hormone receptors. Histol Histopathol 13: 255-270.
75. Hager GL, Lim CS, Elbi C, Baumann CT, 2000 Trafficking of nuclear receptors in living cells. J Steroid Biochem Mol Biol 74: 249-254.
76. Dauvois S, White R, Parker MG, 1993 The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. J Cell Sci 106: 1377-1388.
77. Sebastian T, Sreeja S, Thampan RV, 2004 Import and export of nuclear proteins: focus on the nucleocytoplasmic movements of two different species of mammalian estrogen receptor. Mol Cell Biochem 260: 91-102.
78. Fornerod M, Ohno M, Yoshida M, Mattaj IW, 1997 CRM1 is an export receptor for leucine-rich nuclear export signals. Cell 90: 1051-1060.
79. DeFranco DB 2001 Nuclear export: DNA-binding domains find a surprising partner. Curr Biol 11: R1036-1037.
80. Nonclercq D, Journe F, Laios I, et al, 2007 Effect of nuclear export inhibition on estrogen receptor regulation in breast cancer cells. J Mol Endocrinol 39: 105-118.
81. Castoria G, Migliaccio A, Auricchio F, 2009 Signaling-dependent nuclear export of estradiol receptor controls cell cycle progression in breast cancer cells. Mol Cell Endocrinol 308: 26-31.
82. Leclercq G, Lacroix M, Laios I, Laurent G, 2006 Estrogen receptor alpha: impact of ligands on intracellular shuttling and turnover rate in breast cancer cells. Curr Cancer Drug Targets 6: 39-64.
83. Laios I, Journe F, Nonclercq D, et al, 2005 Role of the proteasome in the regulation of estrogen receptor alpha turnover and function in MCF-7 breast carcinoma cells. J Steroid Biochem Mol Biol 94: 347-359.
84. Laios I, Journe F, Laurent G, et al, 2003 Mechanisms governing the accumulation of estrogen receptor alpha in MCF-7 breast cancer cells treated with hydroxytamoxifen and related antiestrogens. J Steroid Biochem Mol Biol 87: 207-221.
85. Reid G, Hubner MR, Metivier R, et al, 2003 Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. Mol Cell 11: 695-707.
86. Rodriguez AL, Tamrazi A, Collins ML, Katzenellenbogen JA, 2004 Design, synthesis, and in vitro biological evaluation of small molecule inhibitors of estrogen receptor alpha coactivator binding. J Med Chem 47: 600-611.
87. Carlberg C, Seuter S, 2010 Dynamics of nuclear receptor target gene regulation. Chromosoma 119: 479-484.
88. Govind AP, Thampan RV, 2001 Proteins interacting with the mammalian estrogen receptor: proposal for an integrated model for estrogen receptor mediated regulation of transcription. J Cell Biochem 80: 571-579.
89. Edwards DP, 2000 The role of coactivators and corepressors in the biology and mechanism of action of steroid hormone receptors. J Mammary Gland Biol Neoplasia 5: 307-24
90. Lieberman BA, 1997 The estrogen receptor activity cycle: dependence on multiple protein-protein interactions. Crit Rev Eukaryot Gene Expr 7: 43-59.
91. Savkur RS, Burris TP, 2004 The coactivator LXXLL nuclear receptor recognition motif. J Pept Res 63: 207-212.
92. Jacquot Y, Laios I, Cleeren A, et al, 2007 Synthesis, structure, and estrogenic activity of 4-amino-3-(2-methylbenzyl)coumarins on human breast carcinoma cells. Bioorg Med Chem 15: 2269-2282.
93. Metzger D, Ali S, Bornert JM, Chambon P, 1995 Characterization of the amino-terminal transcriptional activation function of the human estrogen receptor in animal and yeast cells. J Biol Chem 270: 9535-9542.
94. Houtman R, de Leeuw R, Rondaij M, et al, 2012 Serine-305 Phosphorylation Modulates Estrogen Receptor Alpha Binding to a Coregulator Peptide Array, with Potential Application in Predicting Responses to Tamoxifen. Mol Cancer Ther 11: 805-816.
