Università della Calabria

Facoltà di Farmacia e Scienze della Nutrizione e della Salute

Dipartimento Farmaco-Biologico

(MED 04/ Patologia Generale)

Dottorato di Ricerca in "Biochimica Cellulare ed Attività dei Farmaci in Oncologia" (XXV ciclo)

Estrogen Receptor beta, through Sp1, recruits a corepressor complex to the Estrogen Receptor alpha gene promoter in breast cancer cells

Docente Tutor

Ch.mo Prof. Sebastiano Andò

Dottorando

Dott. Domenico Zito

Coordinatore

Ch.mo Prof. Diego Sisci

Anno Accademico 2011/2012

Università della Calabria

Facoltà di Farmacia e Scienze della Nutrizione e della Salute

Dipartimento Farmaco-Biologico

(MED 04/ Patologia Generale)

Dottorato di Ricerca in "Biochimica Cellulare ed Attività dei Farmaci in Oncologia" (XXV ciclo)

Estrogen Receptor beta, through Sp1, recruits a corepressor complex to the Estrogen Receptor alpha gene promoter in breast cancer cells

Docente Tutor

Ch.mo Prof. Sebastiano Andò

Dottorando

Dott. Domenico Zito

Coordinatore

Ch.mo Prof. Diego Sisci

Anno Accademico 2011/2012

INDEX

Scientific Publications

1. Panno ML, Giordano F, Rizza P, Pellegrino M, Zito D, Giordano C, Mauro L, Catalano S, Aquila S, Sisci D, De Amicis F, Vivacqua A, Fuqua S.A.W., Andò S (2012). Bergapten induces ER depletion in breast cancer cells through SMAD4-mediated ubiquitination. Breast Cancer Res Treat; 136 (2): 443-55.

2. Bartella V, Rizza P, Barone I, Zito D, Giordano F, Giordano C, Catalano S, Mauro L, Sisci D, Panno ML, Fuqua S.A.W., Andò S (2012). Estrogen receptor beta binds Sp1 and recruits a corepressor complex to the estrogen receptor alpha gene promoter. Breast Cancer Res Treat; 134 (2): 569-81.

Abstract

Human Estrogen receptors alpha (α) and beta (β) are essential components of a complex signal transduction pathway that regulates mammary growth and development. Several studies have reported that normal breast tissues display a relative higher expression of ERβ than ERα, which drastically changes during breast tumorogenesis. Thus, it is reasonable to suggest that a dysregulation of the two estrogen receptor subtypes may induce breast cancer development. However, the molecular mechanisms involved in the potential opposing roles played by the two estrogen receptors on tumor cell growth still needs to be elucidated.

In the present study, we have demonstrated that ERβ overexpression in breast cancer cells decreases cell proliferation and down-regulates ERα mRNA and protein content, with a concomitant repression of estrogen-regulated genes. Transient transfection experiments, using a vector containing the human ERα promoter region, showed that elevated levels of ERβ down-regulated basal ERα promoter activity. Furthermore, site-directed mutagenesis and deletion analysis revealed that the proximal GC-rich motifs at −223 and -214 are critical for the ERβ-induced ERα down-regulation in breast cancer cells. This occurred through ERβ-Sp1 protein-protein interactions within the ERα promoter region and the recruitment of a corepressor complex containing the nuclear receptor corepressor NCoR, accompanied by hypoacetylation of histone H4 and displacement of RNA polymerase II. Silencing of NCoR gene expression by RNA interference reversed the down-regulatory effects of ERβ on ER α gene expression and cell proliferation.

Collectively, these results suggest a novel mechanism by which overexpression of ERβ, through NCoR, is able to down regulate ERα expression, thus repressing ERα's main role on breast cancer cell growth.

Introduction

Estrogens and breast cancer

We have known for many years that estrogens are more than the female hormones. They have an essential role, together with other hormones, in the development of the female sex organs and secondary sex characteristics, the regulation of the menstrual cycle and reproduction, and in both sexes, estrogens have also functions in the skeleton and central nervous system, on behaviour, and in the cardiovascular and immune systems (Fig.1).

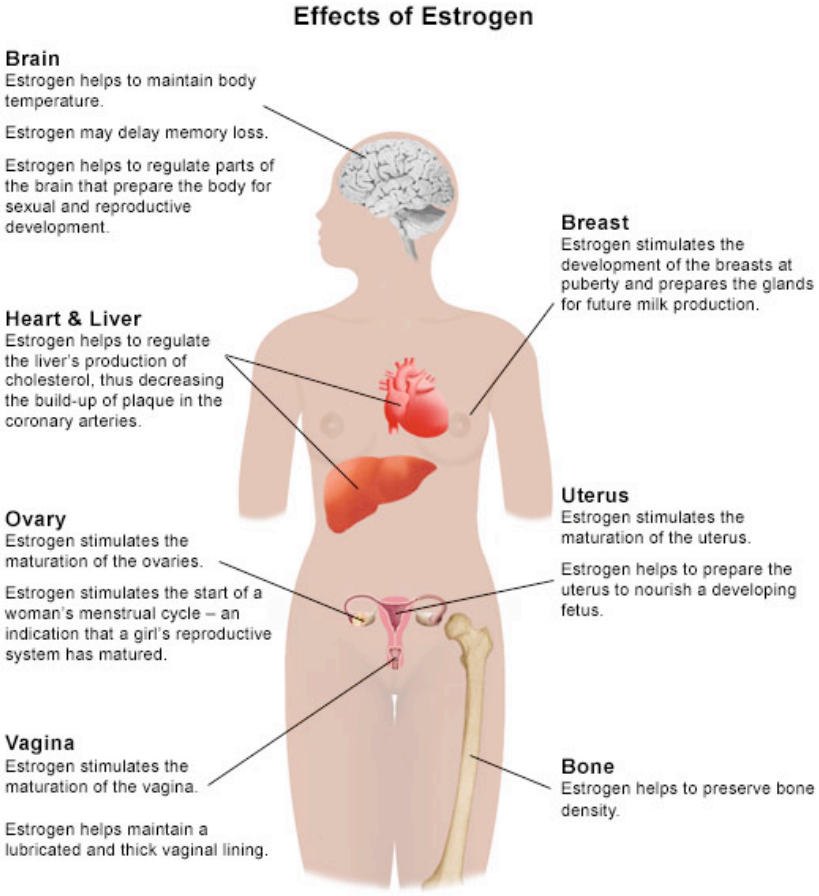


Figure 1. Effects of Estrogens on organs and tissues

In addition to their role in physiology, estrogens are also associated with the development and progression of breast cancer (1). An association between these hormones and breast cancer is important because breast cancer is diagnosed in one million women in the world each year, and estrogens are used for contraception or menopause treatment by >10% of reproductive age and post-menopausal women respectively. In 1896, Beatson discovered that removal of the ovaries resulted in breast cancer remission, connecting for the first time hormones with breast cancer, decades prior to the discovery of estrogens or estrogen receptors (ERs). Seventy years later, O'Malley observed changes in hybridizable RNA upon estrogen stimulation of the chick oviduct, indicating that estrogens regulate transcription (2). Again several years later a specific estrogen-binding protein was discovered that was present in breast tumors and its expression level could predict the response to endocrine disruption, thereby making the link between cancer and estrogens that was described almost a century before (3, 4). The subsequent cloning of the ERα gene and the identification of specific domains within the protein demonstrated that ERα functions as a ligand-dependent transcription factor.

Recently, Epidemiological and experimental evidences implicate estrogens in the aetiology of breast cancer. Most established risk factors for breast cancer in humans are thought to influence risk through hormone-related pathways (5), increased concentrations of endogenous estrogens are strongly associated with increased risk for breast cancer in postmenopausal women (6), and trials have shown that the anti-estrogens tamoxifen and raloxifene reduce the incidence of breast cancer (7). Furthermore, experimental studies in animals have shown that estrogens can promote mammary tumours, and a decrease in exposure to estrogens, by performing an oophorectomy or giving an anti-oestrogenic drug, has the reverse effect (8).

However, the effects of estrogen alone do not fully account for the relationships observed between breast cancer and hormone-related risk factors. Other hormones, such as progesterone (1), prolactin (9) and testosterone (10), may also be important.

Although risk for breast cancer increases with age, there is a marked decline in the rate of increase in risk with age following the loss of ovarian function, either as a result of a bilateral oophorectomy or due to the menopause (11,12), showing that hormone production by the ovaries is a crucial risk factor for breast cancer in humans. The duration of exposure to ovarian hormones seems to be closely related to breast cancer risk: a 1-year delay in the onset of menarche is associated with a 5% reduction in risk for developing breast cancer in later life (13), and each 1-year delay in the onset of menopause is associated with a 3% increase in risk (11, 14). Epidemiological studies have also firmly established associations between risk for breast cancer and other reproductive factors, including nulliparity (having no children) or low parity, late age at first birth, and breast feeding (15). After a transient increase in risk for breast cancer, peaking at about 5 years after giving birth (16), having at least one child is associated with a decrease in the long-term risk of developing breast cancer compared with risk among the nulliparous, and this protective effect increases with number of children (17). Each birth reduces the relative risk of breast cancer by an average of 7% (18). The reduction in risk per birth is greater for births at young ages than older ages, such that women who have their first birth before the age of 20 years have a 30% lower risk than women with a first birth after the age of 35 years (19).

A mechanism involving oestrogens, and probably other hormones, has been proposed to explain both the transient increase in risk and the reduced risk in the long term associated with pregnancy. The very high serum levels of oestrogens and

progesterone during pregnancy stimulate growth of the mammary epithelium and also promote the differentiation of epithelial tissue, reducing the number of epithelial structures most vulnerable to malignant transformation (20). Thus, the short-term effect of pregnancy may be to promote the growth of cancer if a malignant transformation is present in the breast, but in the longer term the risk for breast cancer is reduced. In contrast, malignant transformations are more likely to have accumulated in the breast tissue of older women, and there might therefore be a higher risk of cancer developing in these women when breast cells are stimulated to divide during pregnancy. The effect of age at first birth highlights the importance of timing of exposure as a critical determinant of the effects of steroid hormones such as oestrogen. Breast-feeding is associated with a modest decrease in risk for breast cancer, above and beyond that associated with multiple pregnancies (4% for every 12 months of breast feeding) (17). This effect might be due to the suppression of ovulation, reducing exposure to ovarian hormones.

Estrogen Receptors

Estrogens exert their biological effects via interaction with the two different isoforms of estrogen receptors, ERα, and ERβ, each encoded by unique genes, but with a common structural and functional organization. The two receptor subtypes belong to the nuclear receptor (NR) superfamily, that function as ligand-regulated transcription factors, regulating the expression of genes contributing to growth, differentiation and metabolism. Both of these protein have a high degree of homology in the DBD, but differ considerably in the N-terminal domain and in the AF-1 (activation function) and to a lesser extent in the LBD.

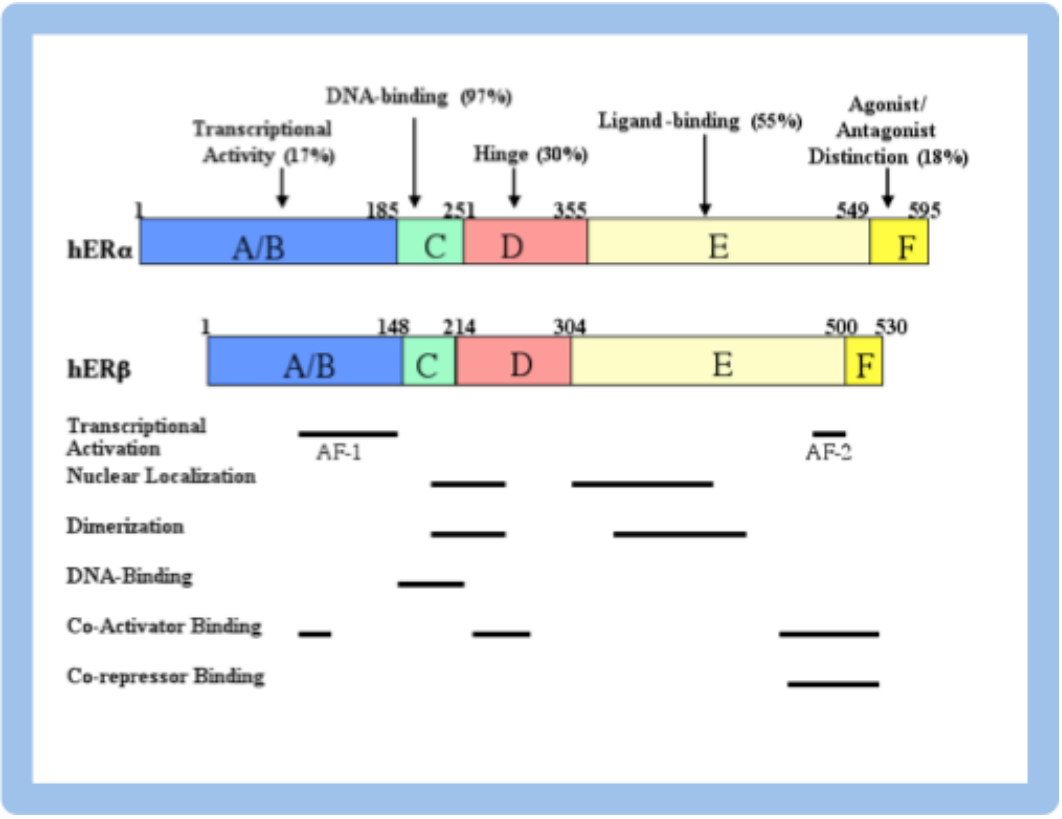


Figure 2. Domain organization and sequence homology of human ERα and ERβ.

Steroid receptors are composed of five domains (Fig. 2) denoted A–F (21). The DNA-binding domain (domain C) is highly conserved between ERα and ERβ with an amino acid identity of 97%, whereas the homology in the ligand-binding domain (domain E) is only 55% (Kuiper et al. 1996). By way of perspective, this level of identity is also seen between the ligand-binding domains of the androgen, glucocorticoid, mineralocorticoid, and progesterone receptors, and is associated with both unique and shared ligand binding. The N-terminal (domains A/B), hinge (domain D), and C-terminal regions (domain F) have the greatest sequence diversity (22). ER contains two 'activation functions' (AF) (Fig. 2) that interact with coactivators. AF-1 that is ligand-independent lies within the N-terminal domain,

whereas for AF-2 that lies in the ligand binding domain (LBD), its activity is dependent on ligand-induced conformational changes. The relative contribution of each AF is cell and promoter dependent.

ER ligands interact with ER subtypes in various parts of the human body (Fig.3). The abundance and distribution of the receptors will, in part, determine whether a ligand will have a particular effect. ERα and ERβ are known to be localized in the breast, brain, cardiovascular system, urogenital tract and bone (23). ERα is the main ER subtype in the liver, whereas ERβ is the main ER in the colon. ERα and ERβ may also localize to distinct cellular subtypes within each tissue. For example, within the ovary, ERα is largely present in the thecal and interstitial cells, whereas ERβ is predominantly in the granulosa cells (24,25). In the prostate, ERβ localizes to the epithelium, whereas ERα localizes to the stroma (26).

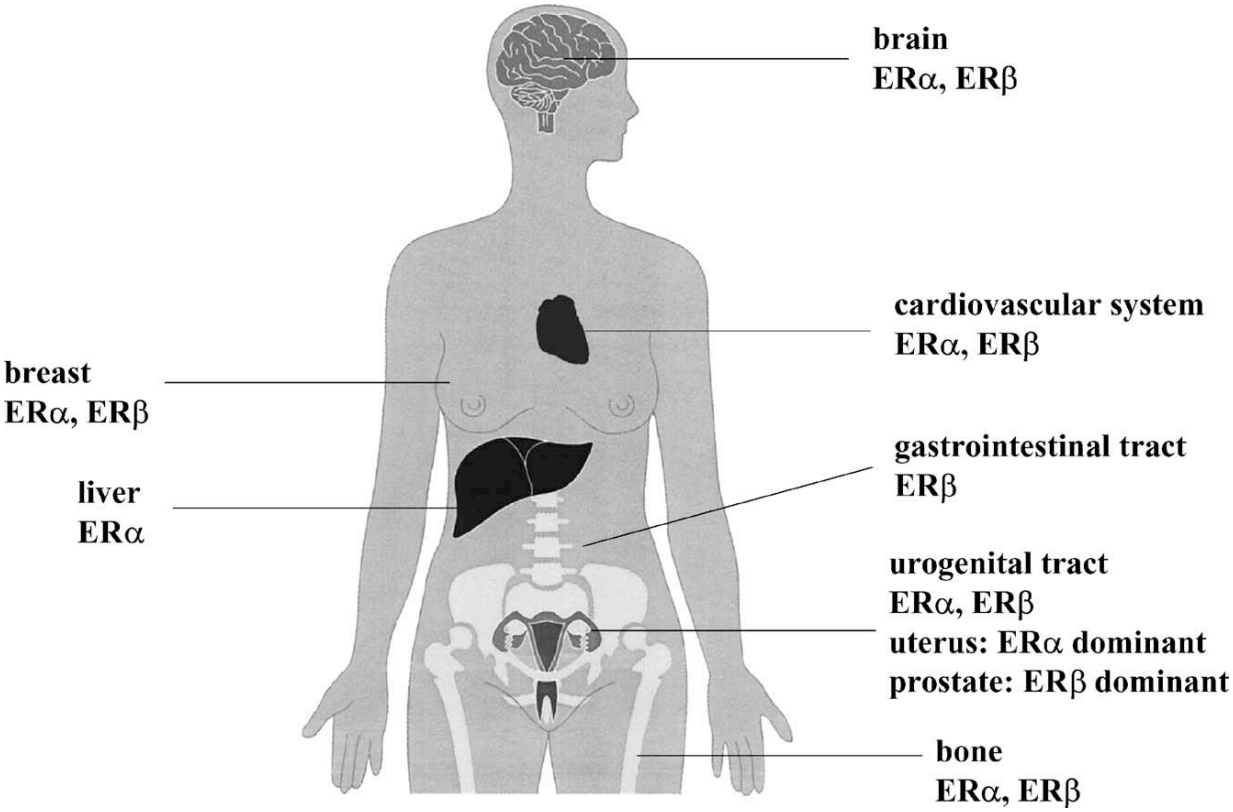


Figure 3. Distribution of ERα and ERβ in the human body. [34].

Estrogen Receptor alpha

ERα was the first estrogen receptor cloned and it was isolated from MCF-7 human breast cancer cells in the late 1980s (27, 28, 29). In accordance with its role as a transcription factor, this 66 kDa Erα, encoded by ESR1 gene on chromosome 6q25.1, localizes primarily to the nucleus. A 46 kDa isoform (hERα46) that lacks the first 173 amino acids of the 66 kDa form of ERα has also been preliminarily characterized (Fig.4) (30). In addition, several ERα splicing variants have been described (31,32), but whether they are expressed as proteins that have a biological function remains unknown. Another source of variability in receptor function, and perhaps also dysfunction, is ERα gene polymorphisms. ERα polymorphisms have been linked to

increased litter size in pigs (33), breast cancer susceptibility (34), bone mineral density and osteoporosis (35), hypertension (36), spontaneous abortion (37), and body height (38).

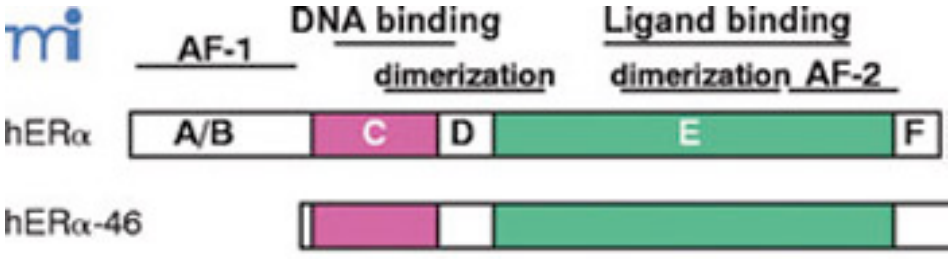


Figure 4. Estrogen Receptor alpha isoforms (Matthews J, Gustafsson JA, 2003 Molecular Interventions 3:281-292)

Estrogen receptor beta

The transcriptional responses to estrogen signaling depend on ligand identity and availability, the cellular concentration and localization of ERs, levels of various coregulator proteins, other signal transduction components, and the chromatin state (39). The discovery of a second ER, ERβ, in 1996 (22) prompted renewed efforts to investigate the mechanisms of action of estrogenic molecules. Recently, global analysis of gene expression profiles and identification of protein-DNA interactions have begun to reveal the molecular architecture of ERβ binding to DNA and the subsequent effects on gene regulatory networks.

ERβ is a member of the nuclear receptor superfamily and shares common structural characteristics with the other members of this family, including five distinguishable domains denoted A–F (Fig. 1) (40). The human ERβ gene (ESR2) is located on chromosome 14q23.2, spanning ~61.2 kb. The ERβ protein is produced from eight exons. Additionally, there are two untranslated exons, 0N and 0K, in the 5′-region

and an exon at the 3′-end that can be spliced to exon 7 to produce the alternative ERβ isoform, ERβ2 (also called ERβcx) (Fig.5) (41). Thus, ERβ2 has a unique C terminus, where the amino acids corresponding to exon 8 are replaced with 26 unique amino acids. The full-length human ERβ (also named ERβ1) protein includes 530 amino acids with an estimated molecular mass of 59.2 kDa, whereas ERβ2 encodes a protein of 495 amino acid residues with a predicted molecular mass of 55.5 kDa. ERβ2 has undetectable affinity for E2 and other tested ligands. ERβ2 was suggested to be a dominant-negative inhibitor of ERα (41). Further mechanistic study revealed that ERβ2 induces proteasome-dependent degradation of ERα, leading to suppression of ERα signaling (42). Although additional mRNA isoforms of ERβ arising from differential splicing have been described, only ERβ2 has been identified at the protein level (Fig.6) (43, 44).

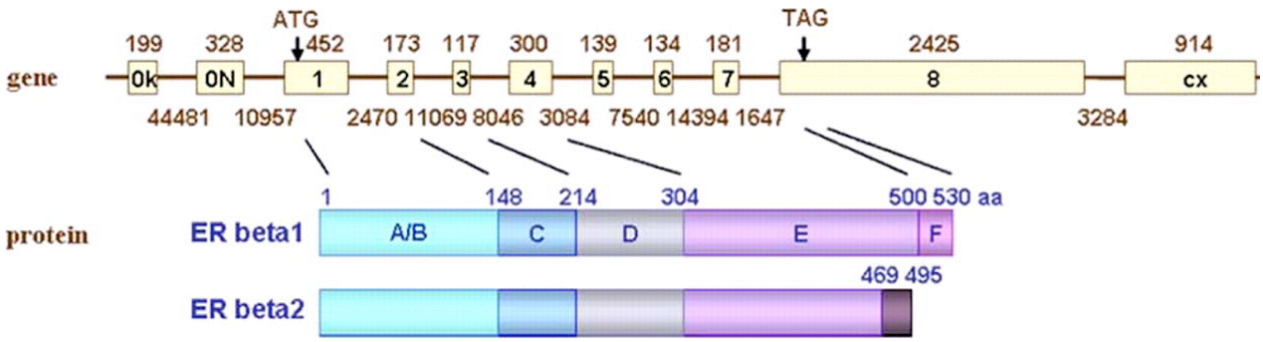


Figure 5. Genomic organization of the human ERβ gene, protein, and functional domains.

Sequencing data suggested that multiple ERβ isoforms exist as a result of alternative splicing of the last coding exon (exon 8) (45). This is also supported by the availability of multiple ERβ isoform transcripts in the human genome project in the NCBI AceView database. With regard to nomenclature, the original ERβ is also called ERβ1. So far, four other ERβ isoforms (ERβ2, ERβ3, ERβ4, and ERβ5) have

been identified (Fig. 6). It has been shown their existence as full-length transcripts, which have in common exon 1 through 7 plus one isoform- specific exon 8 (46,47). The molecular weights of ERβ1, 2, 4, and 5 have been determined as 59, 56, 54 and 53 kDa, respectively, according to protein sequence prediction programs, as well as ectopic protein-expression experiments (47). Since all isoforms share exons 1 through 7, they all have the same AF1 domain, DBD, hinge domain, and LBD, leaving the AF2 domain (C-terminus) specific to each of the isoforms.

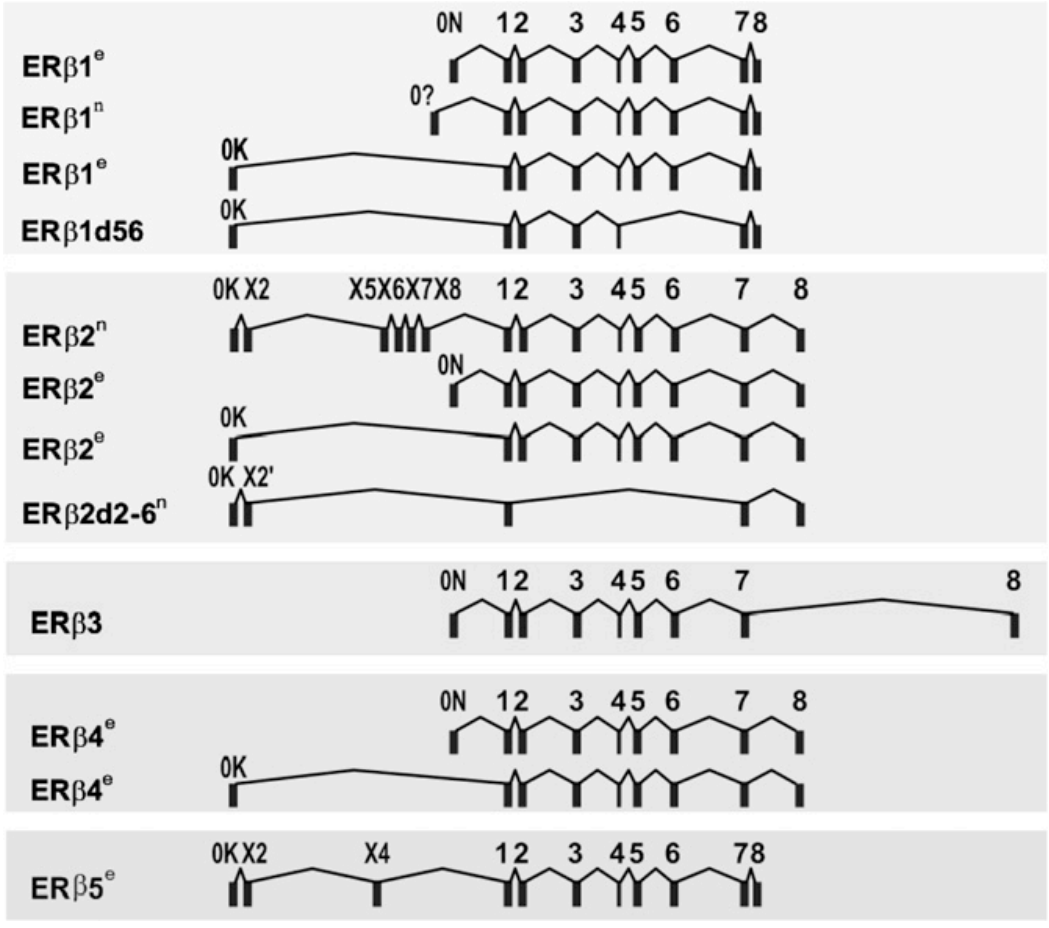


Figure 6. Genomic arrangement of ERβ isoforms

Estrogen Receptors Signaling

Binding of estrogen ligands to ERs in the nucleus results in a cascade of events; the receptor dissociates from the chaperone proteins, dimerizes and, after its phosphorylation, associates with chromatin leading to 2 genomic mechanisms. The first mechanism is represented by the direct DNA binding via a Estrogen Responsive Element (ERE) and may involve the recruitment of specific coregulator proteins, which can enhance binding of the receptor complex to the promoter regions of target genes and augment receptor's transcriptional activity (Fig.7). The second mechanism involves the recruitment of transcription factors through which the ER might affect the expression of estrogen target genes

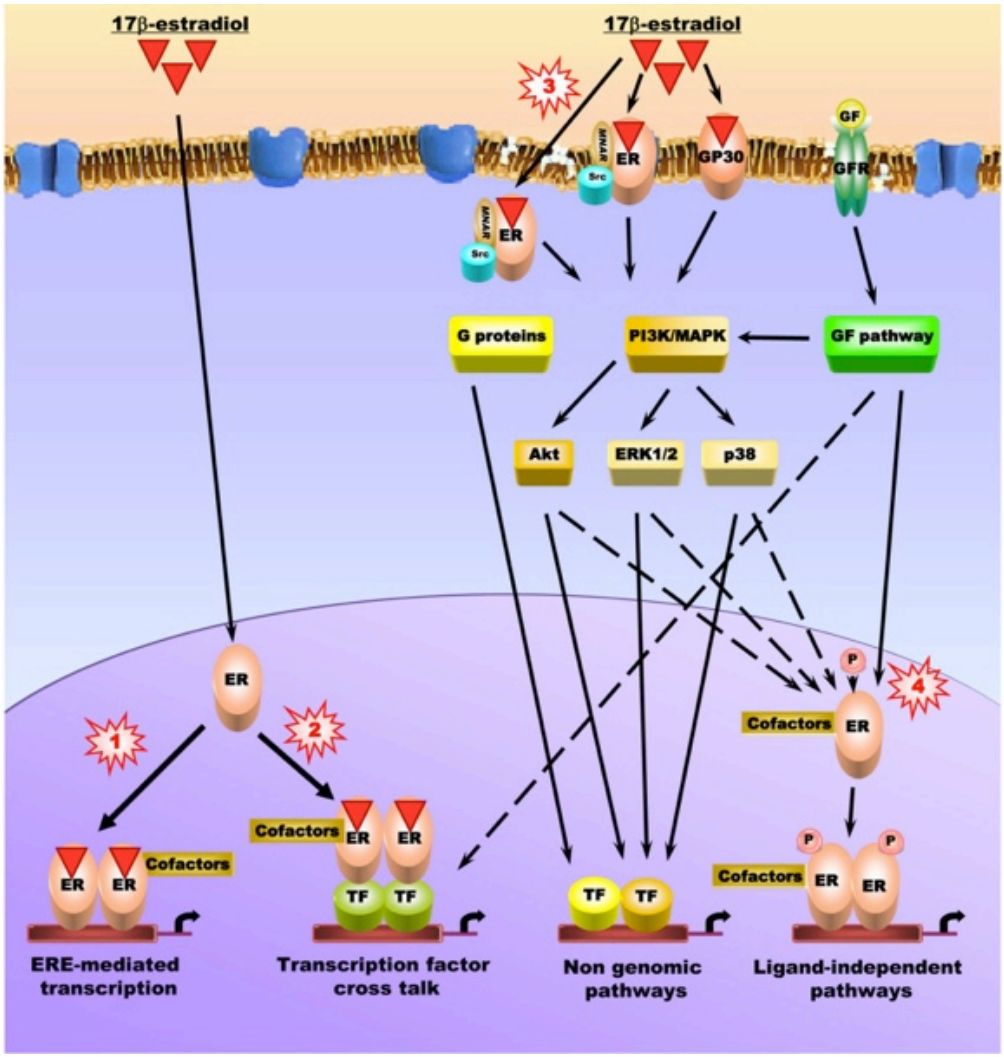


Figure 7. Pathways of Estrogens action

The different anatomy of EREs, the two subtypes of ERs involved (ERα and ERβ), the variety of interacting nuclear co-regulatory proteins and the substantial crosstalk between nuclear transcription factors can yield various responses to estrogen stimulation. Generally, ERα and ERβ can form both homo- and heterodimers before attaching to DNA (48). Both receptor subtypes have different affinities for different response elements and can therefore yield different transcriptional effects at the same site (49). Highly conserved regions in the DBD of ERα and ERβ are the two cysteine–cysteine zinc fingers which allow contact between the major groove of

DNA and the sugar– phosphate backbone. The resulting ER–ERE complex is stabilized by ligand binding [50] and the high mobility group proteins 1 and 2 (51), which are architectural proteins that facilitate chromatin function. Highly estrogen-responsive and perfectly palindromic

sequences have been found in the African clawed frog Xenopus laevis genes encoding vitellogenin A1, A2, B1 and B2 (52). From these natural EREs and similar sequences (53) a minimal consensus sequence for EREs has been derived (Fig. 8). Three specific amino acids within the proximal box (P-Box) of the first zinc finger of the ER bind to the ERE in a sequence-specific manner (54). The second zinc finger is involved in receptor molecule dimerization and ERE half site spacing recognition.