95. Leduc AM, Trent JO, Wittliff JL, et al, 2003 Helix-stabilized cyclic peptides as selective inhibitors of steroid receptor-coactivator interactions. Proc Natl Acad Sci U S A 100: 11273-8
96. Willmer EN, 1961 Steroids and cell surfaces. Biol Rev Camb Philos Soc 36: 368-398.
97. Seyle H, 1952 Correlation between the chemicalstructure and the pharmacological actions of steroid. Endocrinology 30: 437-453.
98. Szego CM, Davis JS, 1967 Adenosine 3',5'-monophosphate in rat uterus: acute elevation by estrogen. Proc Natl Acad Sci USA 58: 1711-1718.
99. Milgrom E, Atger M, Baulieu EE, 1973 Studies on estrogen entry into uterine cells and on estradiol-receptor complex attachment to the nucleus-is the entry of estrogen into uterine cells a protein-mediated process? Biochim Biophys Acta 320: 267-283.
100. Harrison RW, Fairfield S, Orth DN, 1974 Evidence for

glucocorticoid transport through the target cell membrane. Biochem Biophys Res Commun 61: 1262-1267.
101. Suyemitsu T, Terayama H, 1975 Specific binding sites for natural glucocorticids in plasma membranes of rat liver. Endocrinology 96: 1499-1508.
102. Pietras RJ, Szego CM, 1977 Specific binding sites of estrogen at the outer surface of isolated endometrial cells. Nature 265: 69-72.
103. Baulieu E, 1978 Cell membrane a target for steroid hormone. Mol Cell Endocrinol 12: 247-254.
104. Muller RE, Johnston TC, Traish AM, Wotiz HH, 1979 Studies on the mechanism of estradiol uptake by rat uterine cells and on estradiol binding to uterine plasma membranes. Adv Exp Med Biol 117: 401-421.
105. Nenci I, Fabris G, Marchetti E, Marzola A 1980 Cytochemical evidence for steroid binding sites in the plasma membrane of target cells. In: Bresciani F, (ed) Perspectives in Steroid Receptor Reasearch. New York: Raven Press.
106. Pietras RJ, Szego CM, 1980 Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes. Biochem J 191: 743-760.
107. Schaeffer JM, Stevens S, Smith RG, Hsueh AJ, 1980 Binding of 2-hydroxyestradiol to rat anterior pituitary cell membranes. J Biol Chem 255: 9838-9843.
108. Giorgi EP, Stein WD 1981 The transport of steroids into animal cells in culture. Endocrinology 108: 688-697.
109. Zanker KS, Prokscha GW, Blumel G, 1981 Plasma membrane-integrated estrogen receptors in breast tissue: possible modulator molecular for intracellular hormone level. J Cancer Res Clin Oncol 100: 135-148.
110. Srivastava AK, Dey SB, Roy SK, 1983 Interaction of cyproterone acetate with sex hormone binding globulin of monkey plasma. Exp Clin Endocrinol 82: 232-234.
111. Diez A, Sancho MJ, Egana M, Trueba M, Marino A, Macarull JM, 1984 An interaction of testosterone with cell membranes. Horm Metab Res 16: 475-477.
112. Hryb DJ, Khan MS, Rosner W, 1985 Testosterone-estradiol-binding globulin binds to human prostatic cell membranes. Biochem Biophys Res Commun 128: 432-440.
113. Berthois Y, Pourreau-Schneider N, Gandilhon P, Mittre H, Tubiana N, Martin PM, 1986 Estradiol membrane binding sites on human breast cancer cell lines. Use of a fluorescent estradiol conjugate to demonstrate plasma membrane binding systems. J Steroid Biochem 25: 963-972.
114. Pourreau-Schneider N, Berthois Y, Gandilhon P, Cau P, Martin PM, 1986 Early alterations at the plasma membrane of breast cancer cell lines in response to estradiol and hydroxytamoxifen. Mol Cell Endocrinol 48: 77-88.
115. Tubiana N, Mishal Z, le Caer F, et al, 1986 Quantification of oestradiol binding at the surface of human lymphocytes by flow cytofluorimetry. Br J Cancer 54: 501-504.
116. Warner M, Gustafsson JA, 2006 Nongenomic effects of estrogen: why all the uncertainty? Steroids 71: 91-95.
117. Kampa M, Castanas E, 2006 Membrane steroid receptor signaling in normal and neoplastic cells. Mol Cell Endocrinol 246: 76-82.
118. Nakajima T, Kitazawa T, Hamada E, Hazama H, Omata M, Kurachi Y, 1995 17beta-Estradiol inhibits the voltage-dependent L-type Ca2+ currents in aortic smooth muscle cells. Eur J Pharmacol 294: 625-635.
119. Alexaki VI, Charalampopoulos I, Kampa M, et al, 2004 Estrogen exerts neuroprotective effects via membrane estrogen receptors and rapid Akt/NOS activation. FASEB J 18: 1594-1596.
120. Nadal A, Ropero AB, Fuentes E, Soria B, Ripoll C, 2004 Estrogen and xenoestrogen actions on endocrine pancreas: from ion channel modulation to activation of nuclear function. Steroids 69: 531-536.
121. Raz L, Khan MM, Mahesh VB, Vadlamudi RK, Brann DW, 2008 Rapid estrogen signaling in the brain. Neurosignals 16: 140-153.
122. Leclercq G, 2012 Calcium-induced activation of estrogen receptor alpha - New insight. Steroids: 77: 924-927.
123. Yang X, Chen G, Papp R, Defranco DB, Zeng F, Salama Gm 2012 Oestrogen upregulates L-type Ca(2)(+) channels via oestrogen-receptor- by a regional genomic mechanism in female rabbit hearts. J Physiol 590: 493-508.
124. Kelly MJ, Ronnekleiv OK, Ibrahim N, Lagrange AH, Wagner EJM, 2002 Estrogen modulation of K(+) channel activity in hypothalamic neurons involved in the control of the reproductive axis. Steroids 67: 447-456.
125. Kelly MJ, Qiu J, Wagner EJ, Ronnekleiv OK, 2002 Rapid effects of estrogen on G protein-coupled receptor activation of potassium channels in the central nervous system (CNS). J Steroid Biochem Mol Biol 83: 187-193.
126. Andersen HK, Urbach V, Van Kerkhove E, Prosser E, Harvey BJ, 1995 Maxi K+ channels in the basolateral membrane of the exocrine frog skin gland regulated by intracellular calcium and pH. Pflugers Arch 431: 52-65.
127. Muchekehu RW, Harvey BJ, 2009 Estradiol rapidly induces the translocation and activation of the intermediate conductance calcium activated potassium channel in human eccrine sweat gland cells. Steroids 74: 212-217.
128. Donnellan F, Keating N, Geoghegan P, Murray FE, Harvey BJ, Keely SJ, 2010 JNK mitogen-activated protein kinase limits calcium-dependent chloride secretion across colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 298: G37-44.
129. Alzamora R, O'Mahony F, Bustos V, et al, 2011 Sexual dimorphism and oestrogen regulation of KCNE3 expression modulates the functional properties of KCNQ1 K(+) channels. J Physiol 589: 5091-5107.

130. O'Mahony F, Alzamora R, Betts V, et al, 2007 Female gender-specific inhibition of KCNQ1 channels and chloride secretion by 17beta-estradiol in rat distal colonic crypts. J Biol Chem 282: 24563-24573.
131. Saint-Criq V, Rapetti-Mauss R, Yusef YR, Harvey BJ, 2012 Estrogen regulation of epithelial ion transport: Implications in health and disease. Steroids 77: 918-923.
132. Sirotkin AV, Mlyncek J, Lavrincik J, Bulla J, Hetenyi L, 1995 The ability of steroid hormones to control cAMP and cGMP production by human granulosa cells in culture. Cell Signal 7: 61-65.
133. Schreiber V, Nedvidkova J, Jahodova J, 1993 Anterior pituitary weight, cAMP, cGMP and prolactin levels after combined treatment with estradiol and methylene blue. Physiol Res 42: 171-174.
134. Lacasa D, Agli B, Giudicelli Y, 1992 Hormonal activation of the cGMP-inhibited low-Km cyclic AMP phosphodiesterase of rat adipocytes from different sites: influence of ovariectomy. Biochim Biophys Acta 1136: 99-104.