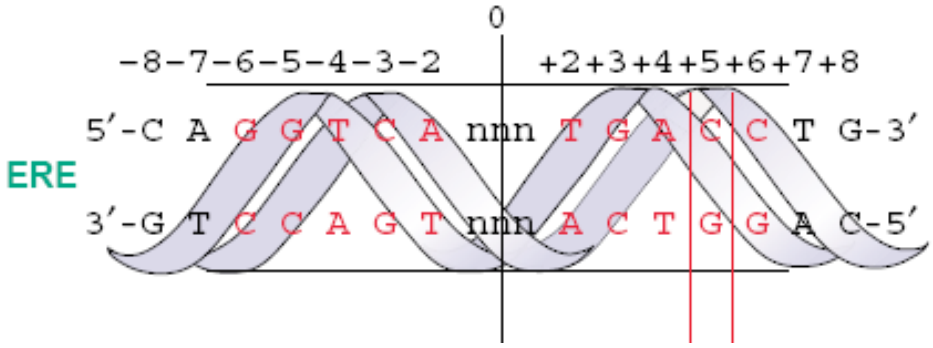


Figure 8. Sequence of the Estrogen Responsive Element ERE

Recent studies indicate that both ERα and ERβ contact the same nucleotides in the consensus ERE [55]. It is therefore assumed at this time that the two ER subtypes interact with EREs in a similar mode. As mentioned previously, ER conformation differs when occupied by different ligands. Also, the structure of the ERE alters the conformation of the receptor. The ERα reacts to a specific single nucleotide alteration within the ERE by changing its DBD conformation by means of a side-

chain rearrangement (56). With these modifications, a rearrangement in the local hydrogen bond network between DNA bases and receptor amino acids is achieved and alternative base contacts enabled. Thus, ER conformation is dependent on two factors: (i) the ligand and (ii) the specific ERE sequence.

In addition, ERs may bind to DNA in a non-classical way through its interaction with other transcription factors, for instance, Sp1 and AP-1, through binding GC-rich and AP-1 motifs. Over to these genomic actions, ERs, located in the cell membrane or cytoplasm, may initiate rapid cellular signaling, and crosstalk with growth factor pathways on the same cascade events (57–58). The last mechanism occur in the absence of natural ligand, in a cell type and gene dependent context (59–60) and may also involves recruitment of coregulators (Fig.7) (50, 53).

Once bound to a regulatory element, ERs interact with adjacent transcription factors, such as SP1 and recruit a variety of coregulators that result in the activation or repression of target genes, by modifying chromatin structure (Fig.9). These bipolar transcriptional activity is mediated through the interaction with two distinct classes of auxiliary proteins: co-activators, such as CBP/p300, and co-repressor, such as NCoR and SMRT, acting through histone de-acetylation to inhibit basal cell transcription machinery. Depending on the promoter structure, type of ligand and receptor subtype, they recruit coactivator or corepressor. ERα and ERβ exert different transcriptional activities and produce different biological effects. In many instances ERα and ERβ exhibit oppositing actions in the regulation of several promoters and specific response elements. These differences and similarities can be ascribed, at list, in part to their protein sequences, in part to their distinct expression pattern. Nuclear receptors usually bind the corepressors N-CoR and SMRT in the absence of ligand or in the presence of antagonists. Agonist binding leads to

corepressor release and coactivator recruitment. A recent study (61) demonstrated that, in vitro and in vivo, ERβ binds to N-CoR and SMRT in the presence of ER agonists such as estradiol and phytoestrogens like genistein, but not in the presence of antagonists. ERα and ERβ present completely distinct modes of action with coregulators, which could be of major importance in terms of potential effects on physiological behaviour (61).

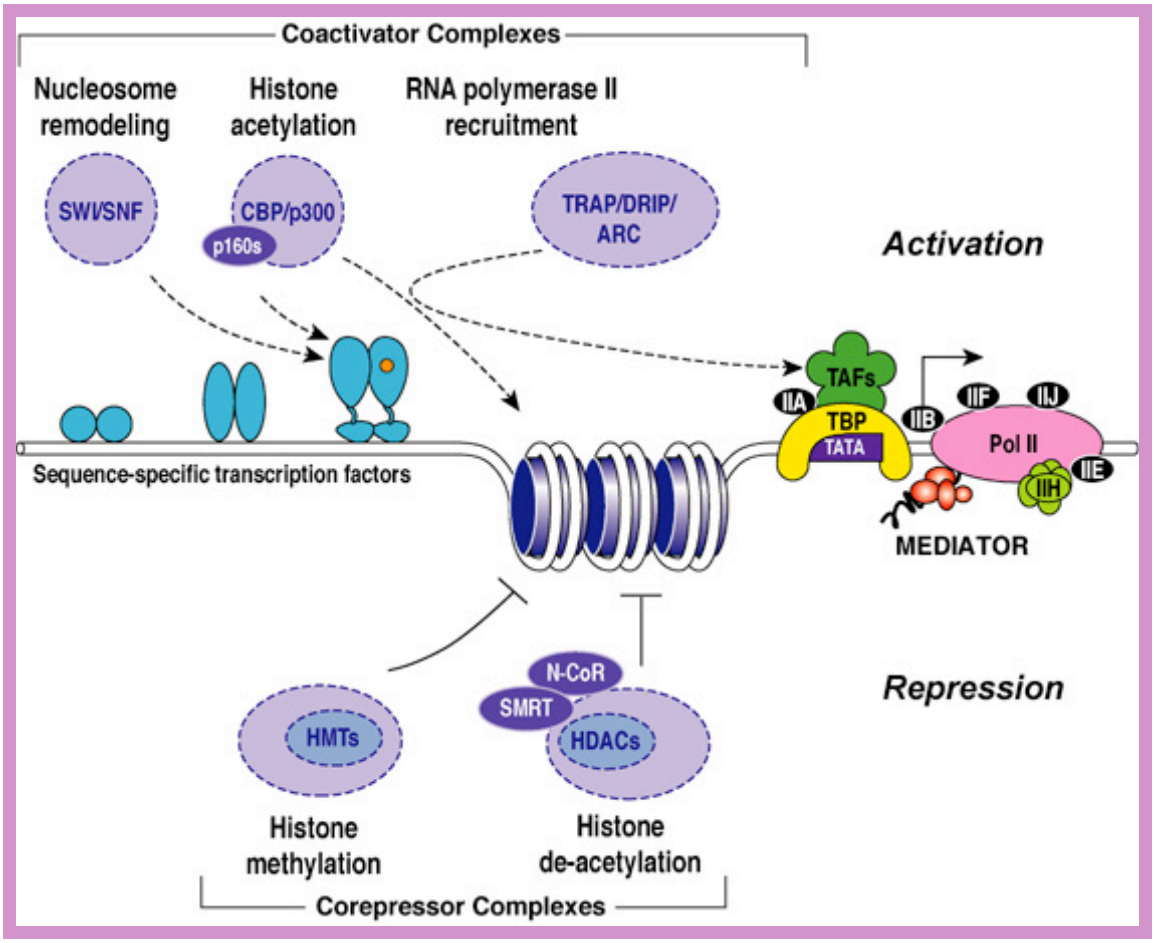


Figure 9. Coactivator and Corepressor Complexes

Coactivators

Coactivators are proteins that enhance transcription. The contact between coactivators and the ER is made through the LXXLL motif present in the coactivator (the "NR box) [23], although the site on the ER required for this interaction varies. Coactivators include steroid receptor coactivator 1 (SRC-1), SRC-2 and SRC-3, which are members of the p160 family. p300 and CREB-binding protein (CBP) are cointegrators, in that they do not themselves bind DNA, but are recruited to promoters by other transcription factors, such as SRC-1. Local chromatin structure is remodeled to allow for gene transcription (62). Chromatin remodeling factors include ATP-dependent nucleosome remodeling complexes and proteins that contain acetyltransferase activity. Histone acetylation correlates with transcription, p300/CBP-associated factor (PCAF), p300/CBP, SRC-1 and SRC-3 contain intrinsic acetyl-transferase activity. Coactivators can also interact preferentially with a particular activation function region. For example, p68 RNA helicase is a coactivator specific for the ERα AF1 region (63). p68 binds CBP, so p68 may serve as a bridge to associate AF1 with AF2 coactivators. p68 enhances the transcriptional activity of the 4-OHT–ERα complex, and the phosphorylation of S118 site of ERα is required for the ability of p68 to enhance transcription. In addition to interacting with both ERα and ERβ or a particular activation function, coactivators can interact selectively with ERα or ERβ. For example, SRC-3 enhances ERα and progesterone receptor (PR) stimulated transcription, but has no effect on ERβ-mediated transcription (64). Therefore, coregulators provide an additional layer of specificity and regulation to the transcriptional activity of the ER. In addition to being a general ER coregulator, this could be accomplished by targeting ERα or ERβ specifically, or interacting with AF1 or AF2.

Corepressors

The first nuclear receptor corepressors identified, SMRT and N-CoR, were isolated in yeast 2-hybrid screens as interacting partners of retinoid X or thyroid hormone receptor (RXR, TR) (65). SMRT and N-CoR are large, homologous proteins (ca. 2500 aa) sharing approximately 45% amino acid sequence identity (66) and both are subject to extensive alternative mRNA splicing, generating multiple isoforms (67). These two corepressors likely share some similar functions while exerting other, distinct influences within cells and organisms. While many interaction partners are shared between the two corepressors, other interaction partners are specific to each corepressor.

Given their role to regulate epigenetic events that underlie transcriptional inactivation, the structurally diverse yet phenotypically related co-repressor proteins have emerged as key players in cancer aetiology Analysis of the pattern of conservation between human SMRT and NCoR shows that there are regions of high conservation separated by regions of much lower conservation. The largest region of high conservation spans a stretch of ~300 amino acids with 83% identity between the two proteins. Other regions of high conservation are smaller and generally span between 20 and 50 amino acids. Both proteins contain multiple repression domains (RDs), Swi3/Ada2/N-CoR/TFIIID (SANT) motifs (68) and nuclear receptor interaction domains (NRIDs) (Fig.10). SANT motifs in corepressors have been shown to be histone-binding modules [10,11], although specific mechanisms underlying this are unclear. The first of the SANT-like domains, has been shown to both recruit and activate HDAC3 and has been termed the deacetylase activation domain (DAD) (Fig. 10) (68). The second SANT-like domain has been reported to

interact directly with histone tails (the enzymatic substrate of HDAC3) and has been termed the histone interaction domain (HID) (Fig.10) (69). SMRT contains two NRIDs, while N-CoR contains three NRIDs. The NRIDs in each can be removed by alternative splicing. The RDs (Repressor Domains) likely serve as binding platforms for the various silencing enzymes recruited to repress gene promoters, including the histone deacetylases (HDACs). Thus, both SMRT and N-CoR are part of larger complexes. These corepressor complexes can be considered to be large docking surfaces to tether repression machinery to transcription factors. An overall picture is emerging in which SMRT and NCoR are largely unstructured platform proteins that act as a scaffold upon which the enzymatic machinery of the repression complex is built.

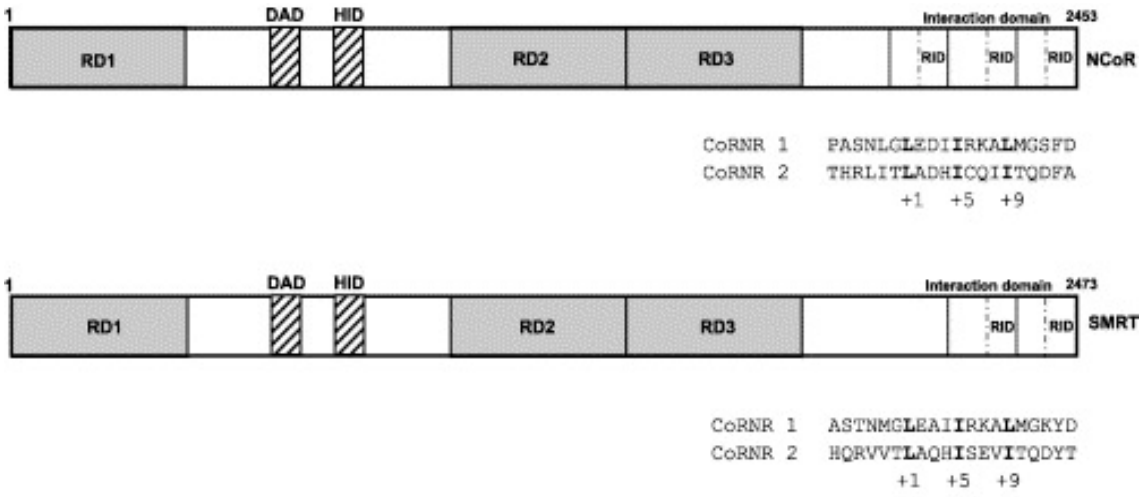


Figure 10. Structural organization of corepressors, NCoR and SMRT. The N-terminal region contains multiple repression domains shown in gray (RDs). The RIDs in the C-terminal region each contain a conserved CoRNR box sequence as denoted by the dashed lines in the RIDs. The specific amino acid sequences of the CoRNR boxes are shown with the conserved LXXXIXXXL sequence in bold. The N-terminal portion of the corepressors also harbors the two matched SANT domains designated by diagonal boxes in the DAD and the HID, respectively.

The repression mediated by SMRT and N-CoR is modulated in part through deacetylation of lysines on histone tails by histone deacetylases contained in large corepressor complexes. Deacetylated histones may serve as preferred binding sites for corepressor complexes in what has been described as a "feed-forward mechanism" (70). Current models indicate that corepressor complexes initially recognize acetylated chromatin and deacetylate the histone tails. These complexes may then show increased affinity for the deacetylated chromatin, thus enhancing gene repression by increased association. HDAC3 is hypothesized to be the primary histone deacetylase in SMRT/NCoR complexes. SMRT and NCoR interact with the ligand binding domanins (LBDs) of Nuclear Receptors in the absence of a bound ligand. These interactions are mediated by short receptor interaction motifs, multiple copies of which are found within many coactivators and co-repressors. Indeed the co-repressors contains sequences that are similar to the NR box of the co-activators (LXXLL motif where L is an hydrophobic amino-acid and x any amino-acid, but the motif is longer and requires additional flanking sequences) and are repeated in each of two NR interaction domains (the CoRNR box) (Fig.10) (71). SMRT and NCoR have two conserved, corepressor motifs containing, nuclear receptor interaction domains called ID1 and ID2 (71). Mapping the co-repressor binding sites on the surface of the LBD showed that the motifs bind to overlapping surfaces. It was suggested that ligand binding would cause a conformational or dynamic change in helix 12 resulting in displacement of the co-repressor and formation of a suitable co-activator binding surface (72). Within NCoR a third receptor interaction domain has been identified (ID3) (61). The thyroid hormone receptor (TR) and Rev-erb-α nuclear receptors have been shown to interact specifically with the ID3 and ID2 domains of NCoR, with the ID3 being proposed as the major determinant for the

interaction of TR with NCoR (61, 73). It has been shown that alternative splicing generates multiple isoforms of SMRT, some of which also include a third receptor interaction domain. Thus splicing may differentially regulate interaction of SMRT with nuclear receptors (74). It has also been suggested that parts of the N- and C-terminal regions of co-repressors, distinct from the "classical" interaction domains, may bind to the DNA binding domain (DBD) of nuclear receptors (75).

Estrogen Receptors and Breast Cancer

Both ERs subtypes are expressed in human mammary tissue with only 7–10 % of the epithelial cells expressing ERα but 80–85 % expressing ERβ (76). In contrast, expression of ERα is increased in breast cancer cells, where it acts as a mediator of cell proliferation and has been shown to be an effective therapeutic target for decades (Fig.11) (77). The role of ERβ in breast cancer is less clear and its prognostic value is still under debate. It is estimated that ERβ is expressed in approximately one-half of human primary breast cancers, but its expression is lost during breast cancer progression, most likely due to promoter hypermethylation (22). Moreover, ERβ protein levels have been linked to good prognosis, prolonged disease-free survival and response to anti-estrogen treatment (78,79). Many cell-based studies suggest that ERβ acts as a negative modulator of ERα action and can negatively regulate breast cancer proliferation (80).

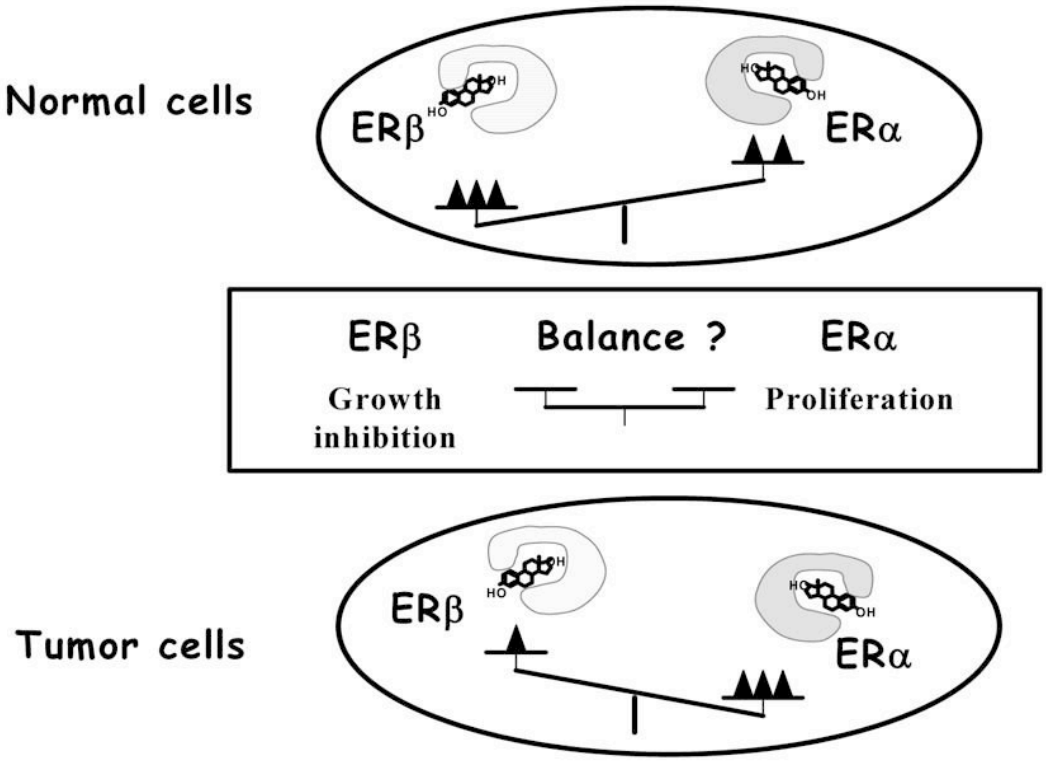


Figure 11. Schematic representation of ERα and ERβ imbalance in estrogen dependent tumor progression

Indeed, inducible expression of ERβ in ERα-positive breast cancer cells inhibited estrogen-stimulated proliferation, tumour angiogenesis, and growth in xenograft experiments (81). Overexpression of ERβ or ERβ cx isoforms also decreased ERα transcriptional activity concomitantly with a reduced expression of estrogen-regulated genes, such as vascular endothelial growth factor (VEGF) or progesterone receptor (PR) (82). In ER-negative cells ectopically expressing the two ERs, ERβ reduced the sensitivity of the cells to estrogen treatment on growth and inhibited cyclin D1 gene activation (83). ERβ might have a modulating effect in breast cancer because it is expressed in normal and malignant breast tissue, binds 17b-estradiol and ERβ can heterodimerize with ERα (81). Many studies suggest a protective role for ERβ in breast cancer and tumors that expressed both ERα and ERβ were node positive and of a higher grade (84). ERβ mRNA levels were also elevated in tumors

that displayed tamoxifen resistance (85).

Overall, the majority of studies suggest that the presence of ERβ is a good prognostic marker for breast cancer. However, the relative amounts of ERα and ERβ must be considered.

Dysregulation of the cell cycle and the apoptotic pathway are key-components in breast cancer tumorigenesis. However, breast cancer cells as well as cell lines in vitro, are most often dependent on estrogen to proliferate. Hence, treatment of breast cancer cells with anti-estrogens leads to cell-cycle arrest or apoptosis (86). In all breast cancer cells studied in vitro, the proliferative effects of E2 is mediated by ERα. However, ERα induces proliferation by regulating multiple cell-cycle factors, which makes the mechanism complex (87). Several data clearly demonstrate that ERβ has an anti-proliferative function when re-introduced into ERα+ breast cancer cells. Also in clinical specimens there is a significant downregulation of ERβ in breast cancer epithelium compared to normal breast epithelium, supporting the notion that ERβ plays a role in tumorigenesis. However, there is no unequivocal correlation of ERβ with proliferation associated parameters such as Ki67-staining. Previous in vitro data indicate that ERβ could act as a dominant negative regulator of ERα activity (88), moreover, expression of adenoviral infection of MCF-7 cells caused tumor regression in a xenograft model (80). The cell cycle is divided into four different phases: Gap 1 (G1), DNA synthesis (S), Gap 2 (G2) and the Mitosis (M-phase). In the presence of E2, ERα stimulates proliferation by regulating numerous cell-cycle genes. ERβ on the other hand binds to the same promoter elements but sometimes modulates gene expression differently. Therefore it is likely that ERβ inhibits proliferation of cancer cells by affecting several other cell-cycle factors than ERα. When ERβ is expressed in ERα+ breast cancer cells, the expression of

important G1-phase regulators is changed, causing a G1 phase cell-cycle arrest (Fig.12) (84). Another important study in ERβ−/− mice showed that ERβ is implicated in the regulation of epithelial growth, and its absence results in hyperplasia of the prostatic epithelium (89). The inhibition of ERα transcriptional activity could be a second molecular mechanism by which ERβ has antiproliferative effects (Fig.12).

Nevertheless, large studies that can correlate tumor characteristics with precise determinations of ERβ mRNA and protein are needed to identify specific situations where ERβ may be a critical player in either carcinogenesis or disease progression.

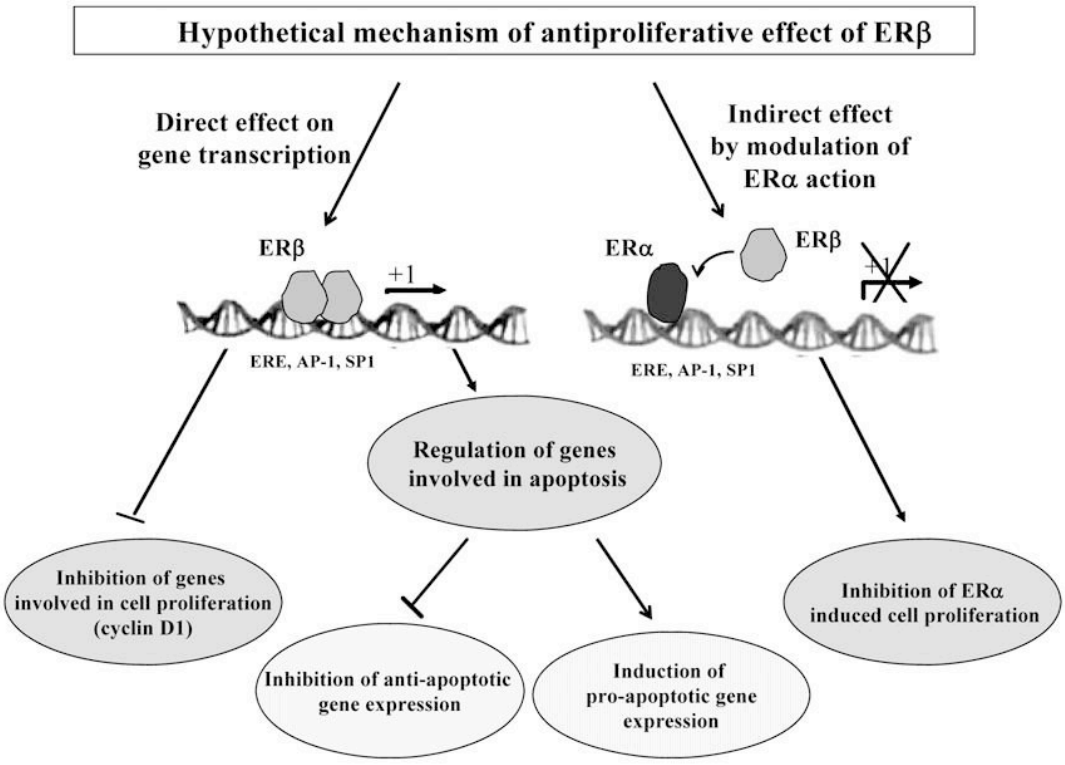


Figure 12 Hypothetical mode of ERβ action on cell proliferation pathways.

Aims

Given the markedly enhanced ratio of ERα/ERβ in early breast cancers and the opposite roles of the two ERs in regulating cell proliferation and differentiation, it is imperative to dissect the molecular mechanisms underlying the dysregulation of these processes in cancer cells.

Therefore, the aim of this study was to investigate if ERβ may play a direct inhibitory role on ERα expression and gene promoter activity. Here, we demonstrate that ERβ through its interaction with Sp1 protein recruits NCoR corepressor in the promoter region upstream the transcription start site of ERα gene, thus down-regulating its expression.

Materials and Methods

Reagents and Antibodies

DMEMF-12 Ham and DMEM were purchased from Invitrogen (Carlsbad, CA, USA), FuGENE 6, Sp-1 human recombinant protein from Promega (Madison, WI, USA). The RETROscript kit from Ambion (Austin, TX, USA). MTT, IGF-1 by Sigma (Milan, Italy). Antibodies against ERα/ERβ/IRS1/cyclinD1/pS2/GAPDH/NCoR/Sp1/SMRT/AcH4/ PolymeraseII were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). SYBR Green by Biosystems (Forster City, CA, USA).

Cell cultures

MCF-7 and ZR75 breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained as recommended.

Plasmids

The plasmids containing the human ERα promoter region or its deletions (E: p − 4100/+ 212; D: p − 2769/+ 212; C: p − 1000/+ 212, A: p − 245/+ 212) were provided by Prof Fuqua [37]. Deletion of Sp-1 site in C plasmid was generated by PCR using following primers: forward 5'-GCGGTACCCGAAAGATCGAGTTGTAGGAC-3' and reverse 5'-CGCTCGAGTTATATAGGGAAGACTGGGCTTAAAATA-3'. The amplified DNA fragment was digested with Kpn I and Xho I and ligated into pGL3-

basic vector. The sequence was confirmed by nucleotide sequence analysis. The plasmid encoding the human ERβ was a gift from JA Gustafsson (Karolinska Institute, Sweden).

Site-directed mutagenesis

The mutation of the two half-ERE sites in C plasmid was created by site-directed mutagenesis using QuickChange kit (Stratagene, La Jolla, CA). The mutagenic primers were: forward 5'-CATAATTGCCTTTGCTTTGGTTCGTGGTTTGAGGTTATGTTTGGTATGAAAAG-3', 5'-CGTGACCTGAGGTTATGTTTGGTATGAAAAGACTACATTTTATATTCAGTTTTCTGAAG-3', and reverse 5'-CTTTTCATACCAAACATAACCTCAAACCACGAACCAAAGCTTTGGCAATTATG-3', 5'-CTTCAGAAAACTGAATATAAAATGTAGTCTTTTCATACCAAACATAACCTCAGGTCACG- 3'.

Mutation was confirmed by DNA sequencing.

Western blot analysis

Equal amounts of cell extracts were resolved under denaturing conditions by electrophoresis in 8% to 10% polyacrylamide gels containing SDS (SDS-PAGE) and transferred to nitrocellulose membranes by electroblotting. After blocking the transferred nitrocellulose membranes were incubated with primary antibodies overnight at 4°C. The antigen-antibody complex was detected by incubation of the membranes with peroxidase-coupled goat anti-mouse, goat anti-rabbit, donkey anti-

goat, and revealed using the ECL System. Blots were then exposed to film and bands were quantified by densitometer. Blots are representative of at least three independent experiments.

RT-PCR and qRT-PCR

ERα/ERβ/IRS-1/pS2/CyclinD1/NCoR/GAPDH gene expressions were evaluated by RT-PCR. cDNAs obtained were amplified using the following primers: forward 5'-AGATCCAAGGGAACGAGCT- 3' and reverse 5'-TTCTCCAGGTAGTAGGGCA-3' (ERα); forward 5'-CCTTCCTCCTATGTAGACAGC- 3' and reverse 5'-TCTCTCTGTTTACAGGTAAGGT-3' (ERβ); forward 5'-AGGATATTTAATTTGCCTCGGG-3' and reverse 5'-AAGCGTTTGTGCATGCTCTTG-3' (IRS-1); forward 5'-TTCTATCCTAATACCATCGACG-3' and reverse 5'-TT TGAGTAGTCAAAGTCAGAGC-3' (pS2); forward 5'-TTAAGATGAAGGAGACCATC-3' and reverse 5'-GCGGTAGTAGGACAGGAAGTTGTT-3' (CyclinD1); forward 5'-GCCACTGTATAACCAGCCAT-3' and reverse 5'-CCTCCATAAGCCCATTCATG-3' (NCoR); forward 5'-GACAACTTTGGTATCGTGGA-3' and reverse 5'-TACCAG GAAATGAGCTTGAC-3' (GAPDH). ERα gene expression was also evaluated by Real-time PCR. Primers used for the amplification were: forward 5'-CACCATTG ATAAAAACAGGAGGAA-3' and reverse 5'-CTCCCTCCTCTTCGGTCTTTTC-3' (ERα); forward 5'-CCCACTCCTCCACCTTTGAC-3' and reverse 5'-

TGTTGCTGTAGCCAAATTCGTT-3' (GAPDH). The relative gene expression levels were calculated as described [14].

Transient transfection assays

MCF-7 cells were transfected using FuGENE6 reagent with the indicated constructs for 16 h. Luciferase activities were assayed as described [38].

Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described [39]. The DNA sequences used as probe or as cold competitor are the following:

5'-TCGTGCGCCCCCGCCCCCTGGCCGTG- 3',

5' - CACGGCCAGGGGGCGGGGGCGCACGA-3' (Sp-1);

5'-TCGTGCGCCCCATACCCCT GGCCGTG- 3',

5' -CACGGCCAGGGGTATGGGGCGCACGA- 3' mutated Sp-1. Probe generation and the protein binding reactions were carried out as described [38]. The reaction mixture was incubated with specific antibodies at 4°C for 12 h before addition of labeled probe.

RNA interference

MCF-7 cells were co-transfected with an empty vector or an ERβ expression vector and RNA duplex of stealth RNAi-targeted human NCoR mRNA sequence 5'-

UUGUUUGGCUCUGGAGACCUCUUGC- 3' or with a stealth RNAi-negative control using Lipofectamine2000 as recommended.

ChIP and Re-ChIP assays

The DNA/protein complexes were extracted as previously described [38]. The precleared chromatin was immunoprecipitated with anti-Sp-1/NCoR/SMRT/AcH4/PoymeraseII antibodies. The anti-Sp-1 immunoprecipitated samples were re-immunoprecipitated with an anti-NCoR or anti-ERβ antibodies. Sample and input DNA (5 μl) were used for PCR amplification with the following primers flanking Sp-1 sequence present in the ERα promoter region: 5'-GCACATAAGGCAGCACATTA-3' (forward), and 5'-TGGCTTAAACATCACTCCAG- 3' (reverse). In another set of experiments, each sample and input DNA (5 μl) were used for real-time PCR using the following primers: 5'-T CGTGCGCCCCCGCCCCCTGCCCGTG- 3' and 5' -CCAAAGAGCAGCTTCCCTGA- 3'. Real-time PCR was performed as described above. Final results were calculated using DDCt method, using input Ct values instead of the GAPDH mRNA. The basal sample was used as calibrator.

Cell proliferation assay

Cell proliferation was determined by using 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenylformazan (MTT) assay as described [40]. Data are representative of three independent experiments, performed in triplicate.

Three-dimensional spheroid culture assays

MCF-7 cells plated in 2 % agar-coated plates were transfected as indicated and treated or not with IGF-1. After 48 h, cultures were photographed using a phase-contrast microscope (Olympus, Milan, Italy). Aggregation extent and cell numbers were evaluated as reported [15]. Data represent three independent experiments, performed in triplicate.

Statistical analysis

Data were analyzed for statistical significance using a two-tailed student's Test, performed by Graph Pad Prism 4.

Results

ER beta overexpression down-regulates ER alpha expression in breast cancer cells

Although data demonstrate that ERβ negatively interferes with ERα signaling in breast cancer cells, it still remains unexplored if ERβ actually affects ERα gene expression. To this aim, ERα-positive MCF-7 and ZR75 breast cancer cells were transiently transfected with an ERβ expression vector and ERα expression was evaluated by RT-PCR and western blotting analysis. As shown in Fig. 13A, B, the ectopic expression of ER β reduced ERα levels in terms of mRNA and protein content in both MCF-7 and ZR75 cells. Concomitantly, ERβ overexpression markedly decreased mRNA levels (Fig. 13C) and protein (Fig. 13D) expression of classical estrogen-regulated genes, such as insulin receptor substrate 1 (IRS-1), pS2 and cyclin D1 in both cell lines. These findings correlated well with a dose-dependent inhibition induced by ERβ overexpression on cell proliferation, as revealed by MTT assays (Fig. 13E).

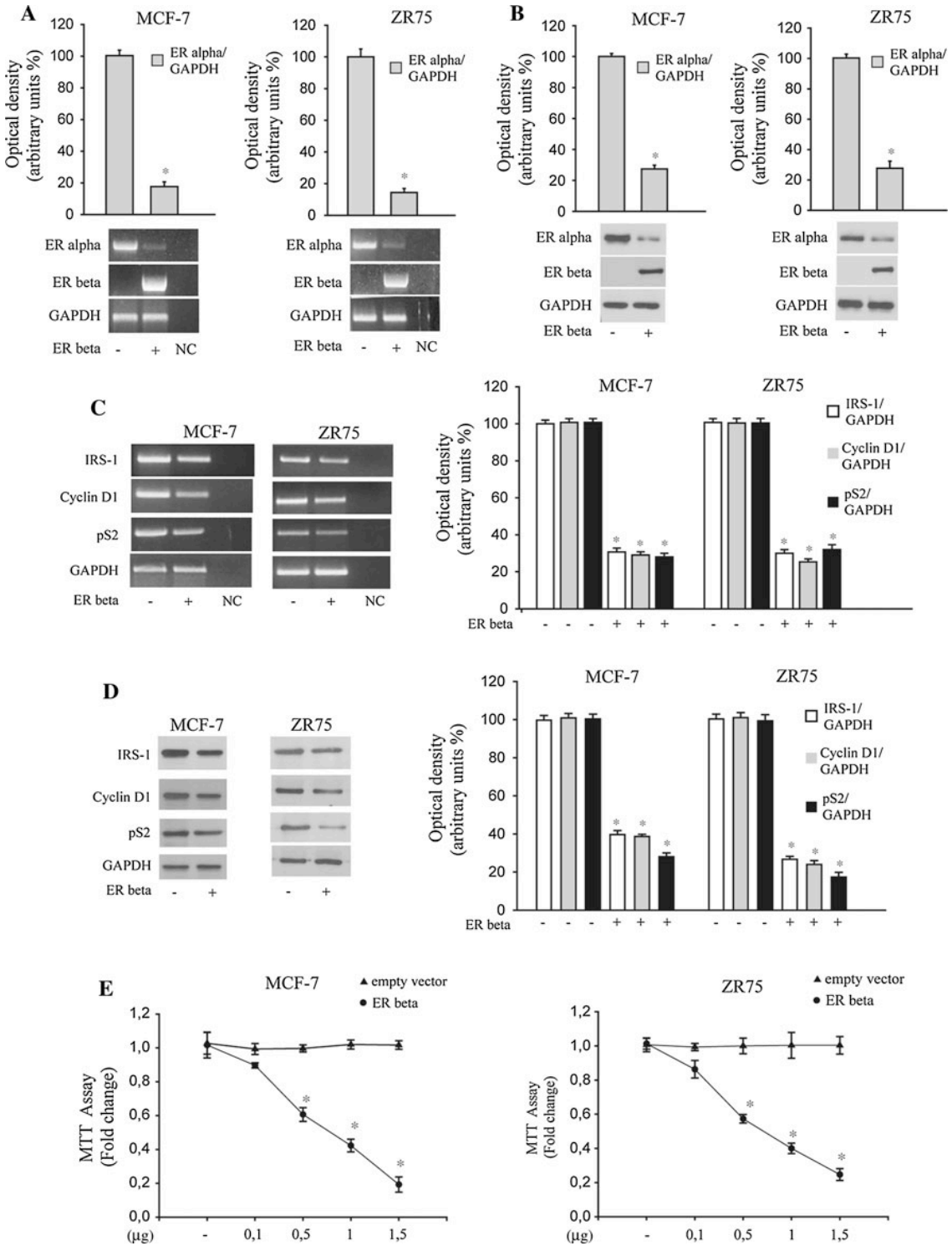


Figure 13. Overexpressed ERβ down-regulates ERα expression in breast cancer cells. **A**. Bottom panel, total RNA was isolated from MCF-7 and ZR75 cells transfected with either empty vector (-) or ERβ expression plasmid and reverse transcribed. cDNA was subjected to PCR using specific primers for ERα, ERβ and GAPDH.NC, negative control, RNA sample without the addition of transcriptase. Upper panel, the histograms represent the mean ± S.D. of three independent experiments in

which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **B**. Bottom panel, western Blot analysis of ERα and ERβ in total protein extracts from MCF-7 and ZR75 cells, transiently transfected with either empty vector (-) or ERβ expression plasmid. GAPDH was used as loading control. Upper panel, the histograms represent the mean ± S.D. of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **C**. Left panel, Total RNA was isolated from MCF-7 and ZR75 cells transfected with either empty vector (-) or ERβ expression plasmid and reverse transcribed. cDNA was subjected to PCR using specific primers for IRS-1, pS2, Cyclin D1, and GAPDH. NC, negative control, RNA sample without the addition of transcriptase. Right panel, the histograms represent the mean ± S.D. of three different experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **D**. Left panel, Western Blot analysis of IRS-1, pS2, Cyclin D1 in total protein extracts from MCF-7 and ZR75 cells transfected with either empty vector (-) or ERβ expression plasmid. GAPDH was used as loading control. Right panel, the histograms represent the mean ± S.D. of three different experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. *, P\0.01 ERβ-transfected cells compared to empty vector (-)-transfected cells. **E**. MTT growth assays in MCF-7 and ZR75 cells transfected with empty vector or ERβ expression vector (0.1–1.5 μg/well) for six days. Cell proliferation is expressed as fold change ± S.D. relative to empty vector (-)-transfected cells and is representative of three different experiments each performed in triplicate. *P\0.05 ERβ-transfected cells compared to empty vector (-)-transfected cells.

Overexpressed ER beta mediates down-regulation of ER alpha via a GC proximal region of its promoter

To analyze how ERβ interferes with ERα gene transcription, we transiently transfected breast cancer cell lines with a luciferase reporter plasmid containing the human ERα promoter region spanning from -4,100 bp to +212 bp. As shown in Fig. 14A, a significant decrease in ERα promoter activity was observed in MCF-7 cells when ERβ was overexpressed. The human ERα promoter contains multiple consensus sites for several transcription factors, including a CAT box, TFIID, AP2c, and Sp-1 motifs (90). To identify the regions within the ERα promoter responsible for ERβ-mediated inhibitory effects, we transiently transfected MCF-7 cell lines with plasmids containing a series of 50 deleted segments of this human ERα promoter. Schematic representation of these constructs is shown in Fig. 14. In transfection experiments performed using p−4100/+ 212 (E), p−2769/+ 212 (D), and p−1000/+ 212 (C) plasmids, the responsiveness to ERβ was still maintained (Fig. 14a), suggesting that the region between −1000 and +212 might be involved in the transrepression mechanisms exerted by ERβ overexpression. Thus, we focused our attention on the latter construct p−1000/+ 212 (C), and we identified, upstream to the initiation transcription site, two half ERE (−867/−861 and −894/−888) and one Sp-1 (−223−214) sites, which are putative effectors of ER signaling. We observed that in MCF-7 cells transiently transfected with the ERα promoter plasmid bearing 2 half ERE-mutated sites (C 2mut half-ERE) or with a deleted construct of ERα promoter containing Sp-1 site (p−245/+ 212, A) that ERβ-mediated down-regulation still persisted. In contrast, deletion of the Sp-1 site (C Sp-1 del) completely abrogated ER

β's effects (Fig. 14B). Similar results were obtained in the ZR75 breast cancer cells (data not shown). Taken together, our findings demonstrated that the down-regulatory effects of ERβ on ERα gene expression requires an Sp1 sequence motif. Since functional domains of Sp1 are involved in protein– protein interactions with other transcription regulatory molecules, such as the corepressors SMRT, NCoR and BCoR (BCL6 corepressor) (91, 92), to inhibit cell transcription machinery, we next addressed whether the same corepressors may be recruited by the ERβ/Sp1 complex to ERα gene promoter.

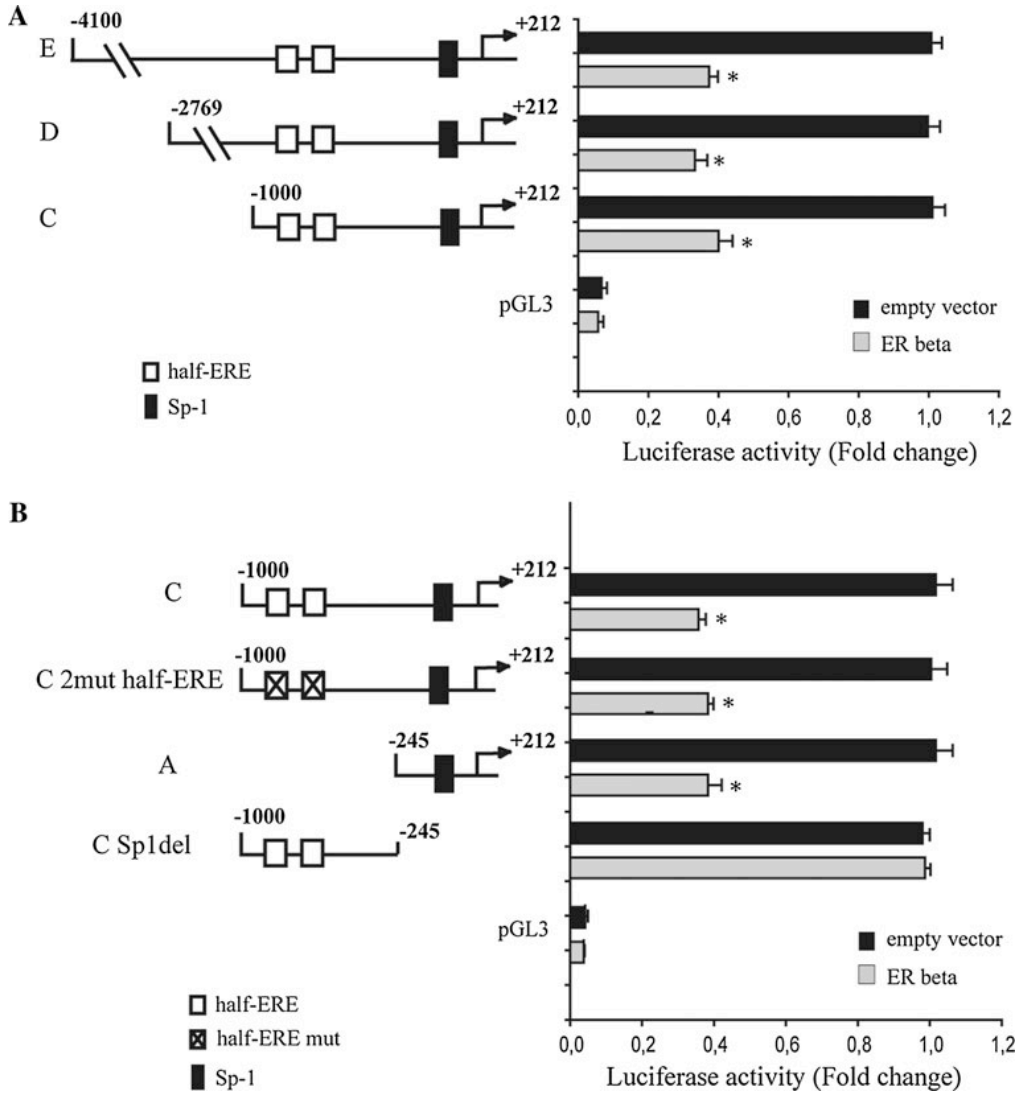


Figure 14. ERα promoter activity is down-regulated by ERβ overexpression, and

deletion of the GC-proximal promoter region abrogates this effect.

A, B. Left panel, schematic representation of constructs of the ERα gene promoter used in this study. Right panel, plasmids containing ERα promoter fragments were transiently cotransfected in MCF-7 cells in presence or absence of ERβ expression plasmid. After 24 h of transfection, luciferase activities were normalized to the Renilla Luciferase as internal transfection control and data where reported as fold change. The values represent the means ± S.D. of three different experiments each performed in triplicate. pGL3: basal activity measured in cells transfected with pGL3 basal vector. *P\0,05 ERβ-transfected cells compared to empty vector transfected cells.

The NCoR corepressor is recruited with Sp-1 to ER alpha promoter region

The specific role of the Sp-1 motif in mediating the inhibitory role of ERβ on ERα gene expression was investigated using electromobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) assays. Using synthetic radiolabeled oligonucleotides bearing the Sp-1 motif present in the ERα promoter region (Fig. 15A, lane 1), we observed the formation of a protein complex in nuclear extracts from MCF-7 cells, which was abrogated by incubation with 100 fold molar excess of unlabeled probe (Fig. 15A, lane 2), demonstrating the specificity of the DNA-binding complex. This inhibition was no longer observed when mutated oligodeoxyribonucleotide probe was used as competitor (Fig. 15A, lane 3). Interestingly, overexpression of ERβ strongly increased the DNA-binding protein complex compared with control samples (Fig. 15A, lane 4). The inclusion of anti-Sp-1, ERβ, NCoR and SMRT antibodies in the reaction immunodepleted the specific band, confirming the presence of these proteins in the complex (Fig. 15A, lanes 5–8). Of note, immunodepletion occurred to a higher extent in the presence of NCoR than of SMRT antibodies. Non-specific IgG did not affect Sp-1

complex formation (Fig. 15A, lane 9). Recombinant Sp-1 protein revealed a complex migrating at the same level as that of nuclear extracts from cells (Fig. 15A, lane 10). Moreover, to better evaluate the involvement of Sp-1 and NCoR/SMRT corepressors in ERβ-mediated ERα down-regulation at the promoter level, ChIP assays were performed. Using specific antibodies against Sp-1, NCoR, SMRT, RNA-polymerase II, and acetyl histone H4, protein-chromatin complexes were immunoprecipitated from MCF-7 cells transfected either with an empty vector or an ERβ expression

vector (Fig. 15B). PCR using primers spanning the Sp-1 binding element in the ERα promoter region clearly showed an enhanced recruitment of Sp-1 and NCoR and slightly of SMRT upon ERβ overexpression. The corepressor DAX-1 was not detected under the same experimental conditions (data not shown). These results were concomitant with a lower association of RNA-Polymerase II and acetyl histone H4 to the ERα regulatory region, indicating that the chromatin in this region is probably in a less permissive environment for gene transcription. Re-ChIP assays confirmed the increased NCoR and ERβ occupancy of the Sp-1-containing region within the ERα promoter in cells overexpressing ERβ (Fig. 15C).

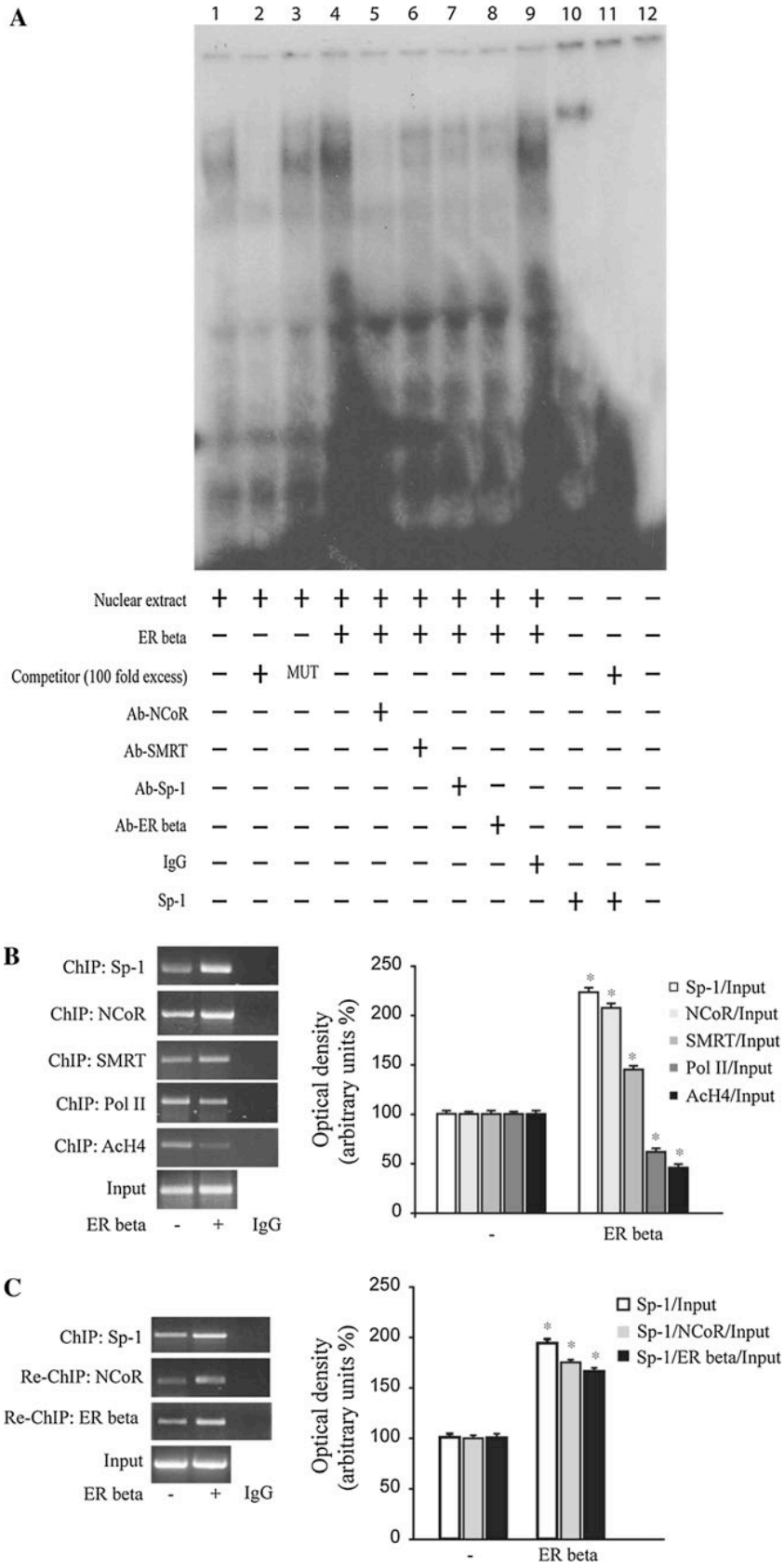


Figure 15. ERβ recruits corepressors to the Sp-1 site in the ERα gene promoter.
A. Nuclear extracts from MCF-7 cells transfected with either empty vector or ER β expression plasmid were incubated with a double-stranded Sp-1 specific sequence

probe labeled with [c32P] ATP and subjected to electrophoresis in a 6 % polyacrylamide gel (lanes 1 and 4). Competition experiments were performed adding as competitor a 100-fold molar excess of unlabeled probe (lane 2 and lane 11) or a 100-fold molar excess of unlabeled oligonucleotide
containing a mutated Sp-1 motif (lane 3). Nuclear extracts from MCF-7 over-expressing ERβ were incubated with anti-NCoR (lane 5) or anti-SMRT (lane 6) or anti-Sp-1 (lane 7) or anti-ERβ (lane 8) or IgG (lane 9) antibodies, in the presence of the probe. Lane 10, Sp-1 protein. Lane 12, probe alone. **B**. Left panel, MCF-7 cells transfected with either empty vector (-) or ERβ expression plasmid were cross-linked with formaldehyde, and lysed. The pre-cleared chromatin was immune-precipitated with specific anti-Sp-1, anti- NCoR, anti-SMRT, anti-Polymerase II, and anti-AcH4 antibodies, and with a normal mouse serum (IgG) as a negative control. A 5 µl volume of each sample and input were analyzed by PCR with specific primers, as detailed in Sect. ‘‘Materials and methods,’’ to amplify ERα promoter sequence containing Sp-1 site. Right panel, the histograms represent the mean ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentage of the control, which was assumed to be 100 %. **C**. Left panel, Chromatin immunoprecipitated with anti-Sp-1 antibody was re-immunoprecipitated with anti-NCoR or anti-ERβ antibodies. A normal mouse serum (IgG) was used as a negative control. A 5 µl volume of each sample and input were analyzed by PCR with specific primers, as detailed in Sect. ‘‘Materials and methods,’’ to amplify ERα promoter sequence containing Sp- 1 site. Right panel, the histograms represent the mean ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentage of the control, which was assumed to be 100 %. *P\0.01 ERβ transfected cells compared to empty vector (-)-transfected cells

NCoR knockdown reverses ER beta's effects on ER alpha down-regulation and cell proliferation

To ascertain the involvement of NCoR on ERβ-related downregulation of ERα, NCoR siRNA knockdown experiments were performed in MCF-7 cells transfected with an ERβ expression vector. Silencing of NCoR gene expression (evaluated by western blot and RT-PCR analysis, Fig. 16A, B) restored both protein and mRNA expression of ERα, while no changes were observed after transfection of cells with a scrambled siRNA control (Fig. 16C, D). We also showed that the increased Sp-1 recruitment to ERα gene promoter was abrogated in presence of NCoR siRNA in MCF-7 cells overexpressing ERβ (Fig. 16E). Concomitantly, the recruitment of RNApolymerase II in the same region was restored (Fig. 16F). Finally, the anti-proliferative effects exerted by ERβ were completely reversed in the presence of NCoR siRNA knockdown in MCF-7 and ZR75 breast cancer cells (Fig. 16G, H), suggesting a crucial role for NCoR in mediating the ERβ-induced inhibitory effects on breast cancer cell proliferation.