135. Minami T, Oomura Y, Nabekura J, Fukuda A, 1990 17 beta-estradiol depolarization of hypothalamic neurons is mediated by cyclic AMP. Brain Res 519: 301-307.
136. Levin ER, Frank HJ, Chaudhari A, Kirschenbaum MA, Bandt A, Mills S, 1989 Decreased atrial natriuretic factor receptors and impaired cGMP generation in glomeruli from the cardiomyopathic hamster. Biochem Biophys Res Commun 159: 807-814.
137. Sanchez-Bueno A, Sancho MJ, Trueba M, Macarulla JM, 1987 An early effect of estradiol at hepatic level, previous to its protein synthesis activation. Int J Biochem 19: 93-96.
138. Fleming H, Blumenthal R, Gurpide E, 1984 Characteristics of cyclic nucleotide dependent regulation of cytoplasmic E2 binders in cultured endometrial and breast cells. J Steroid Biochem 20: 5-9.
139. Flandroy L, Cheung WY, Steiner AL, 1983 Immunofluorescent localization of cGMP, cGMP-dependent protein kinase, calmodulin and cAMP in the rat uterus. Effects of estrogen treatment. Cell Tissue Res 233: 639-646.
140. Flandroy L, Galand P, 1978 Changes in cGMP and cAMP content in the estrogen-stimulated rat uterus: temporal relationship with other parameters of hormonal stimulation. J Cyclic Nucleotide Res 4: 145-58.
141. Flandroy L, Galand P, 1975 Oestrogen-related changes in uterine and vaginal cAMP and cGMP. Arch Int Physiol Biochim 83: 965-967.
142. Aronica SM, Kraus WL, Katzenellenbogen BS, 1994 Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci U S A 91: 8517-8521.
143. Aronica SM, Katzenellenbogen BS, 1991 Progesterone receptor regulation in uterine cells: stimulation by estrogen, cyclic adenosine 3',5'-monophosphate, and insulin-like growth factor I and suppression by antiestrogens and protein kinase inhibitors. Endocrinology 128: 2045-2052.
144. Thomas P, 2012 Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. Gen Comp Endocrinol 175: 367-383.
145. Alexaki VI, Charalampopoulos I, Kampa M, et al, 2006 Activation of membrane estrogen receptors induce pro-survival kinases. J Steroid Biochem Mol Biol 98: 97-110.
146. Szego EM, Barabas K, Balog J, et al, 2006 Estrogen induces estrogen receptor alpha-dependent cAMP response element-binding protein phosphorylation via mitogen activated protein kinase pathway in basal forebrain cholinergic neurons in vivo. J Neurosci 26: 4104-4110.
147. Makarevich AV, Sirotkin AV, Genieser HG, 2004 Action of protein kinase A regulators on secretory activity of porcine granulosa cells in vitro. Anim Reprod Sci 81: 125-136.
148. Shingo AS, Kito S, 2002 Estrogen induces elevation of cAMP-dependent protein kinase activity in immortalized hippocampal neurons: imaging in living cells. J Neural Transm 109: 171-174.
149. Carlstrom L, Ke ZJ, Unnerstall JR, Cohen RS, Pandey SC, 2001 Estrogen modulation of the cyclic AMP response element-binding protein pathway. Effects of long-term and acute treatments. Neuroendocrinology 74: 227-243.
150. Kelly MJ, Lagrange AH, Wagner EJ, Ronnekleiv OK, 1999 Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. Steroids 64: 64-75.
151. Wagner EJ, Ronnekleiv OK, Kelly MJm 1998 Protein kinase A maintains cellular tolerance to mu opioid receptor agonists in hypothalamic neurosecretory cells with chronic morphine treatment: convergence on a common pathway with estrogen in modulating mu opioid receptor/effector coupling. J Pharmacol Exp Ther 285: 1266-1273.
152. Lagrange AH, Ronnekleiv OK, Kelly MJM, 1997 Modulation of G protein-coupled receptors by an estrogen receptor that activates protein kinase A. Mol Pharmacol 51: 605-612.
153. Van Dyke RW, Root KV, Hsi RA, 1996 cAMP and protein kinase A stimulate acidification of rat liver endosomes in the absence of chloride. Biochem Biophys Res Commun 222: 312-316.
154. Fujimoto N, Katzenellenbogen BS, 1994 Alteration in the agonist/antagonist balance of antiestrogens by activation of protein kinase A signaling pathways in breast cancer cells: antiestrogen selectivity and promoter dependence. Mol Endocrinol 8: 296-304.
155. Davies WA, Berghorn KA, Albrecht ED, Pepe GJ, 1993

Developmental regulation of protein kinase-A and -C activities in the baboon fetal adrenal. Endocrinology 132: 2491-2497.
156. Filardo EJ, 2002 Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol 80: 231-238.
157. Simstein R, Burow M, Parker A, Weldon C, Beckman B, 2003 Apoptosis, chemoresistance, and breast cancer: insights from the MCF-7 cell model system. Exp Biol Med (Maywood) 228: 995-1003.
158. Simoncini T, Mannella P, Fornari L, Caruso A, Varone G, Genazzani AR, 2004 Genomic and non-genomic effects of estrogens on endothelial cells. Steroids 69: 537-542.
159. Shupnik MA, 2004 Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 23: 7979-7989.
160. Kalkman HO, 2006 The role of the phosphatidylinositide 3-kinase-protein kinase B pathway in schizophrenia. Pharmacol Ther 110: 117-134.
161. Garcia-Segura LM, Sanz A, Mendez P, 2006 Cross-talk between IGF-I and estradiol in the brain: focus on neuroprotection. Neuroendocrinology 84: 275-279.
162. Cheskis BJ, Greger J, Cooch N, et al, 2008 MNAR plays an important role in ERa activation of Src/MAPK and PI3K/Akt signaling pathways. Steroids 73: 901-905.
163. Prossnitz ER, Oprea TI, Sklar LA, Arterburn JB, 2008 The ins and outs of GPR30: a transmembrane estrogen receptor. J Steroid Biochem Mol Biol 109: 350-353.
164. Fox EM, Andrade J, Shupnik MA, 2009 Novel actions of estrogen to promote proliferation: integration of cytoplasmic and nuclear pathways. Steroids 74: 622-627.
165. Ma CX, Crowder RJ, Ellis MJ, 2011 Importance of PI3-kinase pathway in response/resistance to aromatase inhibitors. Steroids 76: 750-752.
166. Murphy E, 2011 Estrogen signaling and cardiovascular disease. Circ Res 109: 687-696.
167. Makker A, Goel MM, Das V, Agarwal A, 2012 PI3K-Akt-mTOR and MAPK signaling pathways in polycystic ovarian syndrome, uterine leiomyomas and endometriosis: an update. Gynecol Endocrinol 28: 175-181.
168. Miller TW, Balko JM, Arteaga CL, 2011 Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. J Clin Oncol 29: 4452-4461.
169. Haynes MP, Li L, Sinha D, et al, 2003 Src kinase mediates phosphatidylinositol 3-kinase/Akt-dependent rapid endothelial nitric-oxide synthase activation by estrogen. J Biol Chem 278: 2118-2123.
170. Kampa M, Pelekanou V, Gallo D, et al, 2011 ERalpha17p, an ERalpha P295 -T311 fragment, modifies the migration of breast cancer cells, through actin cytoskeleton rearrangements. J Cell Biochem 112: 3786-3796.
171. Simoncini T, Rabkin E, Liao JK, 2003 Molecular basis of cell membrane estrogen receptor interaction with phosphatidylinositol 3-kinase in endothelial cells. Arterioscler Thromb Vasc Biol 23: 198-203.
172. Simoncini T, Varone G, Fornari L, et al, 2002 Genomic and nongenomic mechanisms of nitric oxide synthesis induction in human endothelial cells by a fourth-generation selective estrogen receptor modulator. Endocrinology 143: 2052-2061.
173. Simoncini T, Fornari L, Mannella P, et al, 2002 Novel non-transcriptional mechanisms for estrogen receptor signaling in the cardiovascular system. Interaction of estrogen receptor alpha with phosphatidylinositol 3-OH kinase. Steroids 67: 935-939.