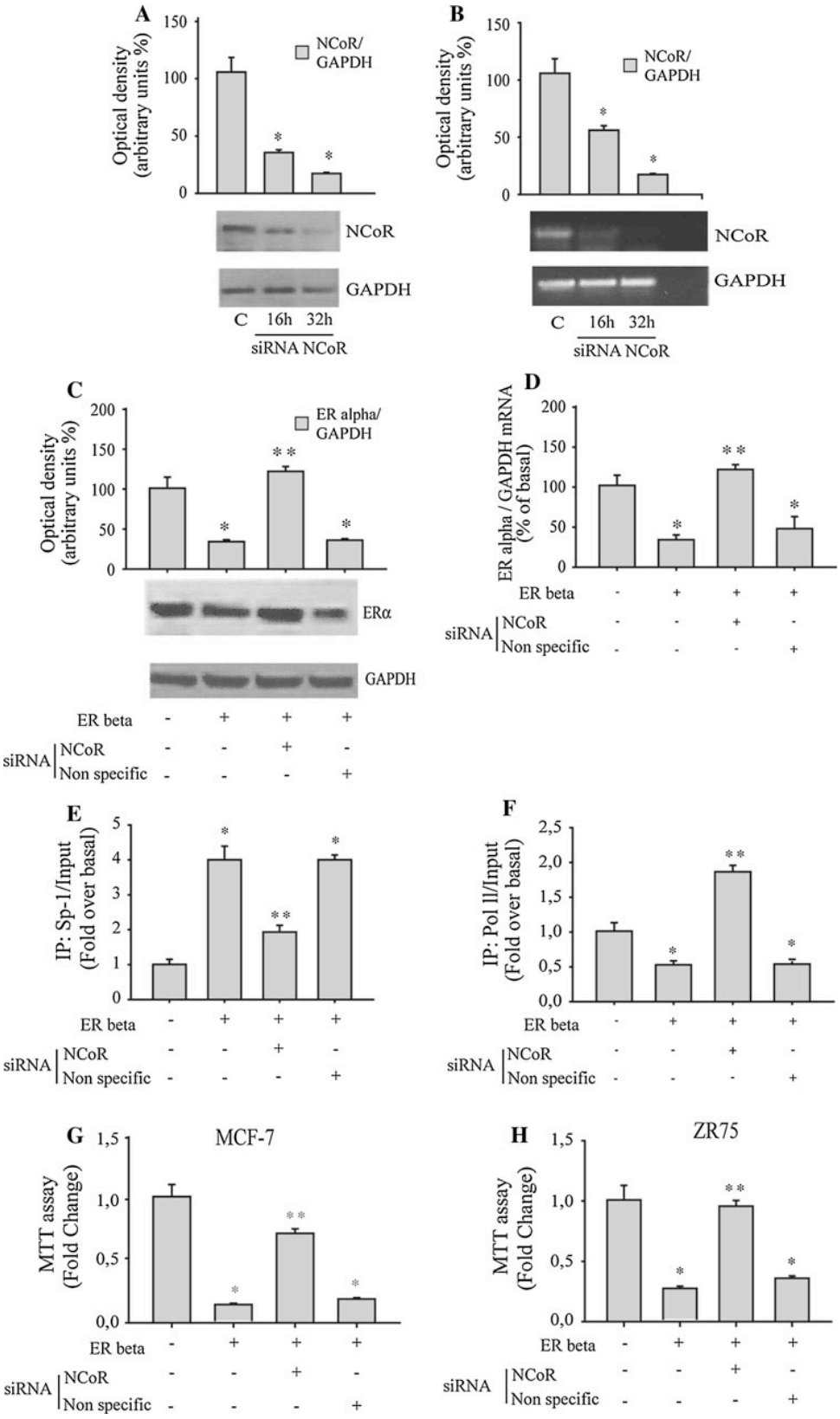
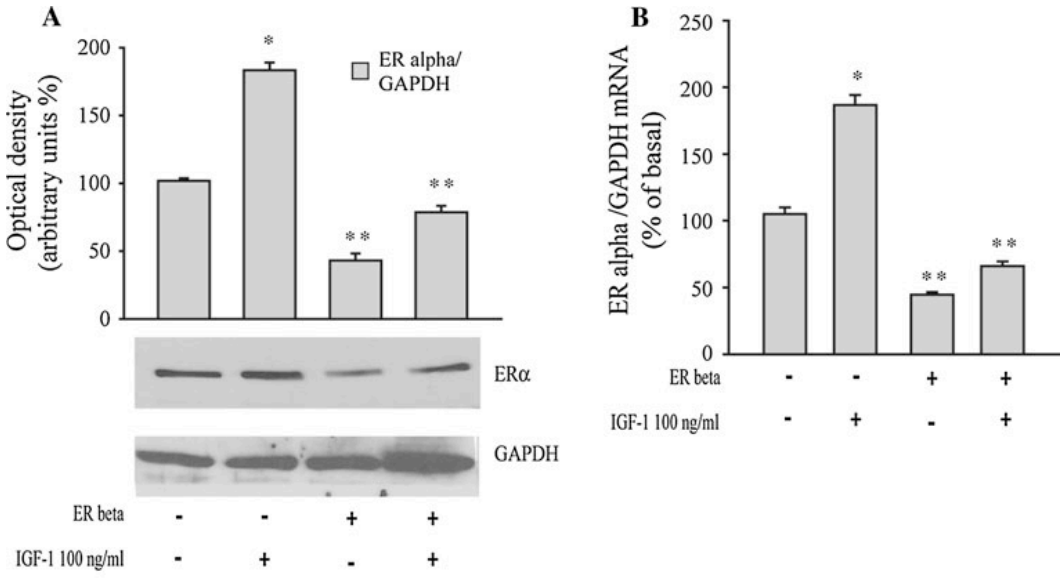


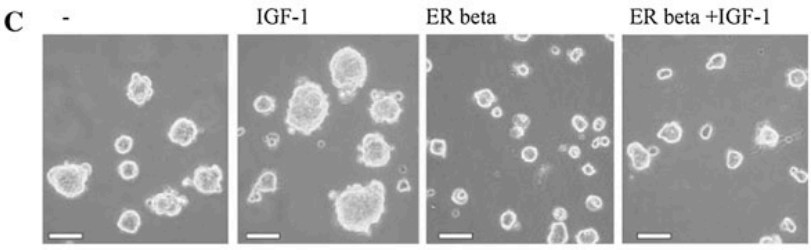
Figure 16. Effects of NCoR silencing on ERβ-mediated downregulation of ERα expression, Sp-1 recruitment to ERα promoter and cell proliferation.

A. Western blot analysis for NCoR in MCF-7 cells transfected with non-specific siRNA (C) or targeted against human NCoR (100 nM) for 16 and 32 h. GAPDH was used as a loading control. **B**. RT-PCR for NCoR or GAPDH in MCF-7 cells transfected as above described. NC: negative control, RNA sample without the addition of reverse transcriptase. The histograms represent the mean ± S.D. of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. *P\0.01 NCoR siRNA-transfected cells compared to nonspecific siRNA (C)-transfected cells. **C**. Western blot analysis for ERα in MCF-7 cells transfected with either empty vector(-) or ERβ expression plasmid in presence of non-specific or NCoR siRNA. GAPDH was used as loading control. The histograms represent the mean ± S.D. of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **D**. RNA was extracted from MCF-7 cells transfected with either empty vector(-) or ERβ expression plasmid in presence of non-specific or NCoR siRNA, reverse transcribed and cDNA was subjected to qRT-PCR for analyzing ERα mRNA levels. Data represent the mean ± S.D. of values from three separate RNA samples expressed as percentage of control (-) assumed to be 100 %. Each sample was normalized to GAPDH mRNA content. **E, F**. MCF-7 cells transfected with either empty vector(-) or ERβ expression plasmid in presence of non-specific or NCoR siRNA, were crosslinked with formaldehyde, and lysed. The precleared chromatin was immunoprecipitated with anti-Sp-1 (e) or anti-RNA-polymerase II (Pol II, f) antibodies. A 5 µl volume of each sample and input was analyzed by qRT-PCR using specific primers to amplify ERα promoter sequence, including the Sp-1 site. Similar results were obtained in two independent experiments. **G, H**. MTT assays in MCF-7 and ZR75 cells transfected as indicated. Results are expressed as fold change ± S.D relative to empty vector-transfected cells and are representative of three different experiments each performed in triplicate. *P\0.01 ERβ-transfected cells compared to empty vector-transfected cells. **P\0.01 NCoR siRNA-transfected cells compared to ERβ-transfected cells.

ER beta antagonizes IGF-1 mediated up-regulatory effects on ER alpha expression and three-dimensional cell growth

It has been previously demonstrated that IGF-1 and insulin can increase ERα expression and stimulate proliferation in breast cancer cells (93-94). Thus, we investigated the ability of ERβ to reverse IGF-1 effects on ERα expression in MCF-7 cells, by western blotting and real time PCR analysis (Fig.17A, B). As expected, IGF-1 enhanced ERα protein and mRNA levels and ERβ overexpression significantly abrogated this increase. Then, the effects of ERβ on IGF-1-induced growth were assessed using three-dimensional MCF-7 cell culture, that simulate ‘‘in vivo’’ the biological features of tumors. Our results showed that ERβ overexpression blocked the IGF-1 induced cell growth, as evidenced by the extent of aggregation scored by measuring the spheroid diameters (Fig. 17C).





	25 µm ≤ 50 µm	50 µm ≤ 100 µm	≥100 µm
-	30 ± 1.0	2.8 ± 1.0	0.0 ± 0.0
IGF-1	5 ± 2.5	34 ± 1.5	85.0 ± 3.1
ER beta	90 ± 3.2	0.6 ± 0.5	0.0 ± 0.0
ER beta + IGF-1	65 ± 1.5	0.8 ± 1.6	0.0 ± 0.0

Figure 17. Overexpressed ERβ reverses IGF-1 enhanced ERα expression and cell–cell adhesion.

A. Bottom panel, MCF-7 cells transiently transfected with either empty vector(-) or ERβ expression plasmid were treated with vehicle or IGF1 (100 ng/ml) for 48 h. Total proteins were extracted and western blot analysis was performed to evaluate the expression of ERα. GAPDH was used as loading control. Upper panel, the histograms represent the mean ± S.D. of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **B**. qRT-PCR for analyzing ERα mRNA levels in cells transfected and treated as indicated. Data represent the mean ± S.D. of values from three separate RNA samples expressed as percentage of control assumed to be 100 %. Each sample was normalized to GAPDH RNA content. *P\0.01 IGF-1 treatment compared to vehicle treated-cells. **P\0.01 ER β overexpressing cells compared to empty vector transfected cells. **C**. MCF-7 cells were transiently transfected with either empty vector (-) or ERβ expression plasmid and growth as three-dimensional cultures in the presence or absence of IGF-1 (100 ng/ml, 48 h). Scale bar = 25 lm. Images are representative of three

independent experiments. The extent of aggregation was scored by measuring the spheroid diameters. The values represent the sum of spheroids in 10 optical fields under 910 magnification.

Discussion

In this study, we show for the first time that ERβ overexpression down-regulates ERα gene expression in a ligand-independent manner in ERα-positive breast cancer cells. This occurs through ERβ interactions with Sp-1 and an enhanced NCoR corepressor recruitment within the human ERα promoter region, up-stream of the initiating transcription site. ERα and ERβ have both overlapping and distinct expression patterns, and mammary gland development in animal models requires ERα signaling. It has been hypothesized that dysregulated ER isoform expression may induce abnormal cell proliferation and survival, thus impacting mammary tumorogenesis. It is also well known that ERα expression is increased and ERβ expression is decreased in early breast cancers, whereas expression of both receptors declines in more invasive cancers (95–96). Expression of ERβ is lost in other early tumor types in respect to normal tissue (97), leading to the hypothesis that ERβ may function as a tumor suppressor [98, 99]. Data coming from cell studies have suggested that ERβ negatively interferes with ERα signaling in breast cancer cells, and mediates antiproliferative effects (99). ERβ over-expression inhibits tumor establishment and growth as well as E2- induced tumor formation ‘‘in vivo’’ in mouse xenografts of ERα-positive MCF-7 and T47D breast cancer cells [100]. Indeed, ERβ induces inhibition of classical estrogen-regulated genes, such as VEGF and PDGFb (101). Recently, Song and Pan (102) demonstrated that ERα-mediated estrogenic activity in the mammary gland can be opposed by ERβ, and it has been suggest that selective agonists such as DPN should be explored for the development of better hormone replacement therapy regimens to reduce or eradicate the risk for breast cancer. In the majority of clinical studies, ERβ expression indicates a

favorable response to adjuvant tamoxifen (Tam) therapy, and patients with ERα +/ERβ + tumors appear to respond at least as well as or better to endocrine therapy than patients with ERα +/ERβ- tumors. In addition, in Tam-treated patients, high ERβ expression correlates with increased overall, and disease-free survival, no disease progression, or no relapse within 5 years (103, 104). Thus, ERβ has emerged as potential marker for predicting response to endocrine therapy. These findings led us to investigate the molecular mechanism through which ER subtypes are regulated in breast cancer cells. Here, we have demonstrated that ERβ overexpression in a ligand-independent manner resulted in inhibition of ERα in terms of mRNA and protein content in breast cancer cells. Similar inhibitory effects were also obtained for the expression of estrogen-dependent genes such as IRS-1, pS2, and cyclin D1. These data underline how ERβ-induced ERα down-regulation might arise via transcriptional mechanisms. Therefore, we focused on the molecular mechanisms by which ERβ mediates repression of ERα gene expression and on the biological consequences of ERβ overexpression on growth of breast cancer cells. ERα and ERβ are transcriptional factors that can regulate gene expression through several different modes including direct DNA-binding (acting as homodimers or as heterodimers) or through tethering to other transcription factors such as activating protein-1 (AP 1) and stimulating protein-1 (Sp-1) (105). This has been most extensively investigated in relationship to protein complexes involving Sp-1 and ERα at GC boxes, which are classic binding sites for members of the Sp-1 family of transcription factors (106, 107). Many studies have observed that ERα is able to enhance binding of Sp1 to its site in several promoter regions (108, 109). The analysis of different functional motifs present within the ERα proximal promoter (90) has identified two half-ERE and one Sp-1 responsive elements, as potential targets of ERβ. Functional

experiments using ERα promoter-deleted or mutated constructs have shown that Sp-1 sequence is an important prerequisite for the down-regulatory effects of ERβ on ERα promoter activity. These results were well supported by electrophoretic mobility shift assays, which revealed a marked increase in a specific DNA-binding complex in nuclear extracts from MCF-7 cells overexpressing ERβ. This complex was immune-depleted by anti-Sp-1 and anti-ERβ antibodies, suggesting the presence of these proteins in the complex. Furthermore, we observed an enhanced recruitment of Sp-1 and ERβ to the ERα promoter, that was concomitant with a decrease in RNA-polymerase II and acetyl histone H4 recruitment, further supporting a negative role for ERβ in modulating ERα gene transcriptional machinery. A recent study reported that the ZFDBD (Zinc Finger DNA-Binding Domain) and ID (Inhibitory Domain) domains of Sp-1 are involved in protein–protein interactions with other transcription regulatory molecules, such as the corepressors SMRT, NCoR, and BCoR (BCL6 corepressor) [91]. These corepressors interact with unliganded nuclear receptors, through an elongated helix of sequence LXXI/HIXXXI/L, alternatively referred to as the CoRNR-box (72, 61). It has been recently documented that NCoR and SMRT are also recruited by both ER and PR in the presence of ligands to regulate transcription of different genes (110, 111). Our results demonstrate that NCoR was the corepressor crucially recruited on the Sp-1 site of the ERα gene promoter together with Sp-1 and ERβ. In general, NCoR and SMRT share the same molecular architecture, interact with many of the same transcription factors, and assemble into similar corepressor complexes (112). We also detected a slight recruitment of SMRT under the same experimental conditions. Finally, the contribution of the NCoR corepressor factor in ERβ-mediated effects emerges from experiments showing that silencing of NCoR gene expression was able to reverse the inhibitory effects of ERβ on ERα mRNA and

protein content, Sp-1 recruitment to the ERα promoter gene and cell growth proliferation. Previous ‘‘in vitro’’ studies have shown that insulin and IGF-1 up-regulate the ERα expression as well as its DNA-binding capacity (93–113). We demonstrated how ERβ reduced the stimulatory effects induced by IGF1 on ERα expression and three-dimensional cell growth, and became a negative modulator of the well-known crosstalk between ERα and IGF1-R signaling pathways. In conclusion, we suggest that inhibition of ERα by ERβ is a critical regulatory pathway occurring in ER-positive cells, addressing prospectively that therapeutic tools which potentiate ERβ action and thereby deplete intratumoral ERα content may be useful to inhibit breast cancer cell growth and progression.

References

1. Anderson E (2002). The role of oestrogen and progesterone receptors in human mammary development and tumorigenesis. Breast Cancer Research; 4: 197-201.

2. O'Malley BW, McGuire WL & Middleton PA (1968). Altered gene expression during differentiation: population changes in hybridizable RNA after stimulation of the chick oviduct with oestrogen. Nature; 218: 1249–1251.

3. Jensen EV, Block GE, Smith S, Kyser K & DeSombre ER (1971). Estrogen receptors and breast cancer response to adrenalectomy. National Cancer Institute Monograph; 34: 55–70.

4. McGuire WL (1973). Estrogen receptors in human breast cancer. Journal of Clinical Investigation; 52: 73–77.

5. Pike MC, Spicer DV, Dahmoush L, Press MF. Rev. (1993). Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol; 15: 17–35.

6. Endogenous Hormones and Breast Cancer Collaborative Group Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies (2002). J Natl Cancer Inst.; 94: 606–616.

7. Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, Boyle P (2003). Overview of the main outcomes in breast-cancer prevention trials. Lancet; 361: 296–300.

8. Dao TL. (1981). The role of ovarian steroid hormones in mammary carcinogenesis. Hormones and Breast Cancer. Cold Spring Harbor; New York: 281–295.

9. Clevenger CV, Furth PA, Hankinson SE, Schuler LA. (2003). The role of prolactin in mammary carcinoma. Endocr Rev.; 24: 1–27.

10. Lillie EO, Bernstein L, Ursin G. (2003). The role of androgens and polymorphisms in the androgen receptor in the epidemiology of breast cancer. Breast Cancer Res.; 5: 164–173.

11. Collaborative Group on Hormonal Factors in Breast Cancer Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52.705 women with breast cancer and 108.411 women without breast cancer. Lancet. (1997); 350: 1047–1059.

12. Eerola H, Aittomaki K, Asko-Seljavaara S, Nevanlinna H, von Smitten K. (2002). Hereditary breast cancer and handling of patients at risk Scand J Surg.; 91: 280–287.

13. Hunter DJ, Spiegelman D, Adami HO, van den Brandt PA, Folsom AR, Goldbohm RA, Graham S, Howe GR, Kushi LH, Marshall JR, Miller AB, Speizer FE, Willett W, Wolk A, Yaun SS. (1997). Non-dietary factors as risk factors for breast cancer, and as effect modifiers of the association of fat intake and risk of breast cancer. Cancer Causes Control.; 8: 49–56.

14. Helmrich et al. (1983). Risk Factors for breast cancer. Am. J. Epidemiol. 1: 35-45.

15. Key TJ, Verkasalo PK, Banks E. (2001). Epidemiology of breast cancer. Lancet Oncol. 2: 133–140.

16. Liu Q, Wuu J, Lambe M, Hsieh SF, Ekbom A, Hsieh CC. (2002). Transient increase in breast cancer risk after giving birth: postpartum period with the highest risk (Sweden) Cancer Causes Control.; 13: 299–305.

17. Collaborative Group on Hormonal Factors in Breast Cancer Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease (2002). Lancet; 360: 187–195.

18. Collaborative Group on Hormonal Factors in Breast Cancer Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease (2002) Lancet; 360: 187–195.

19. Ewertz M, Duffy SW, Adami HO, Kvale G, Lund E, Meirik O, Mellemgaard A, Soini I, Tulinius H. Age at first birth, parity and risk of breast cancer: a meta-analysis of 8 studies from the Nordic countries. (1990). Int J Cancer; 46:597–603.

20. Russo J, Wilgus G, Russo IH. (1979). Susceptibility of the mammary gland to carcinogenesis: I. Differentiation of the mammary gland as determinant of tumor incidence and type of lesion. Am J Pathol; 96:721–736.

21. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. (1995). The nuclear receptor superfamily: the second decade. Cell (6): 835-9.

22. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary (1996). Proc Natl Acad Sci USA 11; 93(12): 5925-30.

23. Pearce ST, V. Craig Jordan. (2004). The biological role of estrogen receptors α and β in cancer. Oncology Hematology; 50: 3-22.

24. Hiroi H, Inoue S, Watanabe T, et al. (1999). Differential immunolocalization of estrogen receptor alpha and beta in rat ovary and uterus. J Mol Endocrinol; 22: 37–44.

25. Sar M, Welsch F. Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary (1999). Endocrinology; 140: 963–71.

26. Weihua Z, Warner M, Gustafsson JA. (2002). Estrogen receptor beta in the prostate. Mol Cell Endocrinol; 193: 1–5.

27. Walter P, Green S, Greene G et al. (1985). Cloning of the human estrogen receptor cdna. Proc Natl Acad Sci USA; 82: 7889–7893.

28. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y and Shine J. (1986). Sequence and expression of human estrogen receptor complementary dna. Science; 231: 1150–1154.

29. Green S, Walter P, Kumar V et al. (1986). Human oestrogen receptor cdna: sequence. Nature; 320: 134–139.

30. Flouriot G, Brand H, Denger S. (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; 19:4688–700.

31. Murphy LC, Dotzlaw H, Leygue E, Douglas D, Coutts A, Watson PH. (1997). Estrogen receptor variants and mutations. J Steroid Biochem Mol Biol; 62:363–72.

32. Poola I, Koduri S, Chatra S, Clarke R. (2000) Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using splice targeted primer approach. J Steroid Biochem Mol Biol; 72: 249–58.

33. Short TH, Rothschild MF, Southwood OI, McLaren DG, De Vries A,Van Der Steen H, Eckardt GR, Tuggle CK, Helm J, Vaske DA, Mileham AJ, Plastow G. (1997). Effect of the estrogen receptor locus on reproduction and production traits in four commercial pig lines. J Anim Sci 75: 3138–3142.

34. Andersen TI, Heimdal KR, Skrede M, Tveit K, Berg K, Borresen A. (1994). Oestrogen receptor (ESR) polymorphisms and breast cancer susceptibility. Hum Genet; 94: 665–670.

35. Mizunuma H, Hosoi T, Okano H, Soda M, Tokizawa T, Kagami I,Miyamoto S, Ibuk i Y, Inoue S, Shiraki M,Ouchi Y (1997) Estrogen receptor gene polymorphism and bone mineral density at the lumbar spine of pre- and postmenopausal women. Bone; 21:379–383.

36. Lehrer S, Rabin J, Kalir T, Schachter B.(1993) Estrogen receptor variant and hypertension in women. Hypertension; 21: 439–441.

37. Schachter BS, Lehrer . (1994). Risk of miscarriage and a common variant of the estrogen receptor gene. Am J Epidemiol; 140:1144–1145.

38. Lehrer S, Rabin J, Stone J, Berkowitz GS (1994) Association of an estrogen receptor variant with increased height in women. Horm Metab Res; 26: 486–488.

39. Marino M., Galluzzo P., Ascenzi P. (2006). Curr. Genomics; 7: 497–508.

40. Gronemeyer H., Laudet V. (1995). Protein Profile; 2: 1173–1308.

41. Ogawa S., Inoue S., Watanabe T., Orimo A., Hosoi T., Ouchi Y., Muramatsu M. (1998) Nucleic Acids Res. 26, 3505–3512.

42. Zhao C., Matthews J., Tujague M., Wan J., Ström A., Toresson G., Lam E. W., Cheng G., Gustafsson J. A., Dahlman-Wright K. (2007) Cancer Res. 67: 3955–3962.

43. Fujimura T., Takahashi S., Urano T., Ogawa S., Ouchi Y., Kitamura T., Muramatsu M., Inoue S. (2001) Biochem. Biophys. Res. Commun.; 289: 692–699.

44. Palmieri C., Lam E. W., Mansi J., MacDonald C., Shousha S., Madden P., Omoto Y., Sunters A., Warner M., Gustafsson J. A., Coombes R. C. (2004). Clin. Cancer Res. 10; 2421–2428.

45. Couse J. F., Korach K. S. (1999) Endocr. Rev.; 20: 358–417.

46. Hodgin J. B., Maeda N. (2002) Endocrinology 143; 4495–4501.

47. Villablanca A. C., Tenwolde A., Lee M., Huck M., Mumenthaler S., Rutledge J. C. (2009) J. Cardiovasc. Transl. Res. 2; 289–299.

48. Pettersson, K. et al. (1997). Mouse estrogen receptor b forms estrogen response element - binding heterodimers with estrogen receptor a. Mol. Endocrinol; 11: 1486–1496.

49. Kulakosky, P.C. et al. (2002) Response element sequence modulates estrogen receptor α and β affinity and activity. J. Mol. Endocrinol; 29: 137–152.

50. Klinge, C.M. (2000) Estrogen receptor interaction with co-activators and co-repressors. Steroids; 65: 227–25.

51. Boonyaratanakornkit V. et al. (1998). High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. Mol. Cell. Biol; 18: 4471–4487.

52. Klein-Hitpass, L. et al. (1988). A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. Nucleic Acids Res; 16: 647–663.

53. Peale, F.V. et al. (1988). Properties of a high-affinity DNA binding site for estrogen receptor. Proc. Natl. Acad. Sci. U. S. A.; 85: 1038–1042.

54. Kuiper, G.G. et al. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology; 138: 863–870.

55. Yi, P. et al. (2002). The effects of estrogen - responsive element - and ligand – induced structural changes on the recruitment of cofactors and transcriptional responses by ERα and ERβ. Mol. Endocrinol; 16: 674–693.

56. Schwabe, J.W. et al. (1995). The oestrogen receptor recognizes an imperfectly palindromic response element through an alternative side- chain conformation. Structure; 3: 201–213.

57. Smith, D.F. and Toft, D.O. (1993). Steroid receptors and their associated proteins. Mol. Endocrinol; 7: 4–11.

58. Wong, C.W. et al. (2001). Structure-function evaluation of ER α and β interplay with SRC family coactivators. ER selective ligands. Biochemistry; 40: 6756–6765.

59. Green, S. and Chambon, P. (1987). Oestradiol induction of a glucocorticoid-responsive gene by a chimaeric receptor. Nature; 325: 75–78.

60. Klein-Hitpass, L. et al. (1988). A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. Nucleic Acids Res.; 16: 647–663.

61. Webb P, Valentine C, Nguyen P, Price RH Jr, Marimuthu A, West BL, Baxter JD & Kushner PJ (2003). ER beta binds NCoR in the presence of estrogens via an LXXLL-like motif in the N-CoR C-terminus. Nuclear Receptor; 1(1): 4.

62. McKenna NJ, Lanz RB, O'Malley BW. (1999). Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev; 20: 321–44.

63. Endoh H, Maruyama K, Masuhiro Y, et al. (1999). Purification and identi-fication of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha. Mol Cell Biol; 19: 5363–72.

64. Suen CS, Berrodin TJ, Mastroeni R, Cheskis BJ, Lyttle CR, Frail DE. (1998). A transcriptional coactivator, steroid receptor coactivator-3, selectively augments steroid receptor transcriptional activity. J Biol Chem; 273: 27645–53.

65. Hong SH, Wong CW, Privalsky ML (1998). Signaling by tyrosine kinases negatively regulates the interaction between transcription factors and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) corepressor. Mol Endocrinol; 12(8): 1161-71.

66. Privalsky ML (2004). The role of corepressors in transcriptional regulation by nuclear hormone receptors. Annu Rev Physiol; 66: 315- 60.

67. Goodson M, Jonas BA, Privalsky MA (2005). Corepressors: custom tailoring and alterations while you wait. Nucl Recept Signal; 3: e003.

68. Codina A., Love J.D., Li Y., Lazar M.A., Neuhaus D., Schwabe J.W.R. (2005). Structural insights into the interaction and activation of histone deacetylase 3 by nuclear receptor corepressors. Proc. Natl. Acad. Sci. USA; 102: 6009–6014.

69. Hartman H.B., Yu J., Alenghat T., Ishizuka T., Lazar M A (2005). The histone-binding code of nuclear receptor co-repressors matches the substrate specificity of histone deacetylase 3. EMBO Rep; 6: 445–451.

70. Yu J, Li Y, Ishizuka T, Guenther MG, Lazar MA (2003). A SANT motif in the SMRT corepressor interprets the histone code and promotes histone deacetylation. Embo J, 22(13): 3403-10.

71. Hu X., Lazar M.A.(1999). The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature.; 402: 93–96.

72. Nagy L., Kao H.Y., Love J.D., Li C., Banayo E., Gooch J.T., Krishna V., Chatterjee K., Evans R.M., Schwabe J.W. (1999). Mechanism of corepressor binding and release from nuclear hormone receptors. Genes Dev.; 13: 3209–3216.

73. Makowski A., Brzostek S., Cohen R.N., Hollenberg A.N. (2003) Determination of nuclear receptor corepressor interactions with the thyroid hormone receptor. Mol. Endocrinol; 17: 273–286.

74. Short S., Malartre M., Sharpe C. (2005). SMRT has tissue-specific isoform profiles that include a form containing one CoRNR box. Biochem. Biophys. Res. Commun; 334:845–852.

75. Varlakhanova N., Snyder C., Jose S., Hahm J.B., Privalsky M.L. (2010). Estrogen receptors recruit SMRT and N-CoR corepressors through newly recognized contacts between the corepressor N terminus and the receptor DNA binding domain. Mol. Cell. Biol.; 30: 1434–1445.

76. Allegra JC, Lippman ME, Green L et al (1979). Estrogen receptor values in patients with benign breast disease. Cancer; 44: 228–231.

77. Yager JD, Davidson NE (2006). Estrogen carcinogenesis in breast cancer. N Engl J Med; 354: 270–282.

78. Zhao C, Dahlman-Wright K et al (2008). Estrogen receptor beta: an overview and update. Nucl Recept Signal; 6: 003-23.

79. Skliris GP, Munot K, Bell SM et al (2003). Reduced expression of estrogen receptor beta in invasive breast cancer and its reexpression using DNA methyl transferase inhibitors in a cell line model. J Pathol; 201: 213–220.

80. Paruthiyil S, Parmar H, Kerekatte V et al (2004). Estrogen receptor b inhibits human breast cancer cell proliferation and tumor formation by causing a G2 Cell Cycle Arrest. Cancer Res; 64: 423–428.

81. Stro¨m A, Hartman J, Foster JS et al (2004). Estrogen receptor b inhibits 17β-estradiol-stimulated proliferation of the breast cance cell line T47D. Proc Natl Acad Sci; 101:1566–1571.

82. Saji S, Omoto Y, Shimizu C et al (2002). Expression of estrogenreceptor (ER) (beta) cx protein in ER (alpha)-positive breast cancer: specific correlation with progesterone receptor. Cancer Res 62: 4849–4853.

83. Pettersson K, Delaunay F, Gustafsson JA (2000). Estrogen receptor b acts as a dominant regulator of estrogen signaling. Oncogene; 19: 4970–4978.

84. Bardin A, Boulle N, Lazennec G, Vignon F, Pujol P. (2004). Loss of ERβ expression as a common step in estrogen-dependent tumor progression. Endocr Relat Cancer; 11(3): 537–551.

85. A Ring, M Dowsett. (2004). Mechanisms of tamoxifen resistance. Endocrine-Related Cancer; 11: 643–658.

86. Gustafsson JA, Warner M. (2000). Estrogen Receptor β in the breast: role in estrogen responsiveness and development of breast cancer. Journal of Steroid Biochemistry and Molecular Biology; 74: 245–248.

87. Dupont J., Karas M., LeRoith D. (2000). The potentiation of estrogen on insulin-like growth factor I action in MCF-7 human breast cancer cells includes cell cycle components. J. Biol. Chem; 275: 35893–35901.

88. Hall JM, McDonnell DP. (1999). The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology; 140:5566–5578.

89. Weihua Z, Makela S, Andersson LC, Salmi S, Saji S, Webster I, Jensen EV, Nilsson S, Warner M & Gustafsson JA (2001). A role for estrogen receptor b in the regulation of growth of the ventral prostate. PNAS; 98: 6330–6335.

90. deGraffenried LA, Hopp TA, Valente AJ et al (2004). Regulation of the estrogen receptor a minimal promoter by Sp1, USF-1 and ERα. Breast Cancer Res and Treat; 85: 111–120.

91. Chen JD, Evans RM (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature; 377: 454–457.

92. Lee JA, Suh DC, Kang JE et al (2005). Transcriptional activity of Sp1 is regulated by molecular interactions between the zinc finger DNA binding domain and the inhibitory domain with corepressors, and this interaction is modulated by MEK. J

Biol Chem; 280: 28061–28071.

93. Panno ML, Salerno M, Pezzi V et al (1996). Effect of estradiol and insulin on proliferative pattern and on estrogen and progesterone receptor contents in MCF-7 cells. J Cancer Res Clin Oncol; 122: 745–74.

94. Lannigan DA (2003). Estrogen receptor phosphorylation. Steroids; 68:1–9.

95. Leygue E, Dotzlaw H, Watson PH et al (1998). Altered estrogen receptor α and β messenger RNA expression during human breast tumorigenesis. Cancer Res; 58: 3197–3201.

96. Roger P, Sahla ME, Makela S et al (2001). Decreased expression of estrogen receptor β protein in proliferative preinvasive mammary tumors. Cancer Res; 61: 2537–2541.

97. Fixemer T, Remberger K, Bonkhoff H (2003). Differential expression of the estrogen receptor beta in human prostate tissue, premalignant changes, and in primary, metastatic, and recurrent prostatic adenocarcinoma. Prostate; 54: 79–87.

98. Chang EC, Frasor J, Komm B et al (2006). Impact of estrogen receptor b on gene networks regulated by estrogen receptor a in breast cancer cells. Endocrinology; 147:4831–4842.

99. Williams C, Edvardsson K, Lewandowski SA et al (2008). A genome-wide study of repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. Oncogene; 27: 1019–1032.

100. Behrens D, Gill JH, Fichtner I (2007). Loss of tumourigenicity of stably Erβ-transfected MCF-7 breast cancer cells. Mol Cell Endocrinol; 274: 19–29.

101. Buteau-Lozano H, Ancelin M, Lardeux B et al (2002). Transcriptional regulation of vascular endothelial growth factor by estradiol and tamoxifen in breast cancer cells: a complex interplay between estrogen receptors α and β. Cancer Res; 62: 4977–4984.

102. Song K, Pan ZZ (2012). Estrogen receptor beta agonist diarylpropionitrile counteracts the estrogenic activity of estrogen receptor alpha agonist propylpyrazole-triol in the mammary gland of ovariectomized Sprague Dawley rats. J Steroid Biochem Mol Biol; 130: 26–35.

103. Esslimani-Sahla M, Simony-Lafontaine J, Kramar A et al (2004). Estrogen receptor beta (ER beta) level but not its ER beta cx variant helps to predict tamoxifen resistance in breast cancer. Clin Cancer Res; 10: 5769–5776.

104. Hopp TA, Weiss HL, Parra IS et al (2004). Low levels of estrogen receptor beta protein predict resistance to tamoxifen therapy in breast cancer. Clin Cancer Res; 10:7490–7499.

105. 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.

106. Porter W, Saville B, Hoivik D et al (1997). Functional synergy between the transcription factor Sp1 and the estrogen receptor. Mol Endocrinol; 11: 1569–1580.

107. Vyhlidal C, Samudio I, Kladde MP et al (2000). Transcriptional activation of transforming growth factor alpha by estradiol: requirement for both a GC-rich site and an estrogen response half-site. J Mol Endocrinol; 24: 329–338.

108. Panno ML, Mauro L, Marsico S et al (2006). Evidence that mouse IRS-1 belongs to the family gene which promoter is activated by ERα through its interaction with Sp-1. J Mol Endocrinol; 36: 91–105.

109. Mauro L, Catalano S, Bossi G et al (2007). Evidences that leptin upregulates E-cadherin expression in breast cancer effects on tumor growth and progression. Cancer Res; 67: 3412–3421.

110. Jiang S, Meyer R, Kang K et al (2006). Scaffold attachment factor SAFB1 suppresses estrogen receptor a-mediated transcription in part via interaction with nuclear receptor corepressor. Mol Endocrinol; 20: 311–320.

111. De Amicis F, Zupo S, Panno ML et al (2009). Progesterone receptor B recruits a repressor complex to a half-TRE site of the estrogen receptor a gene promoter. Mol Endocrinol; 23: 454–465.

112. Ghisletti S, Huang W, Jepsen K et al (2009). Cooperative NCoR/SMRT interactions establish a corepressor-based strategy for integration of inflammatory and anti-inflammatory signalling pathways. Gene Dev; 23: 681–693.

113. Lannigan DA (2003). Estrogen receptor phosphorylation. Steroids; 68: 1–9.

Breast Cancer Res Treat (2012) 136:443–455
DOI 10.1007/s10549-012-2282-3

PRECLINICAL STUDY

Bergapten induces ER depletion in breast cancer cells through SMAD4-mediated ubiquitination

M. L. Panno · F. Giordano · P. Rizza · M. Pellegrino · D. Zito · C. Giordano · L. Mauro · S. Catalano · S. Aquila · D. Sisci · F. De Amicis · A. Vivacqua · S. W. A. Fuqua · S. Andò

Received: 25 September 2012 / Accepted: 27 September 2012 / Published online: 9 October 2012

Abstract ERα function is crucial for the development of normal mammary gland as well as in the process of progression of breast cancer cells. Signals that target receptor levels contribute to regulate estrogens effects in the cells. An intricate cross-regulation has been documented between ERα and TGF-β down-stream molecules: SMAD2, SMAD3, and SMAD4, that can bind ERα and regulate their signaling. Thus, identification of natural anticancer drugs able to influence the latter molecule might provide alternative choices for breast cancer treatment. Taking into account our previous published data we wanted to study the effect of 5-Methoxypsoralen (bergapten) on ERα and on TGF-β pathway. We reported that bergapten, a coumarin containing compound, effectively depletes ERα in MCF-7 breast cancer sensitive cells and in tamoxifen-resistant clone. The decrease of ERα protein after bergapten treatment results from the ubiquitine–proteasome pathway as demonstrated by the use of MG-132. IP experiments with ER antibody, demonstrated that the protein has physical interaction with SMAD4 and poly-ubiquitine and the amount of ubiquitinated receptor, linked to SMAD4, is greater under bergapten. The crucial role played by SMAD4, in this process, emerges from the observation that in breast cancer cells, silencing of SMAD4, resulted in increased expression of endogenous ERα in both control and bergapten-treated cells, compared to wild- type cells. The same results were confirmed in siRNA TGF-β RII cells. The results suggest a novel negative regulation of ERα by TGF-β/SMAD4 in breast cancer cells and indicate that the SMAD4 protein is involved in the degradation of ERα induced by bergapten. We propose that bergapten may efficiently act as a natural antitumoral agent, able to deplete ERα from breast cancer tamoxifen-sensitive and resistant cells, thereby retraining the effect of membrane signals targeting ERα and in such way its mitogenic potentiality.

Keywords ERα-ubiquitination · Bergapten · SMAD4 · Breast cancer cells

Abbreviations

B	Bergapten
5-MOP	5-Methoxypsoralen
ERα	Estrogen receptor α
TGF-β	Trasforming growth factor beta
MAPK	Mitogen-activated protein kinase
Poly Ub	Poly-ubiquitin

M. L. Panno & F. Giordano contributed equally to this work.

M. L. Panno (✉) · F. Giordano · P. Rizza · M. Pellegrino · L. Mauro · S. Andò (✉)
Department of Cellular Biology, University of Calabria, Via P. Bucci, 87036 Arcavacata di Rende (CS), Italy
e-mail: mamissina@yahoo.it

S. Andò
e-mail: sebastiano.ando@unical.it

D. Zito · S. Catalano · S. Aquila · D. Sisci · F. De Amicis · A. Vivacqua · S. Andò
Department of Pharmaco-Biology, Faculty of Pharmacy, Nutritional and Health Science, University of Calabria, 87036 Arcavacata di Rende (CS), Italy

C. Giordano · S. Catalano · S. Aquila · D. Sisci · F. De Amicis · S. Andò
Health Center, University of Calabria, 87036 Arcavacata di Rende (CS), Italy

S. W. A. Fuqua
Lester and Sue Smith Breast Center, Breast Center, Baylor College of Medicine, Houston, TX 77479, USA

Introduction

Estrogens have been recognized as a key carcinogenic factor in breast cancer. Ligands of estrogen receptors (ERs) induce a conformational change that leads the dissociation of HSP90 followed by ERα dimerization, and binding to estrogen response elements in estrogens–responsive genes.

Agonists and antagonists-bound ER recruit either coactivators or corepressors, respectively, regulating gene transcription.

Gene amplification or overexpression of ERα was found in some breast cancer. Approximately 70 % of breast cancers are ERα positive and estrogen dependent. Moreover, the ER status is a basic prognostic marker for primary invasive breast cancer and an indicator for an individual hormonal therapy. The most commonly used anti-estrogens: OH-tamoxifen and ICI 182,780, block estrogen-stimulated tumor growth and have demonstrated efficacy for treatment and prevention of ER-positive breast cancer [1, 2]. However, long-term tamoxifen treatment is associated with estrogen-like action in endometrial tissue leading to a high risk for development of uterine adenocarcinoma. In addition, development of acquired resistance to tamoxifen represents the major clinical problem during endocrine treatment in ER-positive breast cancer. A number of studies have suggested that enhanced growth factor signaling, via various signal transduction pathways, may account for endocrine resistant breast tumor growth [3, 4].

In fact, altered expression and activation of EGFR/HER2, IGF-1R and their key downstream signaling components MAPK/ERK (mitogen activated protein kinases/extracellular signaling regulated kinases) and PI3K/Akt (phosphatidylinositol-3-kinase/protein kinase B) can elicit anti-estrogen resistance through crosstalk with ER signaling [5]. Thus, identification of novel anti-estrogen agents may provide alternative choices for breast cancer treatment. Currently, there is a huge scientific and commercial interest in the discovery of potent, safe, and selective anti-cancer drugs. Coumarins are natural compounds found in many plants that possess medical value by itself and its modified derivatives.

They belong to the flavonoid class of plant secondary metabolites, which have been found to exhibit a variety of biological activities, usually associated with low toxicity addressing considerable interest because of their diverse pharmacological properties like anti-HIV [6], anti-coagulant [7], anti-bacterial [8], anti-oxidant [9], dyslipidemic, and anti-tumoral effects [10]. Among these properties, cytotoxic effects were most extensively examined [11, 12]. Recently, it has been reported that neo-tanshinlactone, a coumarin containing compound, showed significant inhibition against two ER^+ human breast cancer cell lines and was ten-fold more potent and 20-fold more selective than Tamoxifen [13].

In addition, our data have demonstrated how 5-methoxypsoralen (bergapten), exerts both anti-proliferative effects and induces pro-apoptotic responses in human breast cancer cells. Besides, in ER-positive MCF-7 cells 5-methoxypsoralen "per se" is also able to counteract the stimulatory action of IGF-I/E2 on breast cancer cell growth and progression [14].

In addition, in established breast cancer cell lines, a correlation has been observed between ER content and sensitivity to transforming growth factor beta (TGF-β) [15]. The role of TGF-β in breast cancer is ambiguous, since it was shown to display both tumor-suppressing and enhancing effects. However, the downstream signaling components of this growth factor: SMAD2, SMAD3, and SMAD4 have been previously reported to bind ER and to regulate ER signaling [16, 17].

In the present study, we have demonstrated how 5-methoxypsoralen in breast cancer Tamoxifen-sensitive and Tamoxifen-resistant cells is able to deplete ER protein, through a degradative process, that sees the involvement of the SMAD4 protein.

Materials and methods

Materials

DMEM/F12, RPMI 1640, MEM, L-glutamine, penicillin, streptomycin, fetal bovine serum, BSA, and PBS were purchased from Eurobio (Les Ullis Cedex, France). Triazol reagent was obtained from Invitrogen (Carlsbad, CA), and FuGENE 6 was from Roche Applied Science (Indianapolis, IN). Taq DNA polymerase was provided by Promega Corp. (Madison, WI). 5-methoxypsoralen, Estradiol, MG-132 were purchased from SIGMA (Milan, ITALY).

Antibodies against ERα/cyclin, D1/GAPDH/lamin, B/Ub/TGFβ, and RII/phospho-ERK/ERK/phospho-JNK/JNK were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against SMAD2/SMAD3/phospho-SMAD3/SMAD4/phospho-p38 MAPK/p38 MAPK, from EPITOMICS, Inc. (California) and anti-phospho Smad2 from NOVUS BIOLOGICALS, LLC (Littleton, CO, USA).

Cell cultures and treatments

MCF-7 and ZR-75 breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained as recommended. Tamoxifen-resistant MCF7-TR1 cells were generated in the laboratory of Dr. Fuqua as described [18].

Cell proliferation assays

Cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan (MTT) assay as described [19]. Data are representative of three-independent experiments, performed in triplicate.

Transient transfection experiment

MDA-MB231 cells were transfected in SFM with pHEGO (1 μg/well), contains the full-length ERα cDNA, using FuGENE6 for 24 h. Luciferase activities were assayed as described [20].

Immunoprecipitation and western blot analysis

Cytoplasmic and nuclear fractions of cellular protein extract were obtained as previously described [21, 22]. Proteins were resolved on an 8 % sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to a nitrocellulose membrane by electroblotting and probed overnight at 4 °C with the antibody indicated in the figure legends.

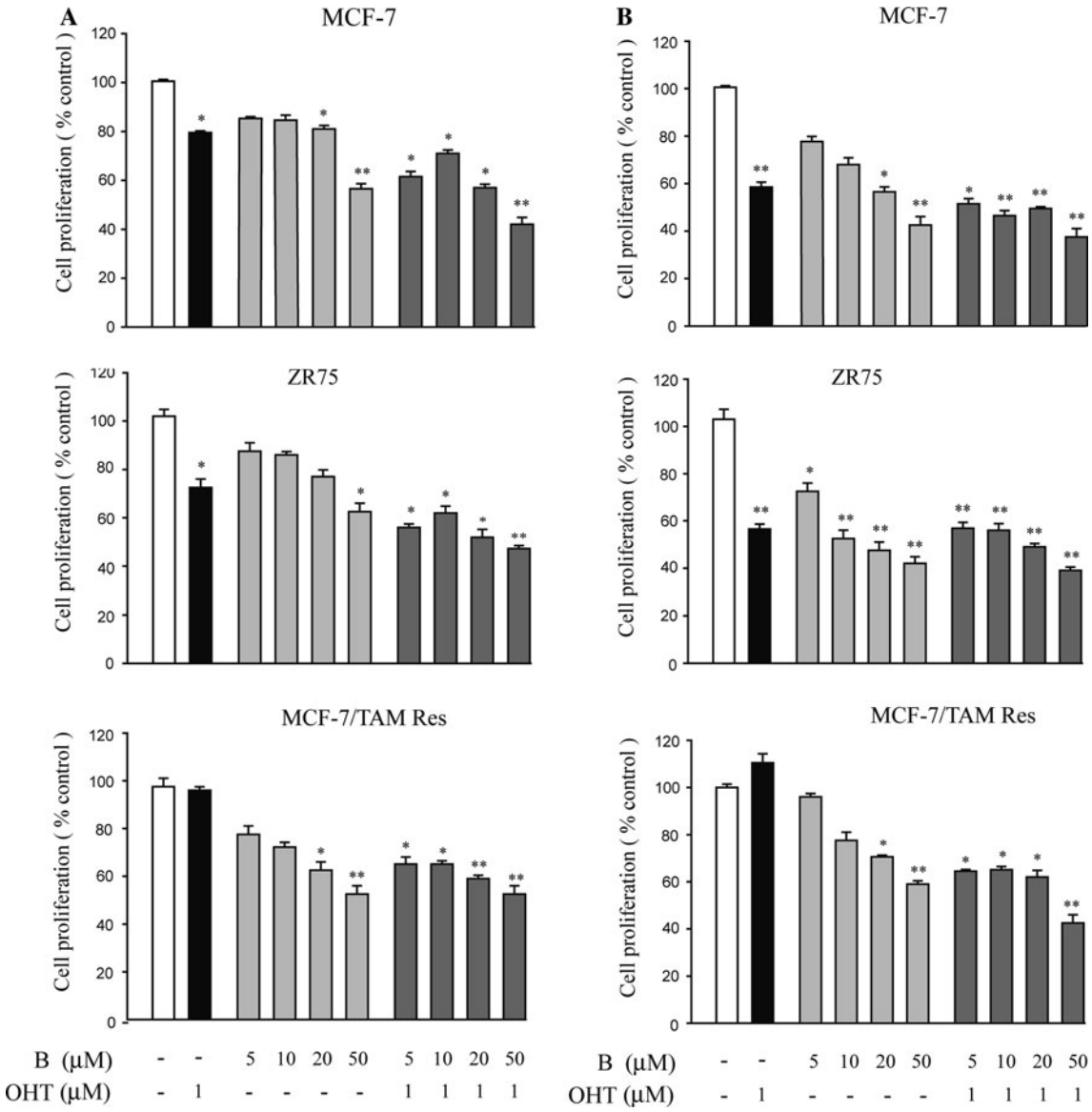


Fig. 1 Cell viability of MCF-7, ZR-75, and MCF-7/TAM Res after bergapten and OH-Tamoxifen treatments. MCF-7, ZR-75, and MCF-7/TAM Res breast cancer cells seeded in six-well plates (100 000 cells/wells) were treated for 48 h (**a**) and 96 h (**b**) with 5, 10, 20, 50 μM of bergapten in a single schedule and in combination with OH-Tamoxifen (1 μM). The values are expressed as percentage of the control, determined by standardizing untreated cells to 100 %. Bergapten (*B*); OH-Tamoxifen (*OHT*). Triplicate results are expressed as mean ±S.D. ($n = 4$). $^{*}P < 0.05$; $^{**}P < 0.01$ as compared to untreated cells

Reverse transcription-polymerase chain reaction (RT-PCR) assay

Total cellular RNA was extracted using TRIZOL reagent (Invitrogen) as suggested by the manufacturer. The evaluation of gene expression was performed by semiquantitative RT-PCR method. For ERα the primers were: 5′-AGATC CAAGGGAACGAGCT-3′ (forward); 5′- TTCTCCAGGT AGTAGGGCA-3′ (reverse); and internal control gene, 36B4, the primers were: 5′-CTCAACATCTCCCCCTTC TC-3′ (forward) and 5′- CAAATCCCATATCCTCGTCC-3′ (reverse).

RNA interference

MCF-7 cells were co-transfected with RNA duplex of stealth RNAi-targeted human SMAD4 or TGF-β type II receptor mRNA or with stealth RNAi-negative control using Lipofectamine 2000 as recommended [23].

Anchorage-independent soft agar growth assays

Soft agar growth assays were conducted as described in the work of Giordano C and colleagues. Data are the mean colony number of three plates and representative of two-independent experiments [24].

Statistical analysis

All data were expressed as the mean ±SD of at least three-independent experiments. The data were analyzed by analysis of variance using the STATPAC computer program.

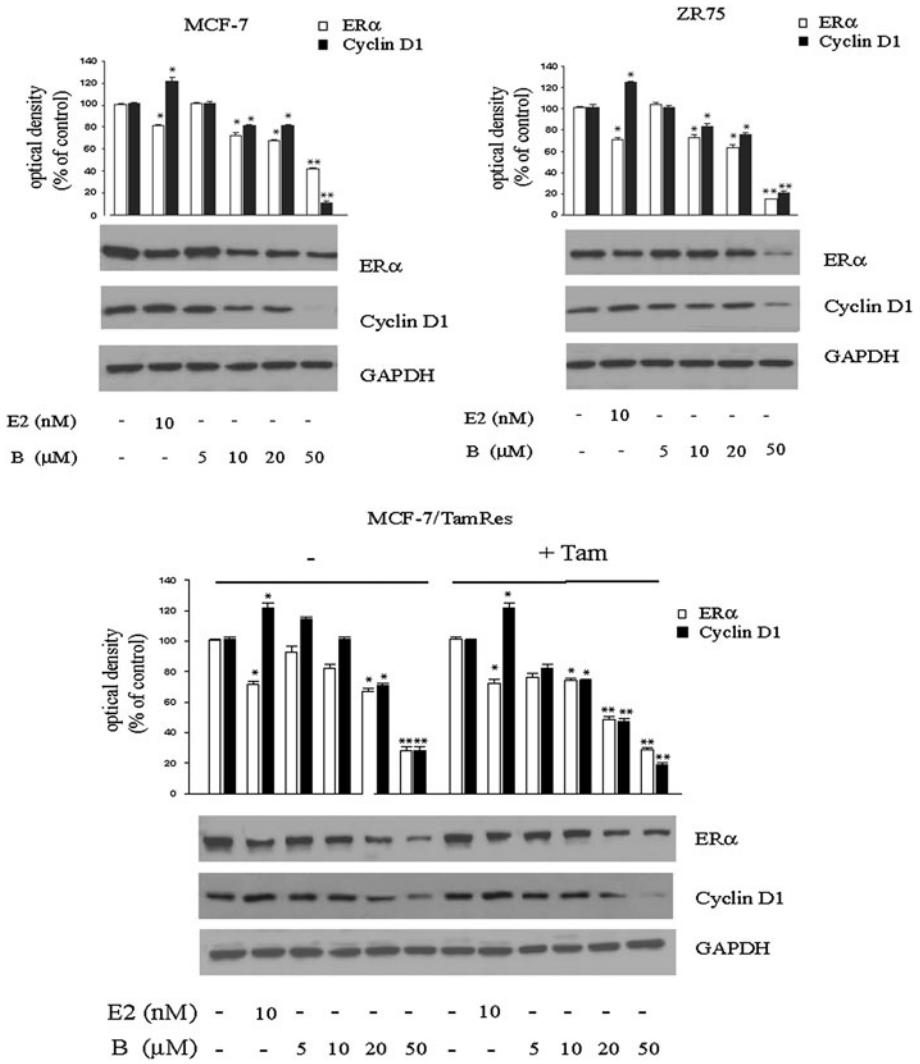


Fig. 2 Bergapten treatment lowers ERα and cyclin D1 in breast cancer cells. MCF-7, ZR-75, and MCF-7/Tam Res cells were treated with E_2 10 nM (taken as positive control) and increasing amounts of bergapten (*B*) (5, 10, 20, 50 μM) for 24 h. A set of MCF-7/Tam Res cells were also maintained with OH-Tamoxifen (T1 μM) during the experimental procedure. GAPDH was used as loading control. Results are representative of three-independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ as compared to untreated cells

Results

Bergapten inhibits breast cancer cell growth and antagonizes the stimulatory action of anti-estrogen in MCF-7 Tamoxifen-resistant cells

To asses the effect of bergapten on cell proliferation: MCF-7, ZR-75 breast cancer cells and MCF-7 Tamoxifen-resistant (MCF-7/TAM Res) cells were treated for 48 and 96 h with different doses of the drug in a single schedule and in combination with OH-Tamoxifen. As shown in Fig. 1, cell growth was assessed by MTT assay. Treatment with bergapten, at both times, inhibited cell growth in a dose-dependent manner. In MCF-7 and ZR-75 cells, the psoralen (20, 50 μM) at 48 h (Fig. 1a) enhances the anti-proliferative activity of OH-Tamoxifen. In addition, in MCF-7/TAM Res clone bergapten, after 96 h, antagonizes the stimulatory action of anti-estrogen even at low doses (5 and 10 μM) (Fig. 1b).

Bergapten down-regulates ER α protein content and decreases estrogen response gene expression

To test the effect of the drug on ERα expression, we treated for 24 h breast cancer cells with the same doses of bergapten used for cell growth. Western Blot analysis of whole–cell lysates of MCF-7 and ZR-75 cells showed that the protein content of ERα was decreased by the highest concentrations of bergapten (Fig. 2). At the same time, we examined the effect of the treatment on ERα-mediated gene expression. The incubation with bergapten reduces cyclin D1 protein in both cell types, taking as positive control the cyclin D1 expression upon E_2 exposure. The same down-regulatory effect was reproduced in MCF-7/TAM Res cells either in the absence or in the presence of OH-Tamoxifen maintained during the experimental time (Fig. 2).

To ascertain if bergapten-mediated ER down-regulation was due to inhibitory effect induced on ER-gene transcription, we performed RT-PCR to detect ERα mRNA level upon the cumarine exposure. As shown in Fig. 3, bergapten does not affect ERα mRNA levels in MCF-7, ZR-75, and MCF-7/TAM Res cells. This reasonably addressed the potential role of post-trascriptional mechanisms in determining the bergapten-induced ERα down-regulation.