174. Pedram A, Razandi M, Aitkenhead M, Hughes CC, Levin ER, 2002 Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. J Biol Chem 277: 50768-50775.
175. Limbourg FP, Huang Z, Plumier JC, et al, 2002 Rapid nontranscriptional activation of endothelial nitric oxide synthase mediates increased cerebral blood flow and stroke protection by corticosteroids. J Clin Invest 110: 1729-1738.
176. Dos Santos EG, Dieudonne MN, Pecquery R, Le Moal V, Giudicelli Y, Lacasa D, 2002 Rapid nongenomic E2 effects on p42/p44 MAPK, activator protein-1, and cAMP response element binding protein in rat white adipocytes. Endocrinology 143: 930-940.
177. Santen RJ, Fan P, Zhang Z, Bao Y, Song RX, Yue W, 2009 Estrogen signals via an extra-nuclear pathway involving IGF-1R and EGFR in tamoxifen-sensitive and -resistant breast cancer cells. Steroids 74: 586-594.
178. Levin ER, 2003 Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol 17: 309-317.
179. Levin ER, 2002 Cellular functions of plasma membrane estrogen receptors. Steroids 67: 471-475.
180. Lichtner RB, 2003 Estrogen/EGF receptor interactions in breast cancer: rationale for new therapeutic combination strategies. Biomed Pharmacother 57: 447-451.
181. Kurebayashi J, Okubo S, Yamamoto Y, Sonoo H, 2004 Inhibition of HER1 signaling pathway enhances antitumor effect of endocrine therapy in breast cancer. Breast Cancer 11: 38-41.
182. Evinger AJ, 3rd, Levin ER, 2005 Requirements for estrogen receptor alpha membrane localization and function. Steroids 70: 361-363.
183. Gee JM, Robertson JF, Gutteridge E, et al, 2005 Epidermal growth factor receptor/HER2/insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer. Endocr Relat Cancer 12: Suppl 1: 99-111.
184. Biswas DK, Iglehart JD, 2006 Linkage between EGFR family receptors and nuclear factor kappaB (NF-kappaB)

signaling in breast cancer. J Cell Physiol 209: 645-652.
185. Carreau S, Bois C, Zanatta L, Silva FR, Bouraima-Lelong H, Delalande C, 2011 Estrogen signaling in testicular cells. Life Sci 89: 584-587.
186. Bordeaux JM, Cheng H, Welsh AW, et al, 2012 Quantitative in situ measurement of estrogen receptor mRNA predicts response to tamoxifen. PLoS One 7: e36559.
187. Smith CL, Onate SA, Tsai MJ, O'Malley BW, 1996 CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. Proc Natl Acad Sci U S A 93: 8884-8888.
188. Razandi M, Pedram A, Levin ER, 2000 Estrogen signals to the preservation of endothelial cell form and function. J Biol Chem 275: 38540-38546.
189. Wada-Hiraike O, Warner M, Gustafsson JA, 2006 New developments in oestrogen signalling in colonic epithelium. Biochem Soc Trans 34: 1114-1116.
190. Kampa M, Pelekanou V, Castanas E, 2008 Membrane-initiated steroid action in breast and prostate cancer. Steroids 73: 953-960.
191. Flamini MI, Sanchez AM, Goglia L, Tosi V, Genazzani AR, Simoncini T, 2009 Differential actions of estrogen and SERMs in regulation of the actin cytoskeleton of endometrial cells. Mol Hum Reprod 15: 675-685.
192. Flamini MI, Sanchez AM, Genazzani AR, Simoncini T, 2011 Estrogen regulates endometrial cell cytoskeletal remodeling and motility via focal adhesion kinase. Fertil Steril 95: 722-726.
193. Levin ER, 2011 Minireview: Extranuclear steroid receptors: roles in modulation of cell functions. Mol Endocrinol 25: 377-384.