Bergapten promotes ERα degradation via the ubiquitine–proteasome pathway

To evaluate the potential molecular mechanism by which bergapten inhibits ER protein expression, we focused on ubiquitin–proteasome pathway. MCF-7, ZR-75, and MCF-7/TAM Res cells were treated with 20 μM and 50 μM

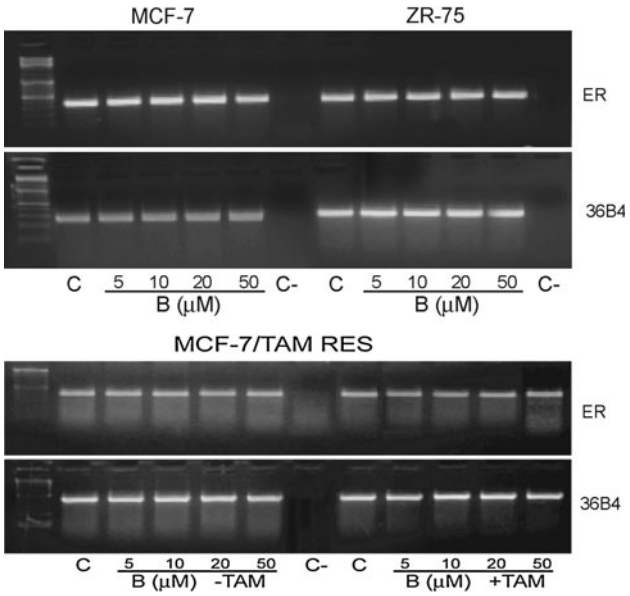


Fig. 3 ERα RT-PCR assay in breast cancer cells under Bergapten. mRNA expression of ERα in MCF-7, ZR-75, and MCF-7/TAM Res cells treated for 24 h with increasing concentrations of bergapten (*B*) as indicated. MCF-7/Tam Res were also treated with bergapten alone or in combination with OH-Tamoxifen. The housekeeping gene 36B4 was determined as control

bergapten in the presence or absence of the proteasomal inhibitor MG-132 (5 μM). In all three cell lines, reduction in ERα by bergapten was prevented by the proteasomal inhibitor MG-132, suggesting that bergapten could induce ERα degradation via the proteasome degradative pathway (Fig. 4).

All these data were confirmed in ER negative MDA-MB 231 cells, overexpressing ectopically the ERα through transient trasfection (Fig. 4). Indeed, MG-132 incubated with bergapten increases the ERα level compared to that obtained by bergapten alone (Fig. 4). Furthermore, in order to evaluate in which cellular compartment ERα degradation occurs we performed Western Blot analysis in extranuclear and nuclear fractions of MCF-7, ZR-75, and MCF-7/TAM Res cells under bergapten treatment. The lowering of ERα induced by psoralen (20 and 50 μM) occurs prevalently in the cytoplasmic fraction of breast cancer cells (Fig. 5).

All results revealed that bergapten affects ERα stability in both wild-type breast cancer cells than in Tamoxifen-resistant clone.

Influence of SMAD4 in the bergapten-induced ERα ubiquitination

ER protein is a cross-road of different intracellular signalings which can modulate ER activity during breast tumorigenesis. Recently, it has been reported how ERα may directly interact with SMADs, the effectors of the TGF-β pathway and, in this respect, to be inversely related to them [16, 17].

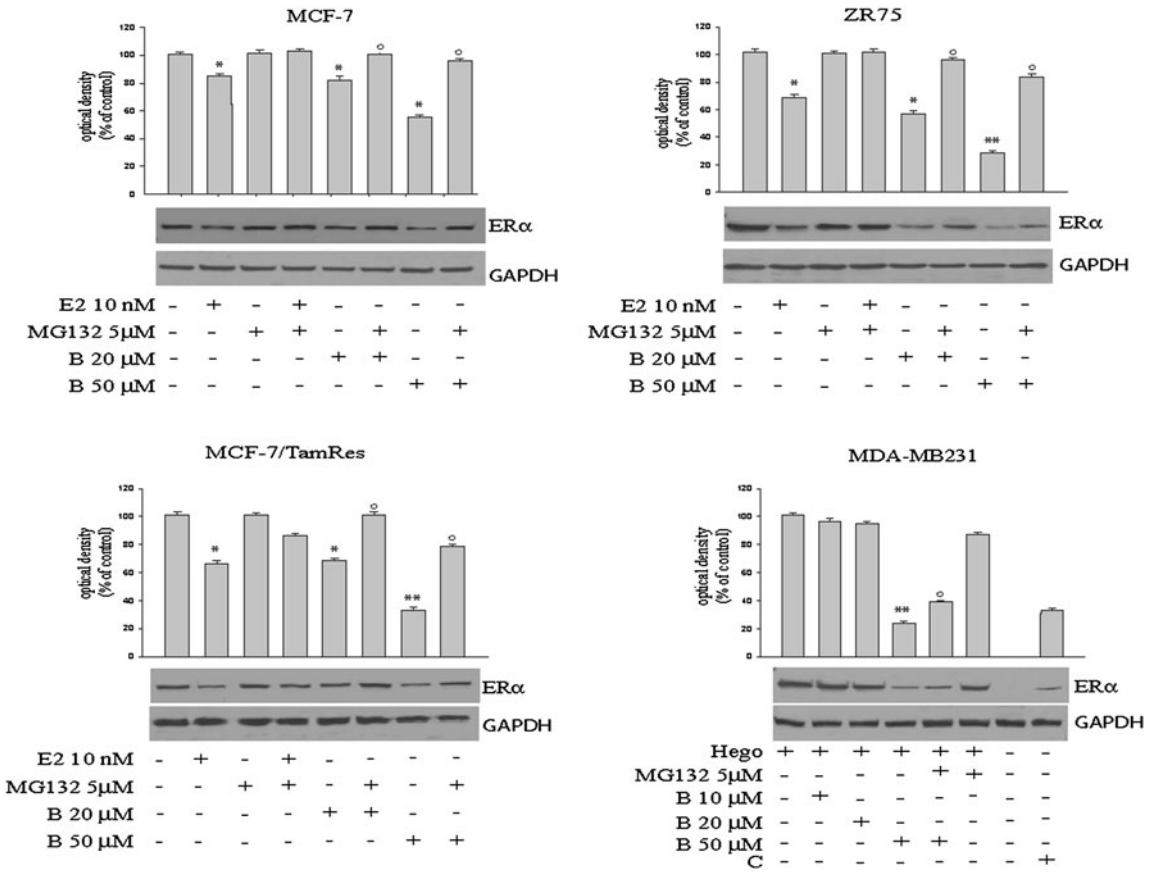


Fig. 4 Bergapten induces ERα degradation via the ubiquitin–proteasome pathway in MCF-7, ZR-75, MCF-7 Tam Res and MDA-MB 231 cells. MCF-7, ZR-75, MCF-7/Tam Res and ER negative MDA-MB 231 cells, transiently overexpressing ERα, were treated with E_2 10nM (taken as positive control) and/or bergapten (*B*) at indicated concentrations in the presence or in the absence of proteasome inhibitor MG-132 (5 μM). This inhibitor was added to the cells 30' before and continued during the bergapten treatment for 4 h. The level of ERα was detected by Western blot with anti-ERα antibody. GAPDH was used as loading control. C: ER + breast cancer cell lysate. Results are representative of four-independent experiments. $*P < 0.05$; $**P < 0.01$ versus untreated cells; $°P < 0.05$ versus bergapten-treated condition

In our study, we wanted to ascertain if bergapten, by depleting ERα, might also affect the expression levels of SMAD proteins. Western Blot analysis performed in MCF-7 and MCF-7/TAM Res cells reveals that, while SMAD3 tends to decrease, SMAD4 protein is maintained upon bergapten treatment, resulting in an increase of the SMAD4/SMAD3 ratio as illustrated in the figure 6 a, b. Furthermore, immunoprecipitation experiments with anti-ERα antibody followed by Immunoblotting with anti-SMAD4 and anti-Poly-Ub antibodies show that ER/SMAD4/Poly-Ub are co-associated in a tripartite complex and that bergapten enhances the amount of SMAD4 and Poly-Ub complexed to ERα (Fig. 7a).

In order to further evaluate the role of SMAD4 in the ER-ubiquitination process we targeted SMAD4 with siRNA. IP experiments of MCF-7 cell lysates with anti-ERα antibody followed by Western Blotting for ERα, showed that SMAD4 knockdown induces a greater retention of ERα, so as to be longer expressed, compared to that one obtained in wild-type cells. This addresses how in the latter circumstance a minor ER-ubiquitination occurs (Fig. 8a, b).

A higher expression of ERα in siRNA SMAD4 MCF-7 cells, compared to wild-type cells, was also confirmed in whole cell lysate by Western Blot (Fig. 8c).

The results confirm that SMAD4 physically interact with ERα and expression of SMAD4 is necessary to mediate the bergapten-induced ER degradation. Taking into account that SMAD4 is a down-stream signaling of TGF-β, we wanted to evaluate the role of TGF-β receptor on ERα protein content both in bergapten-treated and untreated MCF-7 cells. To this aim, we transiently transfected MCF-7 cells with siRNA TGFbeta-RII and we stimulated the cells for short time with the drug to assess the phosphorylation signals down-stream the TGF-β pathway. It is worth to mention how, in this circumstance, as expected, we observed a reduction of SMAD4 and a marked up-regulation of ERα compared to the co

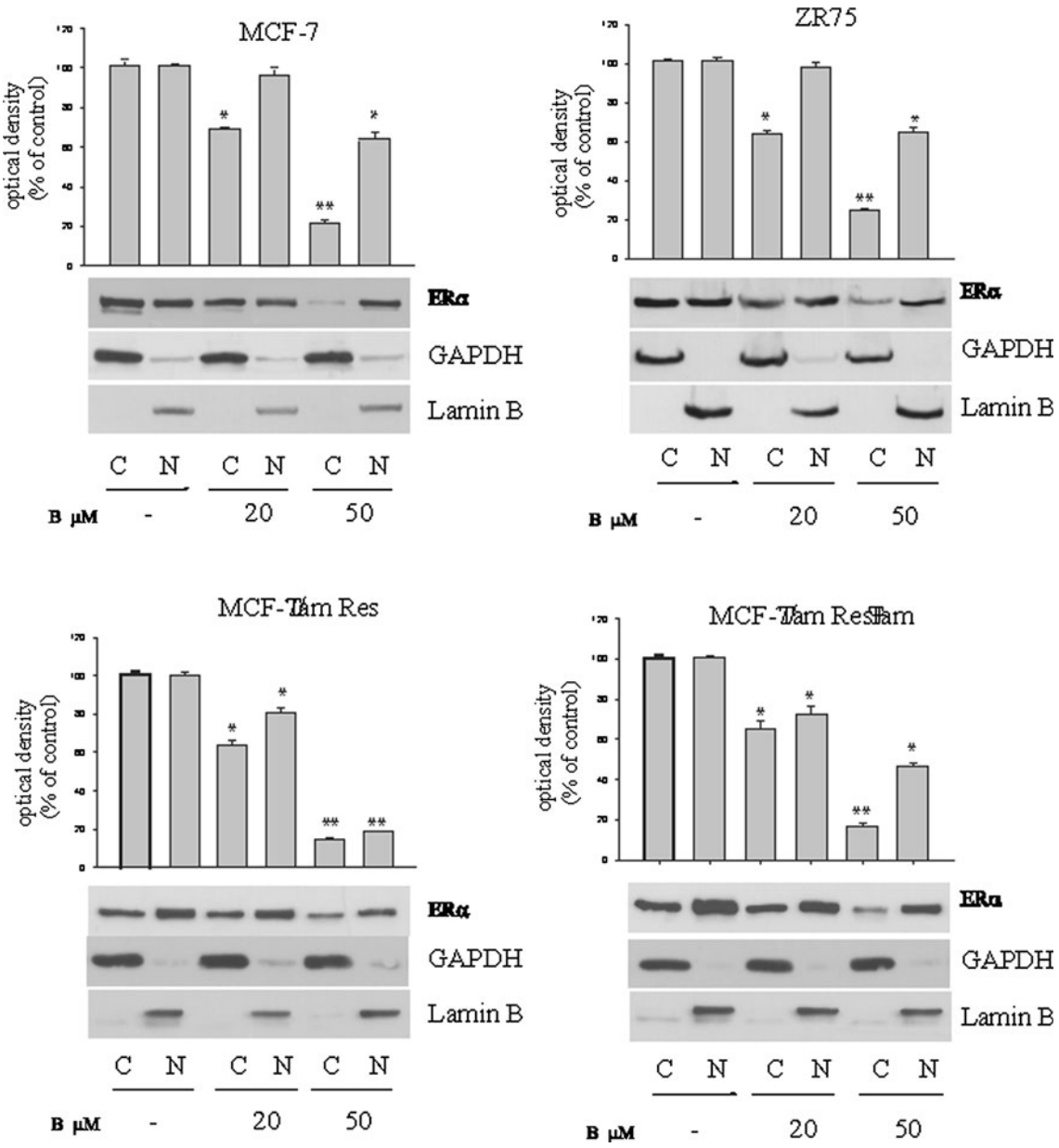


Fig. 5 ER protein in cytosolic and nuclear extracts of breast cancer cells Immunoblot analysis of ERα in cytosolic (*C*) and nuclear (*N*) protein lysates of MCF-7, ZR-75, and MCF-7/Tam Res cells treated for 24 h with bergapten (*B*) as indicated. MCF-7/Tam Res + Tam: cells maintained also with OH-Tamoxifen (T1 μM) during the experimental procedure. Lamin B and GAPDH were used, respectively, as control of nuclear and cytoplasmatic fraction. These results are representative of four- independent experiments. $*P < 0.05$; $**P < 0.01$ versus untreated cells

respective experimental conditions reported in the negative siRNA transfected cells (Fig. 9a).

In addition, in cells bearing siRNA TGF-β RII under bergapten treatment it has been shown a reduction of phospho-SMAD2 and phospho-SMAD3, with respect to scrambled siRNA control (Fig. 9a).

To evaluate the potential involvement of MAPKs in the phosphorylation of SMADs under bergapten treatment, we reprobed the filters for anti-p38 kinase, anti-p-ERK1/2, and for anti-p-JNK.

In these experimental conditions, bergapten was able to up-regulate p-JNK, which appears clearly blunted in the presence of siRNA TGF-β RII, while no substantially modifications were reported under bergapten for p-ERK1/2 and p38MAPK (Fig. 9c).

Indeed, the use of the specific JNK-kinase inhibitor SP600125 reverses the p-SMAD2 and p-SMAD3 activation and leads to an increase of ERα protein (Fig. 9e). Thus, we may conclude that bergapten is able to stimulate TGF-β signaling, which through JNK activation, phosphorylates

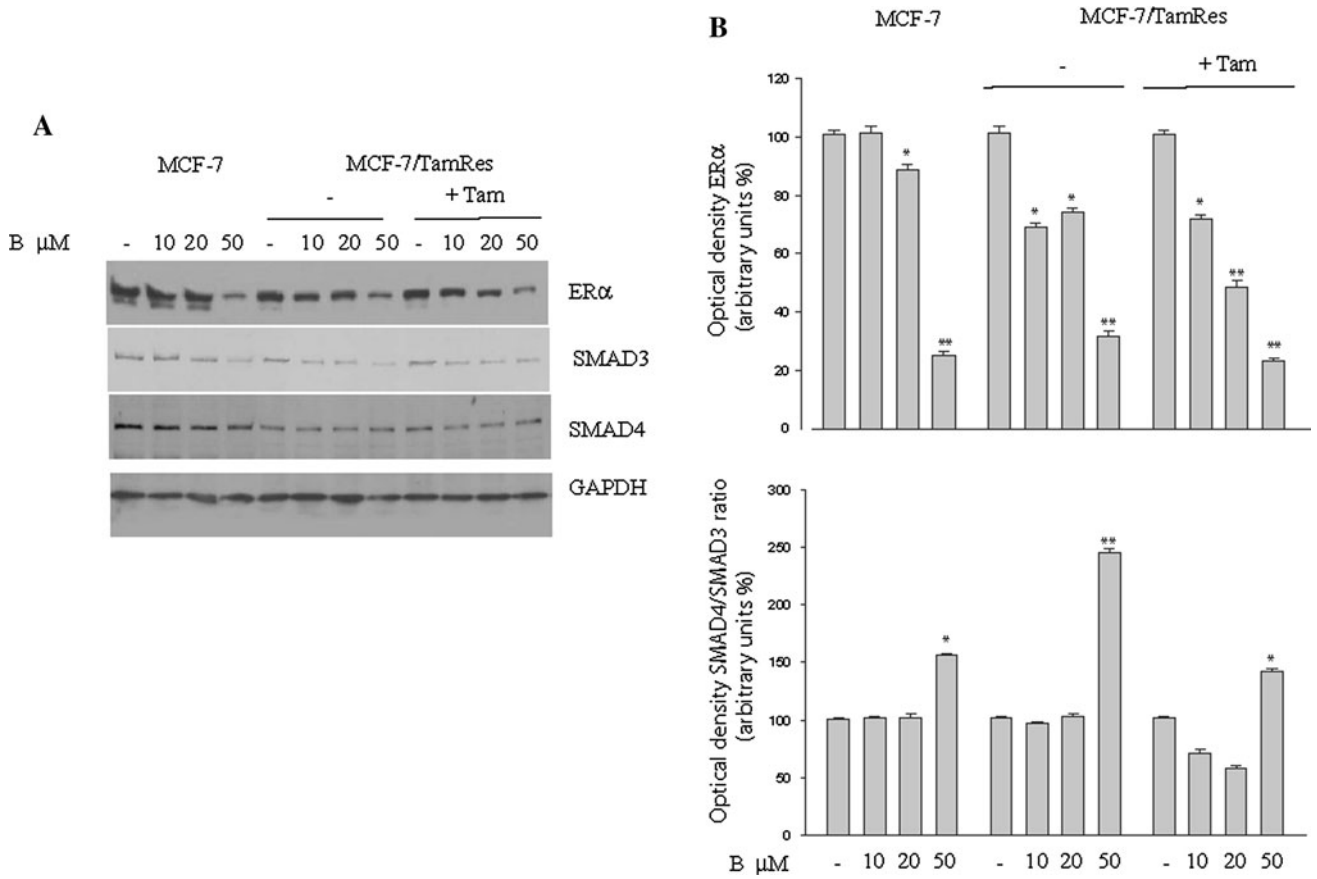


Fig. 6 Immunoblot of ERα and SMAD4/SMAD3 in breast cancer cells. **a** The protein lysates of MCF-7 and MCF-7/Tam Res cells treated for 24 h with different concentrations of bergapten (*B*) as indicated, were immunoblotted with anti-ERα, anti-SMAD3, and anti-SMAD4. GAPDH was used as loading control. MCF-7/Tam Res cells were maintained (+Tam) or not (-Tam) with OH-Tamoxifen (T1 μM) during the experimental procedure. Results are representative of three-independent experiments. **b** Optical density of the ERα and SMAD4/SMAD3 ratio. $*P < 0.05$; $**P < 0.01$ versus untreated cells

SMAD2 and SMAD3. These events lead to an up-regulation of SMAD4 protein, that binds ERα and drives its ubiquitination.

Bergapten influences the anchorage-independent growth induced by Estradiol

We next have evaluated the effects of bergapten on the anchorage-independent growth upon E_2-exposure of MCF-7 and MCF-7/TAM Res clone.

Our data have shown that psoralen is able to antagonize the stimulatory action induced by E_2 on cell growth in soft agar, and, in a higher extent, in Tamoxifen-resistant clone (Fig. 10).

Discussion

ER has become an important target in the treatment of hormones-responsive breast cancer. Unfortunately, most patients initially responding to anti-estrogen therapies develop pharmacological resistance. The potential mechanisms of endocrine resistance are not fully understood, but multifactorial determinants may be involved, such as the growth factor signaling and altered ER regulation.

Therefore, depletion of ERα from breast cancer may give particularly powerful advance to block mitogenic signals, even those coming from the ER/growth factor crosstalk, preventing the development of endocrine resistance.

We showed previously that cumarine-derivate compound: 5-MOP (bergapten) inhibits human breast cancer growth, by increasing p53 and p21 expression, and induces a functional activation of pro-apoptotic response [14]. In this study, we reported that 5-MOP in breast cancer cells is able to down-regulate ERα protein, without affecting mRNA-ERα level, and to decrease estrogen response gene expression such as cyclin D1. This suggests, how, very likely, the down-regulation of ERα upon exposure to bergapten does not involve a transcriptional mechanism. Indeed, the use of MG-132, a proteasome inhibitor,

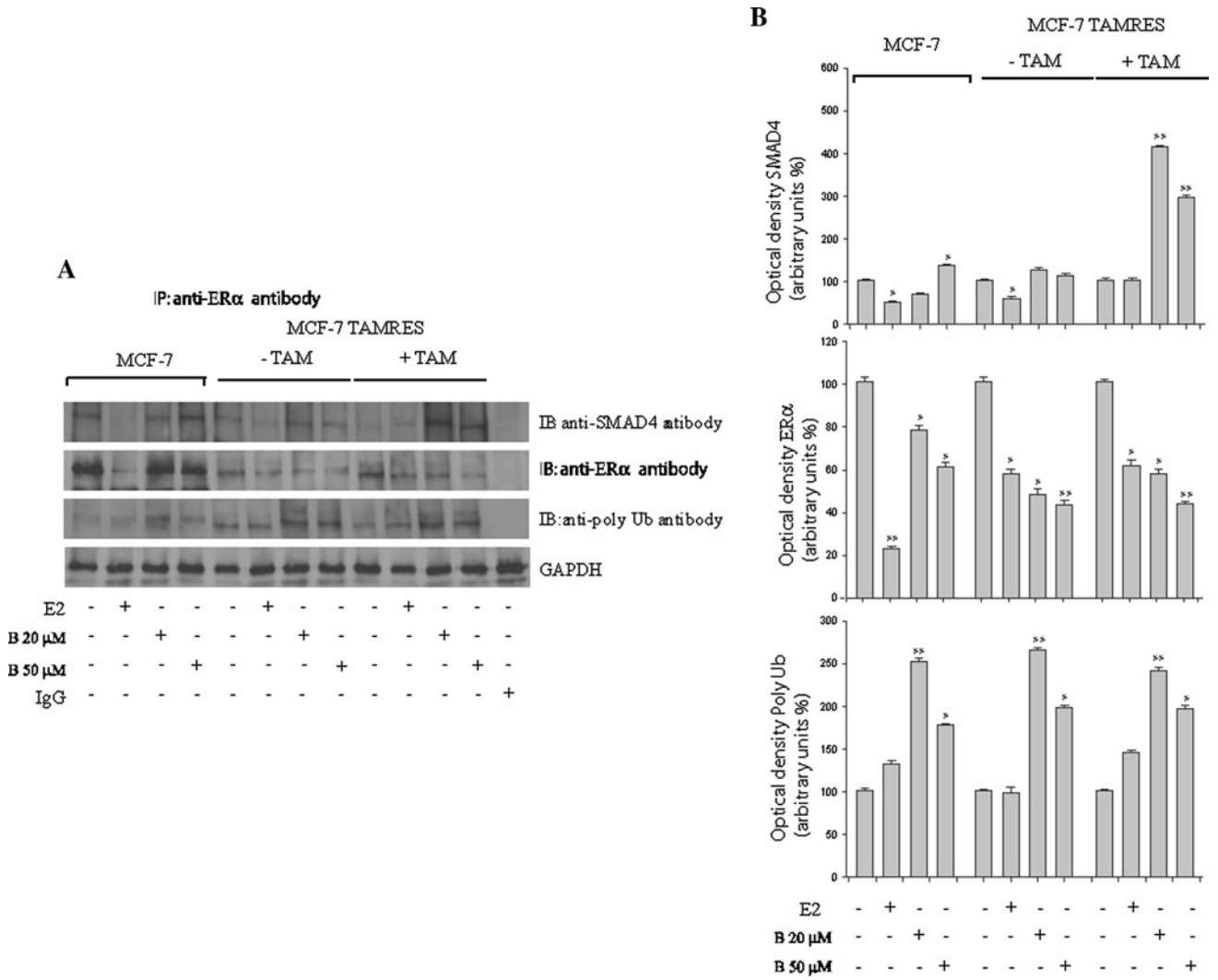


Fig. 7 Co-association between ER Poly-Ub and SMAD4 in breast cancer cells. **a** MCF-7 and MCF-7/Tam Res cells, treated with E_2 10nM and bergapten (*B* 20, 50 μM) for 4 h, were lysated and cellular extracts were immunoprecipitated (*IP*) with anti-ERα antibody, resolved by SDS-PAGE, and immunoblotted (*IB*) with anti-SMAD4, anti-ERα, and anti-ubiquitin antibodies. Prior the immunoprecipitation experiment an aliquote of the lysate corresponding to 30 μg of protein was loaded to determine GAPDH, as loading control. MCF-7/Tam Res cells were maintained (+Tam) or not (-Tam) with OH-Tamoxifen (T1 μM) during the experimental procedure. IgG: negative control of Immunoprecipitation experiment. Results are representative of three-independent experiments. **b** Optical density of the SMAD4, ERα, and Poly Ubiquitin. $*P < 0.05$; $**P < 0.01$ versus untreated cells

reverses the down-regulation of ER under bergapten, addressing the effect of such molecule in enhancing the degradative pathway of the receptor protein. It is worth to mention how these effects were also reproduced in MDA-MB 231 cells overexpressing ectopically the ER alpha through transient transfection experiments. Western Blot analysis of ERα performed respectively in the nuclear and cytosolic cell lysates evidences that under bergapten treatment the decrease of ERα occurs prevalently in the cytoplasmatic fraction.

It is extremely intriguing to observe that the above-reported effect of bergapten on ERα degradation is reproduced in breast cancer Tamoxifen-resistant cells, where we have reported a marked decrease of cell proliferation, evidencing an efficient response of these cells to the effect of psoralen.

It is worth to mention how the enhanced anchorage-independent growth upon E_2 exposure is drastically attenuated in the presence of the combined exposure of bergapten, emphasizing, furthermore, the boostering action of the psoralen on ERα-degradation in breast cancer cells.

These results reflect the anti-tumoral properties of the molecule and call other published data demonstrating how some coumarin derivatives can be potent inhibitors of proliferation of aromatase and ER positive breast cancer cells [25, 26].

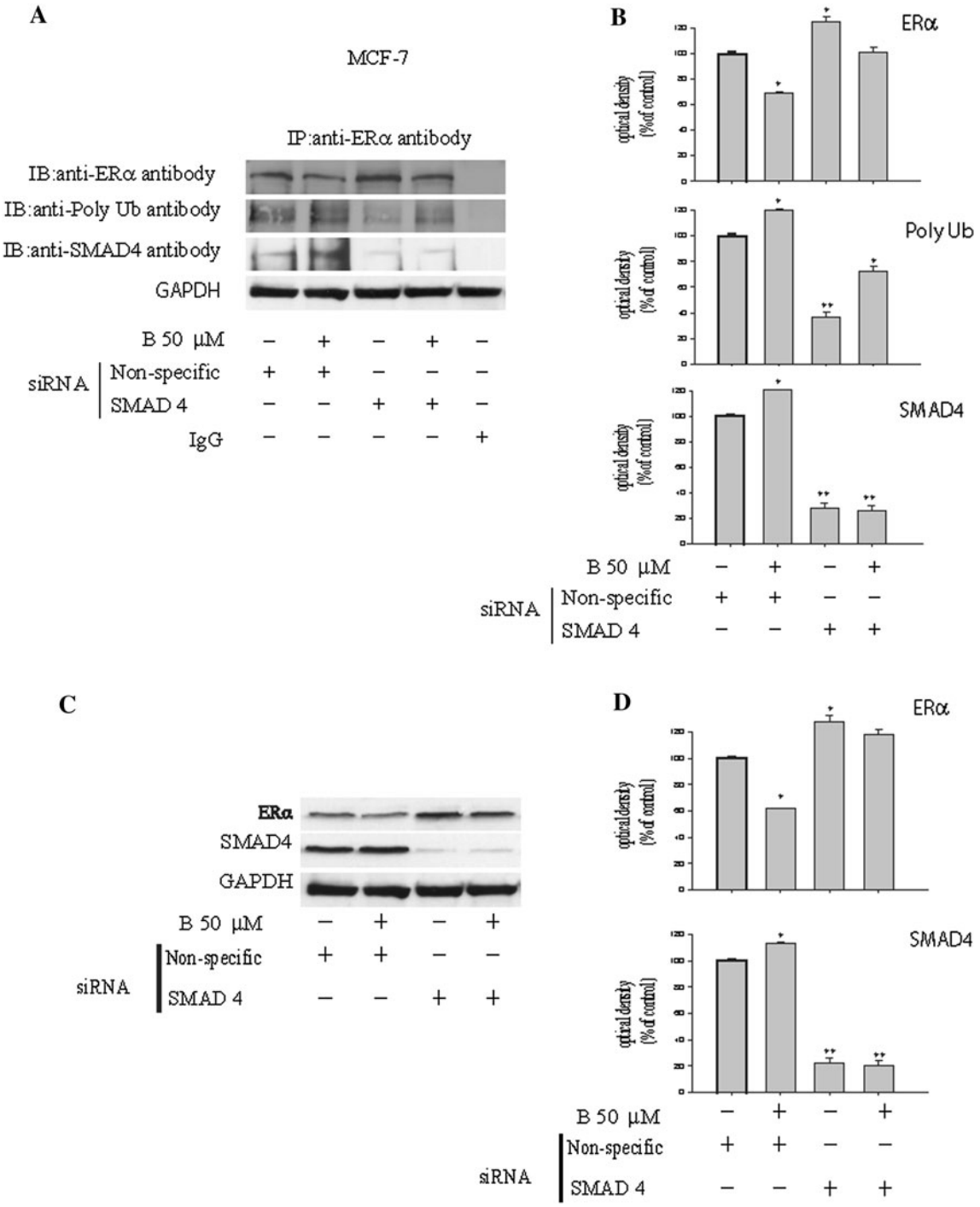


Fig. 8 IP experiment in MCF-7 cells bearing silencing of SMAD4. **a** MCF-7 cells were transfected with either non-targeting siRNA- or SMAD4-specific siRNA. The cells were then untreated or were treated with 50 μM bergapten for 4 h. Cellular extracts were underwent to immunoprecipitation (*IP*) experiments with anti-ERα antibody, resolved by SDS-PAGE, and immunoblotted (*IB*) with anti-ERα, anti-poly-ubiquitin and anti-SMAD4 antibodies. IgG: negative control of Immunoprecipitation experiment. **b, d** Optical density of the proteins. **c** Prior the immunoprecipitation experiment an aliquote of the lysates corresponding to 30 μg of protein was loaded to determine the ERα and SMAD4 protein levels. GAPDH is taken as a loading control. Results are representative of three-independent experiments. $*P < 0.05$; $**P < 0.01$ versus untreated cells

The amount of ERα protein in the cell is a major determinant of the regulation of its own transcriptional activity. Steady state of ERα level is maintained by a dynamic balance between protein synthesis and breakdown. Many factors regulate the endogenous levels of ERα in the cells and this, in turn, influences the

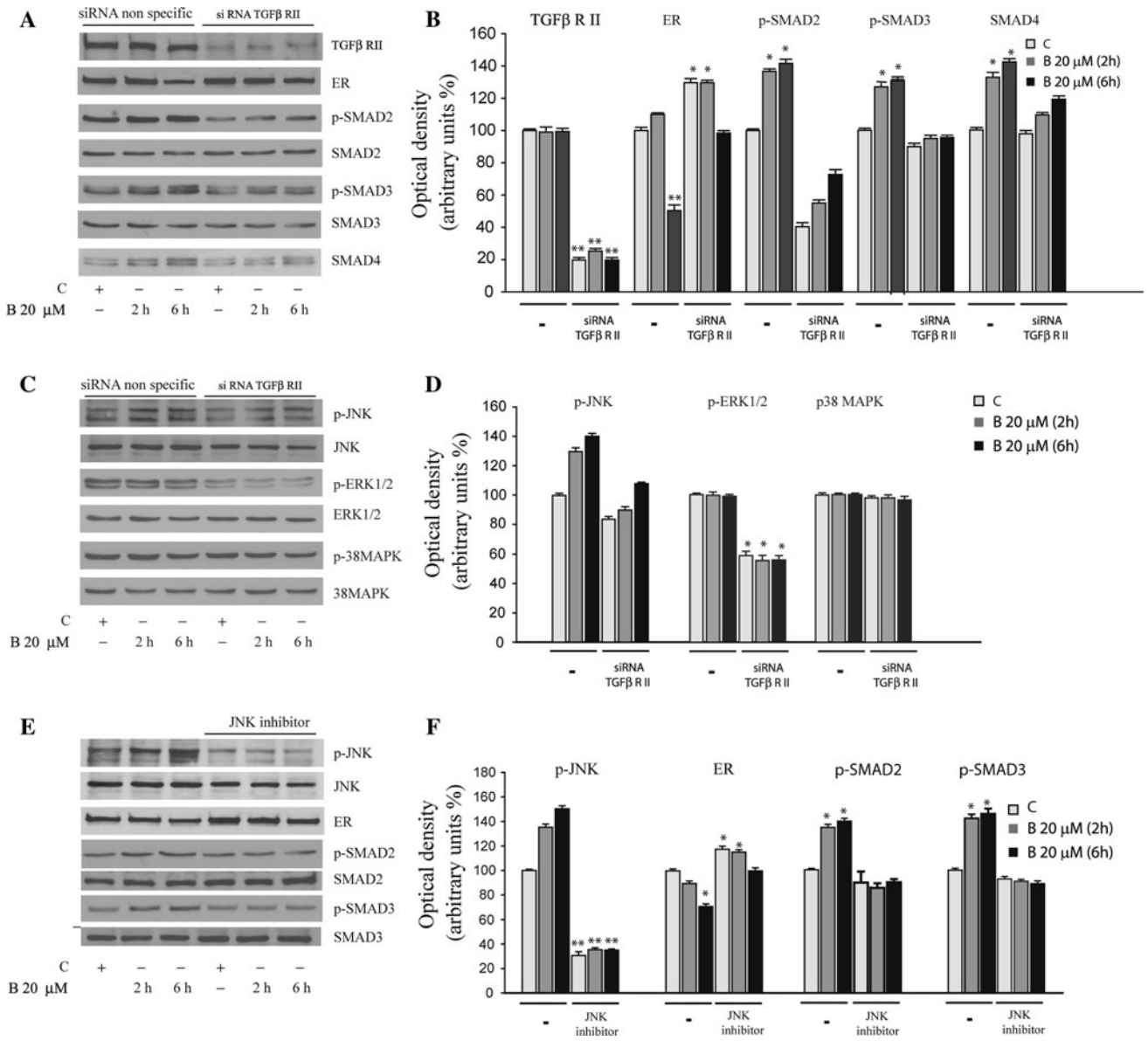


Fig. 9 TGF-β signaling proteins evaluated in MCF-7 cells underwent bergapten treatment MCF-7 cells were transfected with either non-targeting siRNA- or TGF-β RII-specific siRNA. The cells were untreated (*C*) or treated with bergapten (*B*) 20 μM for 2 and 6 h. **a** Immunoblots of TGF-β RII, ER, phospho-SMAD2, phospho-SMAD3, total SMAD2, total SMAD3, and SMAD4 are indicated. **c** Immunoblots of phospho-JNK, JNK, phospho-ERK1/2, ERK1/2, phospho-p38MAPK, 38MAPK. **e** Immunoblots of phospho-JNK, JNK, phospho-SMAD2, phospho-SMAD3, total SMAD2, total SMAD3, and ERα in MCF-7 cells untreated or treated with bergapten and in cells pre-treated for 1 h with the JNK-kinase inhibitor SP600125 (10 μM). **b**, **d**, **f** Densitometric analysis of the corresponding proteins. $*P < 0.05$, $**P < 0.01$ versus untreated cells

interactions of the receptor protein with specific coactivating or corepressing transcription elements. Control of ERα expression is an important mean to modulate cellular responses to growth.

Several observations have documented the crosstalk between ERα-activity and TGF-β signaling. Indeed, previous reports have demonstrated how ERα is able to physically interact with SMAD2, SMAD3, and SMAD4 and to abrogate TGF-β signaling cascade [16, 17, 27].

On the other hand, while TGF-β signaling has been demonstrated to stimulate ERα transcriptional activity, the complex of SMAD3 and SMAD4 inhibits its activity [16, 28].

In our study, we proved that in breast cancer-treated cells the down-regulation of ERα coincides with the increase of SMAD4/SMAD3 ratio, implying a functional relationship between SMAD4 and ER.

Indeed, immunoprecipitation assay reveals that ERα/SMAD4 and poly-ubiquitine co-associated in a tripartite complex.

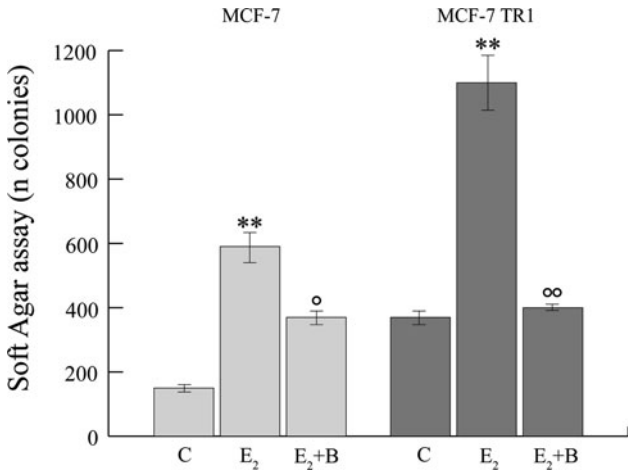


Fig. 10 Soft agar growth assay of MCF- and MCF-7/TAM Res cells MCF-7 and MCF-7/Tamoxifen-resistant cells (MCF-7TR1) were seeded (5,000/well) in 0.35 % agarose and then treated with vehicle (*C*), E_2 (10 nM), bergapten (*B* 50 μM). Cells were allowed to grow for 14 days and the number of colonies ≥50 μm were quantified and the results were graphed. $^{**}P < 0.01$ versus untreated cells; $^{\circ}P < 0.05$; $^{\circ\circ}P < 0.01$ versus E2 condition

As expected, the amount of ERα in the immunoprecipitate is lowered by the treatment, while the levels of SMAD4 and poly-ubiquitine present in the complex are markedly higher, addressing the important role of the latter two proteins in sustaining ERα-degradation.

The crucial role played by SMAD4 in ER-depletion emerges from the evidence that in the presence of its silencing the endogenous ERα is enhanced, being better preserved in both control and treated-cells, than in wild-type cells.

The phenomenon was also observed in MCF-7 cells silenced for TGF-*β* RII. In the same vein, IP experiments confirmed that SMAD4 silenced in MCF-7 cells lowers the ubiquitination of ERα, thereby highlighting a negative relationship between the two proteins. In addition, in the presence of the JNK-specific inhibitor the activation of SMAD proteins was partially reversed, while concomitantly the ERα content was enhanced.

These findings suggest that SMAD4 protein, downstream the TGF-*β* signaling, is crucially involved in the degradative process of the receptor.

In addition, the observation that SMAD4 is up-regulated following bergapten treatment, highlights a new molecular mechanism through which psoralen might affect ERα stability and thereby regulate breast cancer cell progression.

Results in the same direction, have been published regarding the involvement of proteasomal degradation of ERα by TGF-*β* in breast cancer cells. So, all this brings again to a functional interplay between TGF-*β* and ER-signaling in breast cancer [29].

Finally, apart from the apoptotic function of bergapten, as we previously documented in breast cancer cells [14], the present article has highlighted a novel role of the molecule in regulating ERα-protein stability via the ubiquitin–proteasome pathway.

This study, once again, draws attention to the anti-cancer properties of psoralen, emphasizing how the molecule can unfold its functional activity even in the absence of photoactivation.

Based on what we just said, we propose that bergapten may efficiently act as a natural anti-tumoral agent, able to deplete ERα from breast cancer tamoxifen-sensitive and resistant cells thereby preventing crosstalk between the receptor and growth factor mitogenic signaling.

Acknowledgments This study was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC 2011, grant IG 11595) and PRIN grant 2008; we thank Dr. P. Cicirelli for technical assistance, University of Calabria, Cosenza.

Conflict of interest The authors declare no conflict of interests.

References

1. Furr BJ, Jordan VC (1984) The pharmacology and clinical uses of tamoxifen. Pharmacol Ther 2:127–205
2. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, Mc-Cue BL, Wakeling AE, McClelland RA, Manning DL, Nicholson RI (1995) Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. J Natl Cancer Inst 87:746–750
3. Johnston SR, Head J, Pancholi S, Detre S, Martin LA, Smith IE, Dowsett M (2003) Integration of signal transduction inhibitors with endocrine therapy: an approach to overcoming hormone resistance in breast cancer. Clin Cancer Res 9(1 Pt 2):524S–532S
4. Morrow M, Jordan VC (1993) Molecular mechanisms of resistance to tamoxifen therapy in breast cancer. Arch Surg 128: 1187–1191
5. Zhang Y, Moerkens M, Ramaiahgari S, de Bont H, Price L, Meerman J, van de Water B (2011) Elevated insulin-like growth factor 1 receptor signaling induces antiestrogen resistance through the MAPK/ERK and PI3 K/Akt signaling routes. Breast Cancer Res 3:R52
6. Ma T, Liu L, Xue H, Li L, Han C, Wang L, Chen Z, Liu G (2008) Chemical library and structure-activity relationships of 11-demethyl-12-oxo calanolide a analogues as anti-HIV-1 agents. J Med Chem 5:1432–1446
7. Kidane AG, Salacinski H, Tiwari A, Bruckdorfer KR, Seifalian AM (2004) Anticoagulant and antiplatelet agents. Their clinical and device application (s) together with usages to engineer surface. Biomacromolecules 5:798–813
8. Appendino G, Percalli E, Fuzzati N, Arnoldi L, Stavri M, Gibbons S, Ballero M, Maxia AJ (2004) Antimycobacterial coumarins from the sardinian giant fennel (*Ferula communis*). J Nat Prod 12:2108–2110
9. Kontogiorgis CA, Hadjipavlou LD (2004) Synthesis and biological evaluation of novel coumarin derivatives with a 7-azomethine linkage. Bioorg Med Chem Lett 3:611–614

10. Sashidhara KV, Rosaiah JN, Kumar A, Bhatia G, Khanna AK (2010) Synthesis of novel benzocoumarin derivatives as lipid lowering agents. Bioorg Med Chem Lett 10:3065–3069
11. Kostova I (2005) Synthetic and natural coumarins as cytotoxic agents. Curr Med Chem Anticancer Agents 1:29–46
12. Musa MA, Cooperwood JS, Khan MO (2008) A review of coumarin derivatives in pharmacotherapy of breast cancer. Curr Med Chem 26:2664–2679
13. Sashidhara KV, Kumar A, Kumar M, Sarkar J, Sinha S (2010) Synthesis and in vitro evaluation of novel coumarin-chalcone hybrids as potential anticancer agents. Bioorg Med Chem Lett 24:7205–7211
14. Panno ML, Giordano F, Palma MG, Bartella V, Rago V, Maggiolini M, Sisci D, Lanzino M, De Amicis F, Andò S (2009) Evidence that bergapten, independently of its photoactivation, enhances p53 gene expression and induces apoptosis in human breast cancer cells. Curr Cancer Drug Targets 4:469–481
15. Arteaga CL, Tandon AK, Von Hoff DD, Osborne CK (1988) Transforming growth factor beta: potential autocrine growth inhibitor of estrogen receptor-negative human breast cancer cells. Cancer Res 48:3898–3904
16. Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F (2001) Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3. J Biol Chem 276:42908–42914
17. Ito I, Hanyu A, Wayama M, Goto N, Katsuno Y, Kawasaki S, Nakajima Y, Kajiro M, Komatsu Y, Fujimura A, Hirota R, Murayama A, Kimura K, Imamura T, Yanagisawa J (2010) Estrogen inhibits transforming growth factor beta signaling by promoting Smad2/3 degradation. J Biol Chem 19:14747–14755
18. D Amicis F, Giordano F, Vivacqua A, Pellegrino M, Panno ML, Tramontano D, Fuqua SA, Andò (2011) Resveratrol through NF-Y/p53/Sin3/HDAC1 complex phosphorylation, inhibits estrogen receptor alpha gene expression via p38MAPK/CK2 signaling in human breast cancer cells. FASEB J 25(10):3695–3707
19. Bartella V, Rizza P, Barone I, Zito D, Giordano F, Giordano C, Catalano S, Mauro L, Sisci D, Panno ML, Fuqua SAW, Andò S (2012) Estrogen receptor beta binds Sp1 and recruits a corepressor complex to the estrogen receptor alpha gene promoter. Breast Cancer Res Treat 134(2):569–581
20. Guido C, Panza S, Santoro M, Avena P, Panno ML, Perrotta I, Giordano F, Casaburi I, Catalano S, De Amicis F, Sotgia F, Lisanti MP, Andò S, Aquila S (2012) Estrogen receptor beta (ERβ) produces autophagy and necroptosis in human seminoma cell line through the binding of the Sp1 on the phosphatase and tensin homolog deleted from chromosome 10 (PTEN) promoter gene. Cell Cycle 11:2911–2921
21. Sisci D, Middea E, Morelli C, Lanzino M, Aquila S, Rizza P, Catalano S, Casaburi I, Maggiolini M, Andò S (2010) 17 beta-Estradiol enhances alpha(5) integrin subunit gene expression through ER alpha-Sp1 interaction and reduces cell motility and invasion of ER alpha-positivbreast cancer cells. Breast Cancer Res Treat 124:63–770
22. Panno ML, Giordano F, Mastroianni F, Morelli C, Brunelli E, Palma MG, Pellegrino M, Aquila S, Miglietta A, Mauro L, Bonofiglio D, Andò S (2006) Evidence that low doses of Taxol enhance the functional transactivatory properties of p53 on p21 waf promoter in MCF-7 breast cancer cells. FEBS Lett 580: 2371–2380
23. Catalano S, Giordano C, Rizza P, Gu G, Barone I, Bonofiglio D, Giordano f, malivindi R, Faccione D, lanzino M, De Amicis F, Andò S (2009) Evidence that leptin through STAT and CREB signalling enhances cyclin D1 expression and promotes human endometrial cancer proliferation. J Cell Physiol 218(3):490–500
24. Giordano C, Cui Y, Barone I, Andò S, Mancini MA, Berno V, Fuqua SWA (2010) Growth factor-induced resistance to tamoxifen is associated with a mutation of estrogen receptor α and its phosphorylation at serine 305. Breast Cancer Res Treat 119: 71–85
25. Chen S, Cho M, Karlsberq K, Zhou D, Yuan YC (2004) Biochemical and biological characterization of a novel anti-aromatase coumarin derivative. J Biol Chem 46:48071–48078
26. Macaulay VM, Bates PJ, McLean MJ, Rowlands MG, Jenkins TC, Ashworth A, Neidle S (1995) Inhibition of aromatase expression by a psoralen-linked triplex-forming oligonucleotide targeted to a coding sequence. FEBS Lett 372:222–228
27. Wu L, Wu Y, Gathings B, Wan M, Li X, Grizzle W, Liu Z, Lu C, Mao Z, Cao X (2003) Smad4 as a transcription corepressor for estrogen receptor alpha. J Biol Chem 17:15192–15200
28. Ren Y, Wu L, Frost AR, Grizzle W, Cao X, Wan M (2009) Dual effects of TGF-beta on ER alpha-mediated estrogenic transcriptional activity in breast cancer. Mol cancer 8:1–11
29. Petrel TA, Brueggemeier RW (2003) Increased proteasome-dependent degradation of estrogen receptor-alpha by TGF-beta1 in breast cancer cell lines. J Cell Biochem 88:181–190

Breast Cancer Res Treat
DOI 10.1007/s10549-012-2090-9

PRECLINICAL STUDY

Estrogen receptor beta binds Sp1 and recruits a corepressor complex to the estrogen receptor alpha gene promoter

V. Bartella · P. Rizza · I. Barone · D. Zito · F. Giordano · C. Giordano · S. Catalano · L. Mauro · D. Sisci · M. L. Panno · S. A. W. Fuqua · S. Andò

Received: 2 May 2012 / Accepted: 3 May 2012

Abstract Human estrogen receptors alpha and beta are crucially involved in the regulation of mammary growth and development. Normal breast tissues display a relative higher expression of ER beta than ER alpha, which drastically changes during breast tumorogenesis. Thus, it is reasonable to suggest that a dysregulation of the two estrogen receptor subtypes may induce breast cancer development. However, the molecular mechanisms underlying the potential opposing roles played by the two estrogen receptors on tumor cell growth remain to be elucidated. In the present study, we have demonstrated that ER beta overexpression in breast cancer cells decreases cell proliferation and down-regulates ER alpha mRNA and protein content, along with a concomitant repression of estrogen-regulated genes. Transient transfection experiments, using a vector containing the human ER alpha promoter region, showed that elevated levels of ER beta down-regulated basal ER alpha promoter activity. Furthermore, site-directed mutagenesis and deletion analysis revealed that the proximal GC-rich motifs at −223 and −214 are critical for the ER beta-induced ER alpha down-regulation in breast cancer cells. This occurred through ER beta-Sp1 protein–protein interactions within the ER alpha promoter region and the recruitment of a corepressor complex containing the nuclear receptor corepressor NCoR, accompanied by hypoacetylation of histone H4 and displacement of RNA-polymerase II. Silencing of NCoR gene expression by RNA interference reversed the down-regulatory effects of ER beta on ER alpha gene expression and cell proliferation. Our results provide evidence for a novel mechanism by which overexpression of ER beta through NCoR is able to down regulate ER alpha gene expression, thus blocking ER alpha's driving role on breast cancer cell growth.

Keywords Breast cancer · Estrogen receptor alpha · Estrogen receptor beta · NCoR corepressor

V. Bartella and P. Rizza have contributed equally to this study.

V. Bartella · D. Zito · S. Catalano · D. Sisci
Department of Pharmaco-Biology, University of Calabria, Via P. Bucci, 87036 Arcavacata di Rende, CS, Italy

P. Rizza · I. Barone · F. Giordano · L. Mauro · M. L. Panno · S. Andò (✉)
Department of Cellular Biology, Faculty of Pharmacy, Nutritional and Health Sciences, University of Calabria, Via P. Bucci, 87036 Arcavacata di Rende, CS, Italy
e-mail: sebastiano.ando@unical.it

I. Barone · C. Giordano · S. Catalano · D. Sisci · S. Andò
Centro Sanitario, University of Calabria, Arcavacata di Rende, CS, Italy

S. A. W. Fuqua
Lester and Sue Smith Breast Center and Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX, USA

Introduction

Estrogens play an important role in mammary gland development, but they are also involved in mammary carcinogenesis [1, 2]. These steroid hormones exert their biological effects via interaction with the two different isoforms of estrogen receptors, ER alpha, and ER beta, each encoded by unique genes, but with a common structural and functional organization. Binding of estrogen ligands to ERs in the nucleus results in receptor phosphorylation, dimerization, and recruitment of specific coregulator proteins, termed coactivators, which enhance binding of the receptor complex to promoter regions of target genes known as

Published online: 24 May 2012

estrogen response elements (EREs) and augment receptor's transcriptional activity [3, 4]. In addition to these genomic actions, ERs, located in the cell membrane or cytoplasm, may initiate rapid cellular signaling, and are involved in intricate networks of crosstalk with growth factor pathways [5–7]. In addition, ERs may bind to DNA in a non classical way through its interaction with other transcription factors. For instance, ER alpha/Sp1, ER alpha/AP-1, and ER beta/Sp-1, ER beta/AP-1-mediated gene regulation through binding GC-rich and AP-1 motifs have been extensively investigated [8–12]. Indeed, the latter indirect mechanisms may occur in the absence of natural ligand, in a cell type and gene dependent context [12–15], and may also involve recruitment of corepressors such as NCoR and SMRT to inhibit basal cell transcription machinery [16, 17].

Both ERs subtypes are expressed in human mammary tissue with only 7–10 % of the epithelial cells expressing ER alpha but 80–85 % expressing ER beta [18–20]. In contrast, expression of ER alpha is increased in breast cancer cells, where it acts as a mediator of cell proliferation and has been shown to be an effective therapeutic target for decades [21]. The role of ER beta in breast cancer is less clear and its prognostic value is still under debate. It is estimated that ER beta is expressed in approximately one-half of human primary breast cancers, but its expression is lost during breast cancer progression, most likely due to promoter hypermethylation [22]. Moreover, ER beta protein levels have been linked to good prognosis, prolonged disease-free survival and response to anti-estrogen treatment [23, 24]. Many cell-based studies suggest that ER beta acts as a negative modulator of ER alpha action and can negatively regulate breast cancer proliferation [25–27]. Indeed, inducible expression of ER beta in ER alpha-positive breast cancer cells inhibited estrogen-stimulated proliferation, tumor angiogenesis, and growth in xenograft experiments [28, 29]. Overexpression of ER beta or ER beta cx isoforms also decreased ER alpha transcriptional activity concomitantly with a reduced expression of estrogen-regulated genes, such as vascular endothelial growth factor (VEGF) or progesterone receptor (PR) [30–32]. In ER-negative cells ectopically expressing the two ERs, ER beta reduced the sensitivity of the cells to estrogen treatment on growth and inhibited cyclin D1 gene activation [33–36].

Given the markedly enhanced ratio of ER alpha/ER beta in early breast cancers and the opposite roles of the two ERs in regulating cell proliferation and differentiation, it is imperative to dissect the molecular mechanisms underlying the dysregulation of these processes in cancer cells. Therefore, the aim of this study was to investigate if ER beta may play a direct inhibitory role on ER alpha expression and gene promoter activity. Here, we demonstrate that ER beta through its interaction with Sp1 protein recruits NCoR corepressor in the promoter region upstream the transcription start site of ER alpha gene, thus down-regulating its expression.

Materials and methods

Reagents and antibodies

DEMF-12 Ham and DMEM were purchased from Invitrogen (Carlsbad, CA, USA), FuGENE 6, Sp-1 human recombinant protein from Promega (Madison, WI, USA). The RETROscript kit from Ambion (Austin, TX, USA). MTT, IGF-1 by Sigma (Milan, Italy). Antibodies against ER alpha/ER beta/IRS-1/cyclinD1/pS2/GAPDH/NCoR/Sp-1/SMRT/AcH4/PolymeraseII were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). SYBR Green by Biosystems (Forster City, CA, USA).

Cell cultures

MCF-7 and ZR75 breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained as recommended.

Plasmids

The plasmids containing the human ER alpha promoter region or its deletions (E:p − 4100/+ 212; D:p − 2769/+ 212; C:p − 1000/+ 212, A:p − 245/+ 212) were provided by Prof Fuqua [37]. Deletion of Sp-1 site in C plasmid was generated by PCR using following primers: forward 5′-GCGGTACCCGAAAGATCGAGTTGTAGGAC-3′ and reverse 5′-CGCTCGAGTTATATAGGGAAGACTGGGCTTAAAATA-3′. The amplified DNA fragment was digested with Kpn I and Xho I and ligated into pGL3-basic vector. The sequence was confirmed by nucleotide sequence analysis. The plasmid encoding the human ER beta was a gift from JA Gustafsson (Karolinska Institute, Sweden).

Site-directed mutagenesis

The mutation of the two half-ERE sites in C plasmid was created by site-directed mutagenesis using QuickChange kit (Stratagene, La Jolla, CA). The mutagenic primers were: forward 5′-CATAATTGCCTTTGCTTTGGTTCGTGGTTTGAGGTTATGTTTGGTATGAAAAG-3′, 5′-CGTGACCTGAGGTTATGTTTGGTATGAAAAGACTACATTTTATATTCAGTTTTCTGAAG-3′, and reverse 5′-CTTTTCATACCAAACATAACCTCAAACCACGAACCAAAGCTTTGGCAATTATG-3′, 5′- CTTCAGAAAACTGAATATAAAATGTAGTCTTTTCATACCAAACATAACCTCAG

GTCACG-3′. Mutation was confirmed by DNA sequencing.

Western blot analysis

Equal amounts of cell extracts were subjected to SDS-PAGE, as described [38]. Blots are representative of at least three independent experiments.

RT-PCR and qRT-PCR

ER alpha/ER beta/IRS-1/pS2/CyclinD1/NCoR/GAPDH gene expressions were evaluated by RT-PCR. cDNAs obtained were amplified using the following primers: forward 5′-AGATCCAAGGGAACGAGCT-3′ and reverse 5′-TTCTCCAGGTAGTAGGGCA-3′ (ER alpha); forward 5′-CCTTCCTCCTATGTAGACAGC-3′ and reverse 5′-TCTCTCTGTTTACAGGTAAGG T-3′ (ER beta); forward 5′-AGGATATTTAATTTGCCTCGGG-3′ and reverse 5′-AAGCGTTTGTGCATGCTCTTG-3′ (IRS-1); forward 5′-TTCTATCCTAATACCATCGACG-3′ and reverse 5′-TTTGAGTAGTCAAAGTCAGAGC-3′ (pS2); forward 5′-TCTAAGATGAAGGAGACCATC-3′ and reverse 5′-GCGGTAGTAGGACAGGAAGTTGTT-3′ (CyclinD1); forward 5′-GCCACTGTATAACCAGCCAT-3′ and reverse 5′-CCTCCATAAGCCCATTCATG-3′ (NCoR); forward 5′-GACAACTTTGGTATCGTGGA-3′ and reverse 5′-TACCAGGAAATGAGCTTGAC-3′ (GAPDH). ER alpha gene expression was also evaluated by Real-time PCR. Primers used for the amplification were: forward 5′-CACCATTGATAAAAACAGGAGGAA-3′and reverse 5′-CTCCCTCCTCTTCGGTCTTTTC-3′ (ER alpha); forward 5′-CCCACTCCTCCACCTTTGAC3′ and reverse 5′-TGTTGCTGTAGCCAAATTCGTT-3′ (GAPDH). The relative gene expression levels were calculated as described [14].

Transient transfection assays

MCF-7 cells were transfected using FuGENE6 reagent with the indicated constructs for 16 h. Luciferase activities were assayed as described [38].

Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described [39]. The DNA sequences used as probe or as cold competitor are the following: 5′-TCGTGCGCCCCCGCCCCCTGGCCGTG-3′, 5′- CACGGCCAGGGGGCGGGGGCGCACGA-3′ (Sp-1); 5′-TCGTGCGCCCCATACCCCTGGCCGTG-3′, 5′-CACGGCCAGGGGTATGGGGCGCACGA-3 mutated Sp-1. Probe generation and the protein-binding reactions were carried out as described [38]. The reaction mixture was incubated with specific antibodies at 4 °C for 12 h before addition of labeled probe.