194. Pelekanou V, Kampa M, Gallo D, et al, 2011 The estrogen receptor alpha-derived peptide ERalpha17p ($P_{(295)}$-$T_{(311)}$) exerts pro-apoptotic actions in breast cancer cells in vitro and in vivo, independently from their ERalpha status. Mol Oncol 5: 36-47.
195. Sanchez AM, Flamini MI, Polak K, et al, 2012 Actin cytoskeleton remodelling by sex steroids in neurones. J Neuroendocrinol 24: 195-201.
196. Sanchez AM, Flamini MI, Fu XD, et al, 2009 Rapid signaling of estrogen to WAVE1 and moesin controls neuronal spine formation via the actin cytoskeleton. Mol Endocrinol 23: 1193-1202.
197. Sanchez AM, Flamini MI, Baldacci C, Goglia L, Genazzani AR, Simoncini T, 2010 Estrogen receptor-alpha promotes breast cancer cell motility and invasion via focal adhesion kinase and N-WASP. Mol Endocrinol 24: 2114-2125.
198. Simoncini T, Scorticati C, Mannella P, et al, 2006 Estrogen receptor alpha interacts with Galpha13 to drive actin remodeling and endothelial cell migration via the RhoA/Rho kinase/moesin pathway. Mol Endocrinol 20: 1756-1771.
199. Giretti MS, Fu XD, De Rosa G, et al, 2008 Extranuclear signalling of estrogen receptor to breast cancer cytoskeletal remodelling, migration and invasion. PLoS One 3: e2238.
200. Kampa M, Nifli AP, Charalampopoulos I, et al, 2005 Opposing effects of estradiol- and testosterone-membrane binding sites on T47D breast cancer cell apoptosis. Exp Cell Res 307: 41-51.
201. Jacob J, Sebastian KS, Devassy S, et al, 2006 Membrane estrogen receptors: genomic actions and post transcriptional regulation. Mol Cell Endocrinol 246: 34-41.
202. Pelekanou V, Notas G, Sanidas E, Tsapis A, Castanas E, Kampa M, 2010 Testosterone membrane-initiated action in breast cancer cells: Interaction with the androgen signaling pathway and EPOR. Mol Oncol 4: 135-149.
203. Pelekanou V, Kampa M, Kafousi M, et al, 2007 Erythropoietin and its receptor in breast cancer: correlation with steroid receptors and outcome. Cancer Epidemiol Biomarkers Prev 16: 2016-2023.
204. Pelekanou V, Notas G, Kampa M, et al, 2012 ERalpha36, a new variant of the ERalpha is expressed in triple negative breast carcinomas and has a specific transcriptomic signature in breast cancer cell lines. Steroids 77: 928-934.
205. Zhang XT, Kang LG, Ding L, Vranic S, Gatalica Z, Wang ZY, 2011 A positive feedback loop of ER-alpha36/EGFR promotes malignant growth of ER-negative breast cancer cells. Oncogene 30: 770-780.
206. Zhang X, Ding L, Kang L, Wang ZY, 2012 Estrogen receptor-alpha 36 mediates mitogenic antiestrogen signaling in ER-negative breast cancer cells. PLoS One 7: e30174.
207. Lee LM, Cao J, Deng H, Chen P, Gatalica Z, Wang ZY, 2008 ER-alpha36, a novel variant of ER-alpha, is expressed in ER-positive and -negative human breast carcinomas. Anticancer Res 28: 479-483.
208. Shi L, Dong B, Li Z, et al, 2009 Expression of ER-α36, a novel variant of estrogen receptor α, and resistance to tamoxifen treatment in breast cancer. J Clin Oncol 27: 3423-3429.
209. Welsh AW, Lannin DR, Young GS, et al, 2012 Cytoplasmic estrogen receptor in breast cancer. Clin Cancer Res 18: 118-126.
210. Martin PM, Sheridan PJ, 1986 Intracellular localization of steroid hormone receptors. A challenging problem. Autoradiographic and biochemical study. Ann Pathol 6: 115-119.
211. Seo HS, Larsimont D, Querton G, et al, 1998 Estrogenic and anti-estrogenic regulation of estrogen receptor in MCF-7 breast-cancer cells: comparison of immunocytochemical data with biochemical measurements. Int J Cancer 78: 760-765.