RNA interference

MCF-7 cells were co-transfected with an empty vector or an ER beta expression vector and RNA duplex of stealth RNAi-targeted human NCoR mRNA sequence 5′-UUGUUUGGCUCUGGAGACCUCUUGC-3′ or with a stealth RNAi-negative control using Lipofectamine2000 as recommended.

ChIP and Re-ChIP assays

The DNA/protein complexes were extracted as previously described [38]. The precleared chromatin was immunoprecipitated with anti-Sp-1/NCoR/SMRT/AcH4/PoymeraseII antibodies. The anti-Sp-1 immunoprecipitated samples were re-immunoprecipitated with an anti-NCoR or anti-ER beta antibodies. Sample and input DNA (5 µl) were used for PCR amplification with the following primers flanking Sp-1 sequence present in the ER alpha promoter region: 5′-GCACATAAGGCAGCACATTA-3′ (forward), and 5′-TGGCTTAAACATCACTCCAG-3′ (reverse). In another set of experiments, each sample and input DNA (5 µl) were used for real-time PCR using the following primers: 5′-TCGTGCGCCCCCGCCCCCTGCCCGTG-3′ and 5′-CCAAAGAGCAGCTTCCCTGA-3′. Real-time PCR was performed as described above. Final results were calculated using ΔΔCt method, using input Ct values instead of the GAPDH mRNA. The basal sample was used as calibrator.

Cell proliferation assay

Cell proliferation was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan (MTT) assay as described [40]. Data are representative of three independent experiments, performed in triplicate.

Three-dimensional spheroid culture assays

MCF-7 cells plated in 2 % agar-coated plates were transfected as indicated and treated or not with IGF-1. After 48 h, cultures were photographed using a phase-contrast microscope (Olympus, Milan, Italy). Aggregation extent and cell numbers were evaluated as reported [15]. Data represent three independent experiments, performed in triplicate.

Statistical analysis

Data were analyzed for statistical significance using a two-tailed student's Test, performed by Graph Pad Prism 4.

Results

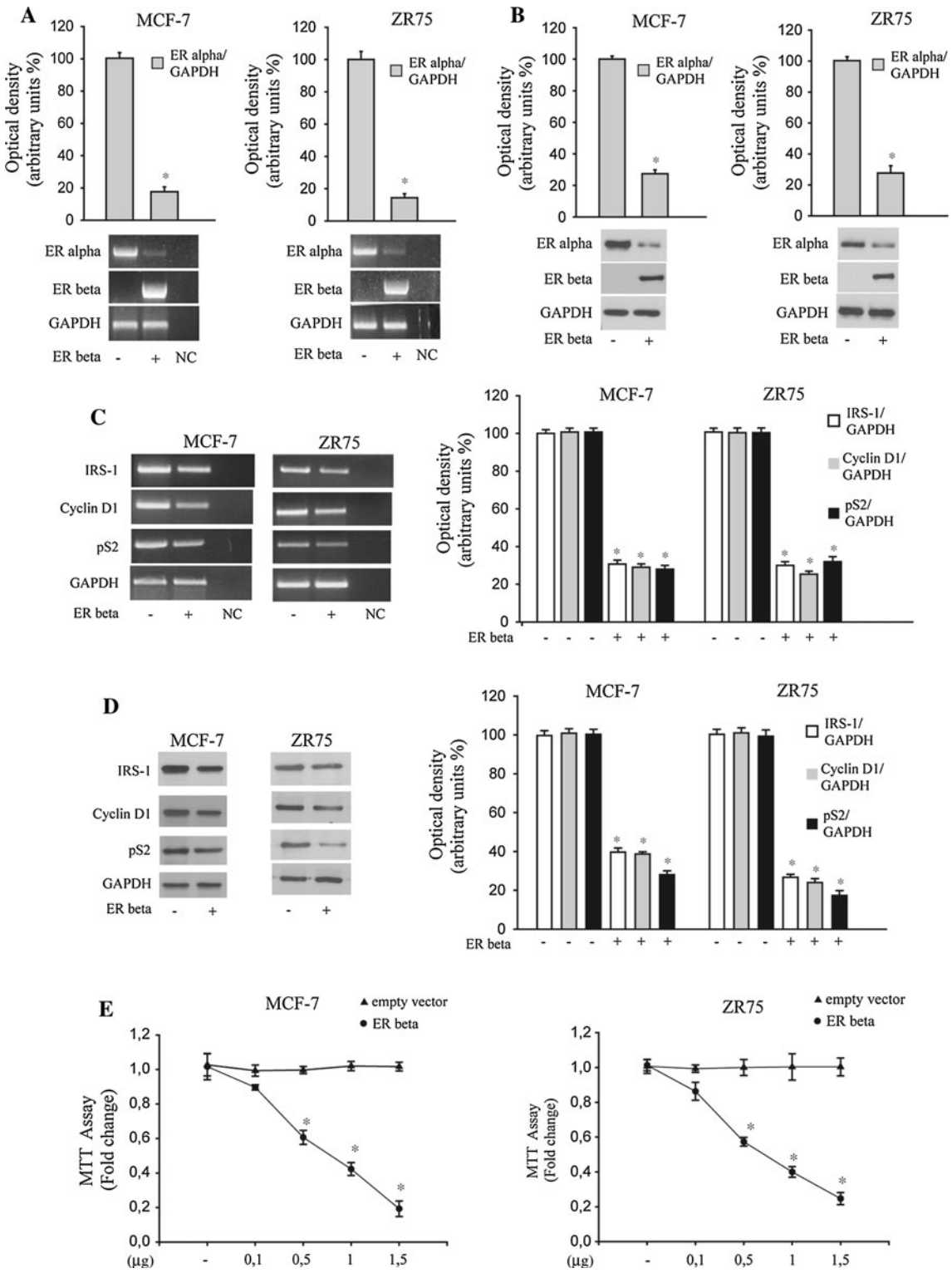
ER beta overexpression down-regulates ER alpha expression in breast cancer cells

Although data demonstrate that ER beta negatively interferes with ER alpha signaling in breast cancer cells, it still remains unexplored if ER beta actually affects ER alpha gene expression. To this aim, ER alpha-positive MCF-7 and ZR75 breast cancer cells were transiently transfected with an ER beta expression vector and ER alpha expression was evaluated by RT-PCR and western blotting analysis. As shown in Fig. 1a, b, ectopically expressed ER beta reduced ER alpha levels in terms of mRNA and protein content in both MCF-7 and ZR75 cells. Concomitantly, ER beta overexpression markedly decreased mRNA levels (Fig. 1c) and protein (Fig. 1d) expression of classical estrogen-regulated genes, such as insulin receptor substrate 1 (IRS-1), pS2 and cyclin D1 in both cell lines. These findings correlated well with a dose-dependent inhibition induced by ER beta overexpression on cell proliferation, as revealed by MTT assays (Fig. 1e).

Fig. 1 Overexpressed ER beta down-regulates ER alpha expression in breast cancer cells. **a** *Bottom panel*, total RNA was isolated from MCF-7 and ZR75 cells transfected with either empty vector (-) or ER beta expression plasmid and reverse transcribed. cDNA was subjected to PCR using specific primers for ER alpha, ER beta and GAPDH. NC, negative control, RNA sample without the addition of transcriptase. *Upper panel*, the histograms represent the mean ± S.D. of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **b** *Bottom panel*, western Blot analysis of ER alpha and ER beta in total protein extracts from MCF-7 and ZR75 cells, transiently transfected with either empty vector (-) or ER beta expression plasmid. GAPDH was used as loading control. *Upper panel*, the histograms represent the mean ± S.D. of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **c** *Left panel*, Total RNA was isolated from MCF-7 and ZR75 cells transfected with either empty vector (-) or ER beta expression plasmid and reverse transcribed. cDNA was subjected to PCR using specific primers for IRS-1, pS2, Cyclin D1, and GAPDH. NC, negative control, RNA sample without the addition of transcriptase. *Right panel*, the histograms represent the mean ± S.D. of three different experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **d** *Left panel*, Western Blot analysis of IRS-1, pS2, Cyclin D1 in total protein extracts from MCF-7 and ZR75 cells transfected with either empty vector (-) or ER beta expression plasmid. GAPDH was used as loading control. *Right panel*, the histograms represent the mean ± S.D. of three different experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. *, $P < 0.01$ ER beta-transfected cells compared to empty vector (-)-transfected cells. **e** MTT growth assays in MCF-7 and ZR75 cells transfected with empty vector or ER beta expression vector (0.1–1.5 μg/well) for six days. Cell proliferation is expressed as fold change ± S.D. relative to empty vector (-)-transfected cells and is representative of three different experiments each performed in triplicate. *$P < 0.05$ ER beta-transfected cells compared to empty vector (-)-transfected cells

Overexpressed ER beta mediates down-regulation of ER alpha via a GC proximal region of its promoter

To analyze how ER beta interferes with ER alpha gene transcription, we transiently transfected breast cancer cell lines with a luciferase reporter plasmid containing the human ER alpha promoter region spanning from −4,100 bp to +212 bp. As shown in Fig. 2a, a significant decrease in ER alpha promoter activity was observed in MCF-7 cells when ER beta was overexpressed. The human ER alpha promoter contains multiple consensus sites for several transcription factors, including a CAT box, TFIID, AP2γ, and Sp-1 motifs [37]. To identify the regions within the ER alpha promoter responsible for ER beta-mediated inhibitory effects, we transiently transfected MCF-7 cell lines with plasmids containing a series of 5′ deleted segments of this human ER alpha promoter. Schematic representation of these constructs is shown in Fig. 2. In transfection experiments performed using p-4100/+ 212 (E), p-2769/+ 212 (D), and p-1000/+ 212 (C) plasmids, the responsiveness to ER beta was still maintained (Fig. 2a), suggesting that the region between −1000 and +212 might be involved in the transrepression mechanisms exerted by ER beta overexpression. Thus, we focused our attention on the latter construct p-1000/+ 212 (C), and we identified, upstream to the initiation transcription site, two half ERE (−867/−861 and −894/−888) and one Sp-1 (−223−214) sites, which are putative effectors of ER signaling. We observed that in MCF-7 cells transiently transfected with the ER alpha promoter plasmid bearing 2 half ERE-mutated sites (C 2mut half-ERE) or with a deleted construct of ER alpha promoter containing Sp-1 site (p-245/+ 212, A) that ER beta-mediated down-regulation still persisted. In contrast, deletion of the Sp-1 site (C Sp-1 del) completely abrogated ER beta's effects (Fig. 2b). Similar results were obtained in the ZR75 breast cancer cells (data not shown). Taken together, our findings demonstrated that the down-regulatory effects of ER beta on ER alpha gene expression requires an Sp1 sequence motif. Since functional domains of Sp1 are involved in protein–protein interactions with other transcription regulatory molecules, such as the corepressors SMRT, NCoR and BCoR (BCL6 corepressor) [41–44], to inhibit cell transcription machinery, we next addressed whether the same corepressors may be recruited by the ER beta/Sp1 complex to ER alpha gene promoter.



The NCoR corepressor is recruited with Sp-1 to ER alpha promoter region

The specific role of the Sp-1 motif in mediating the inhibitory role of ER beta on ER alpha gene expression was investigated using electromobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) assays. Using synthetic radiolabeled oligonucleotides bearing the Sp-1 motif present in the ER alpha promoter region (Fig. 3a, lane 1), we observed the formation of a protein complex in nuclear

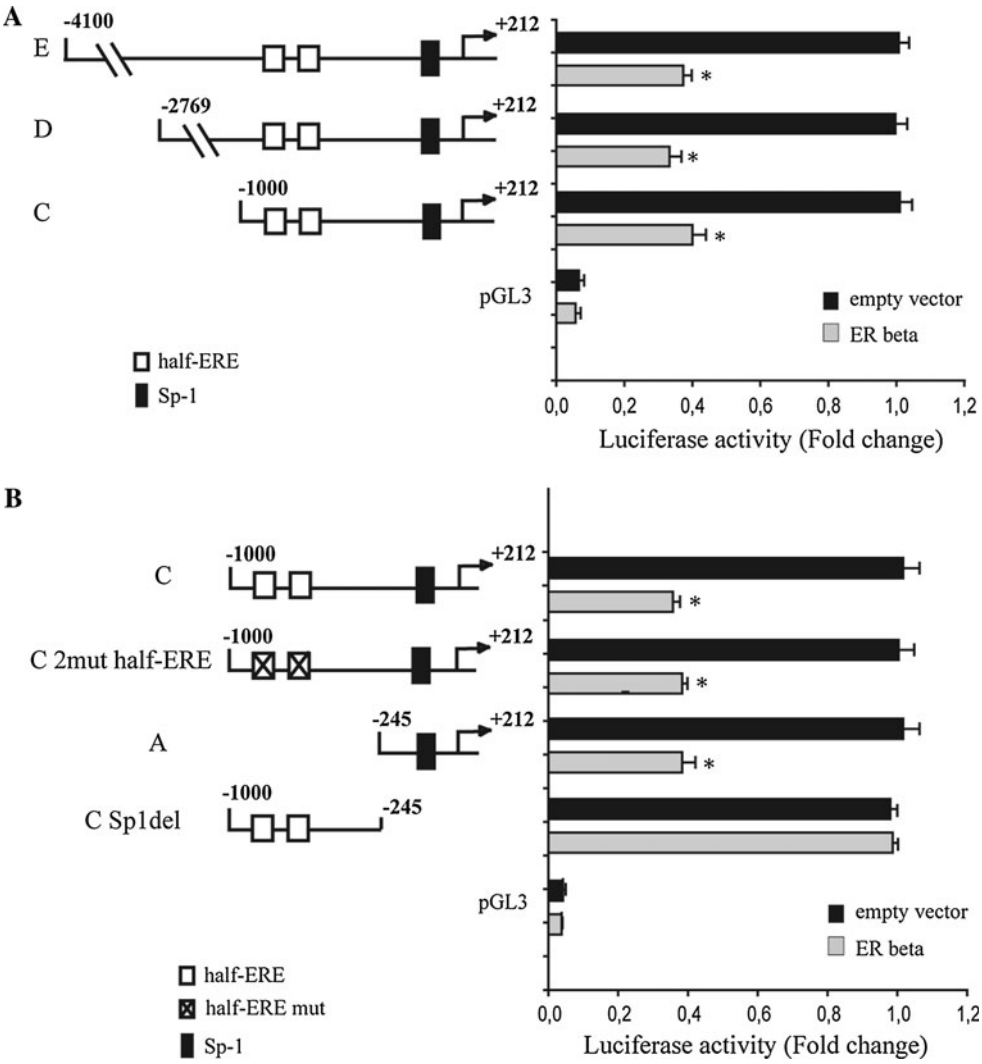
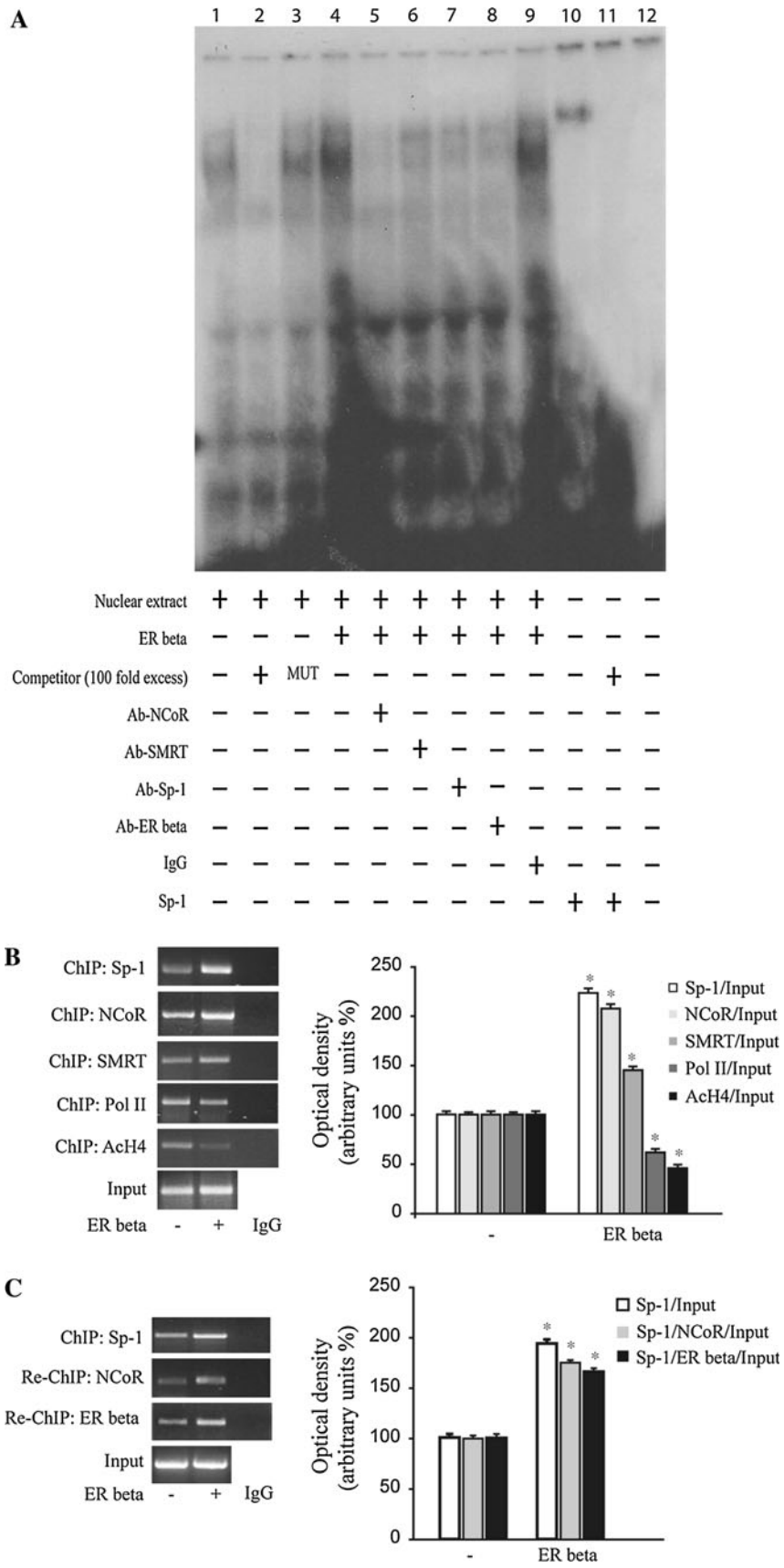


Fig. 2 ER alpha promoter activity is down-regulated by ER beta overexpression, and deletion of the GC-proximal promoter region abrogates this effect. **a, b** *Left panel*, schematic representation of constructs of the ER alpha gene promoter used in this study. *Right panel*, plasmids containing ER alpha promoter fragments were transiently cotransfected in MCF-7 cells in presence or absence of ER beta expression plasmid. After 24 h of transfection, luciferase activities were normalized to the Renilla Luciferase as internal transfection control and data where reported as fold change. The values represent the means ± S.D. of three different experiments each performed in triplicate. pGL3: basal activity measured in cells transfected with pGL3 basal vector. $*P < 0{,}05$ ER beta-transfected cells compared to empty vector-transfected cells

Fig. 3 ER beta recruits corepressors to the Sp-1 site in the ER alpha gene promoter. **a** Nuclear extracts from MCF-7 cells transfected with either empty vector or ER beta expression plasmid were incubated with a double-stranded Sp-1 specific sequence probe labeled with [γ^{32}P] ATP and subjected to electrophoresis in a 6 % polyacrylamide gel (*lanes 1* and *4*). Competition experiments were performed adding as competitor a 100-fold molar excess of unlabeled probe (*lane 2* and *lane 11*) or a 100-fold molar excess of unlabeled oligonucleotide containing a mutated Sp-1 motif (*lane 3*). Nuclear extracts from MCF-7 over-expressing ER beta were incubated with anti-NCoR (*lane 5*) or anti-SMRT (*lane 6*) or anti-Sp-1 (*lane 7*) or anti-ER beta (*lane 8*) or IgG (*lane 9*) antibodies, in the presence of the probe. *Lane 10*, Sp-1 protein. *Lane 12*, probe alone. **b** *Left panel,* MCF-7 cells transfected with either empty vector (-) or ER beta expression plasmid were cross-linked with formaldehyde, and lysed. The pre-cleared chromatin was immune-precipitated with specific anti-Sp-1, anti-NCoR, anti-SMRT, anti-Polymerase II, and anti-AcH4 antibodies, and with a normal mouse serum (IgG) as a negative control. A 5 µl volume of each sample and input were analyzed by PCR with specific ▶ primers, as detailed in Sect. "Materials and methods," to amplify ER alpha promoter sequence containing Sp-1 site. *Right panel,* the histograms represent the mean ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentage of the control, which was assumed to be 100 %. **c** *Left panel*, Chromatin immunoprecipitated with anti-Sp-1 antibody was re-immunoprecipitated with anti-NCoR or anti-ER beta antibodies. A normal mouse serum (IgG) was used as a negative control. A 5 µl volume of each sample and input were analyzed by PCR with specific primers, as detailed in Sect. "Materials and methods," to amplify ER alpha promoter sequence containing Sp-1 site. *Right panel,* the histograms represent the mean ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentage of the control, which was assumed to be 100 %. $*P < 0.01$ ER beta-transfected cells compared to empty vector (-)-transfected cells


A
1 2 3 4 5 6 7 8 9 10 11 12
Nuclear extract + + + + + + + + + − − −
ER beta − − − + + + + + + − − −
Competitor (100 fold excess) − + MUT − − − − − − − + −
Ab-NCoR − − − − + − − − − − − −
Ab-SMRT − − − − − + − − − − − −
Ab-Sp-1 − − − − − − + − − − − −
Ab-ER beta − − − − − − − + − − − −
IgG − − − − − − − − + − − −
Sp-1 − − − − − − − − − + + −
B
ChIP: Sp-1
ChIP: NCoR
ChIP: SMRT
ChIP: Pol II
ChIP: AcH4
Input
ER beta - + IgG
Optical density (arbitrary units %)
250
200
150
100
50
0
Sp-1/Input
NCoR/Input
SMRT/Input
Pol II/Input
AcH4/Input
-
ER beta
C
ChIP: Sp-1
Re-ChIP: NCoR
Re-ChIP: ER beta
Input
ER beta - + IgG
Optical density (arbitrary units %)
250
200
150
100
50
0
Sp-1/Input
Sp-1/NCoR/Input
Sp-1/ER beta/Input
-
ER beta

extracts from MCF-7 cells, which was abrogated by incubation with 100 fold molar excess of unlabeled probe (Fig. 3a, lane 2), demonstrating the specificity of the DNA-binding complex. This inhibition was no longer observed when mutated oligodeoxyribonucleotide probe was used as competitor (Fig. 3a, lane 3). Interestingly, overexpression of ER beta strongly increased the DNA-binding protein complex compared with control samples (Fig. 3a, lane 4). The inclusion of anti-Sp-1, ER beta, NCoR and SMRT antibodies in the reaction immunodepleted the specific band, confirming the presence of these proteins in the complex (Fig. 3a, lanes 5–8). Of note, immunodepletion occurred to a higher extent in the presence of NCoR than of SMRT antibodies. Non-specific IgG did not affect Sp-1 complex formation (Fig. 3a, lane 9). Recombinant Sp-1 protein revealed a complex migrating at the same level as that of nuclear extracts from cells (Fig. 3a, line 10).

Moreover, to better evaluate the involvement of Sp-1 and NCoR/SMRT corepressors in ER beta-mediated ER alpha down-regulation at the promoter level, ChIP assays were performed. Using specific antibodies against Sp-1, NCoR, SMRT, RNA-polymerase II, and acetyl histone H4, protein-chromatin complexes were immunoprecipitated from MCF-7 cells transfected either with an empty vector or an ER beta expression vector (Fig. 3b). PCR using primers spanning the Sp-1 binding element in the ER alpha promoter region clearly showed an enhanced recruitment of Sp-1 and NCoR and slightly of SMRT upon ER beta overexpression. The corepressor DAX-1 was not detected under the same experimental conditions (data not shown). These results were concomitant with a lower association of RNA-Polymerase II and acetyl histone H4 to the ER alpha regulatory region, indicating that the chromatin in this region is probably in a less permissive environment for gene transcription. Re-ChIP assays confirmed the increased NCoR and ER beta occupancy of the Sp-1-containing region within the ER alpha promoter in cells overexpressing ER beta (Fig. 3c).

NCoR knockdown reverses ER beta's effects on ER alpha down-regulation and cell proliferation

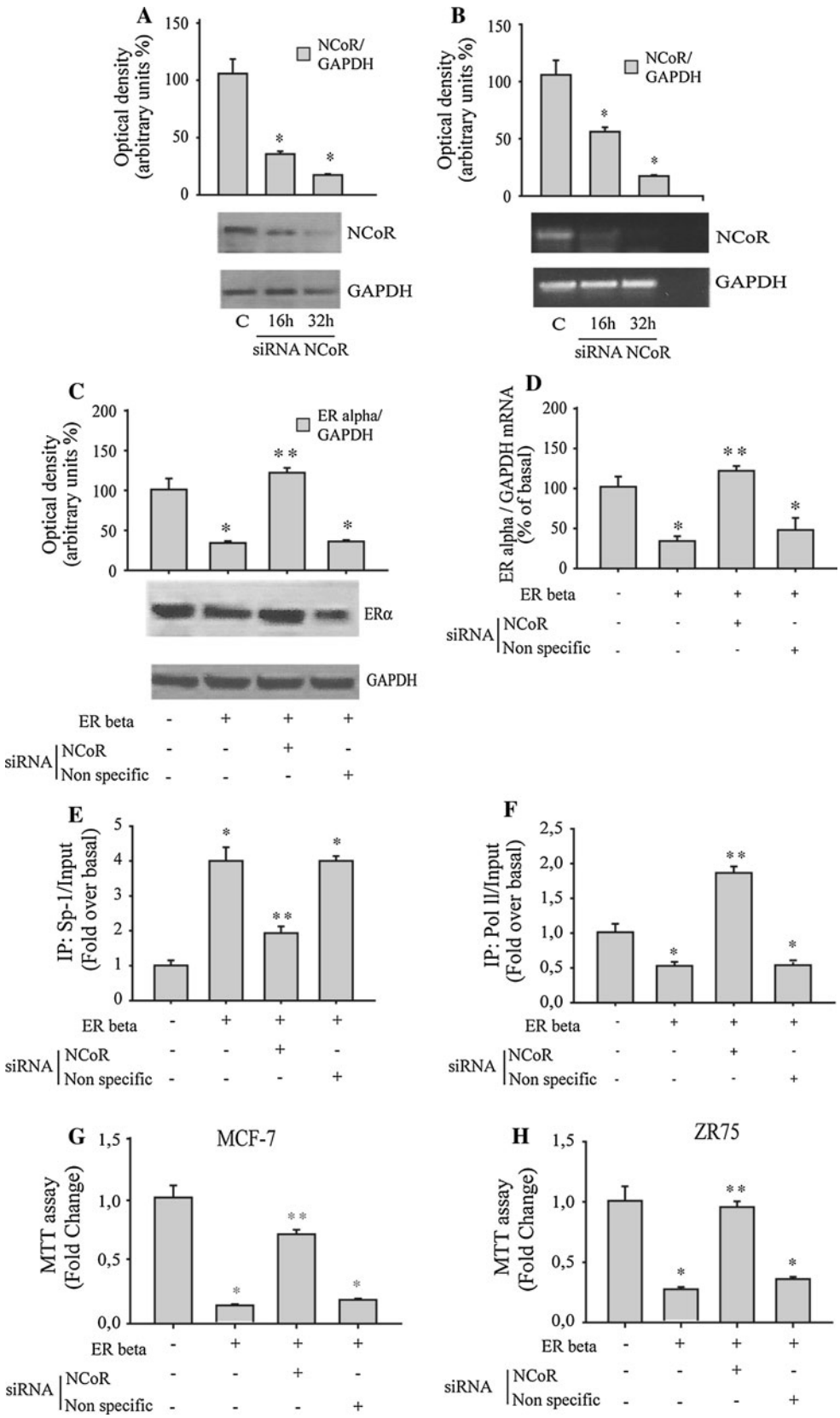
To ascertain the involvement of NCoR on ER beta-related downregulation of ER alpha, NCoR siRNA knockdown experiments were performed in MCF-7 cells transfected with an ER beta expression vector. Silencing of NCoR gene expression (evaluated by western blot and RT-PCR analysis, Fig. 4a, b) restored both protein and mRNA expression of ER alpha, while no changes were observed after transfection of cells with a scrambled siRNA control (Fig. 4c, d). We also showed that the increased Sp-1 recruitment to ER alpha gene promoter was abrogated in presence of NCoR siRNA in MCF-7 cells overexpressing ER beta (Fig. 4e). Concomitantly, the recruitment of RNA-polymerase II in the same region was restored (Fig. 4f).

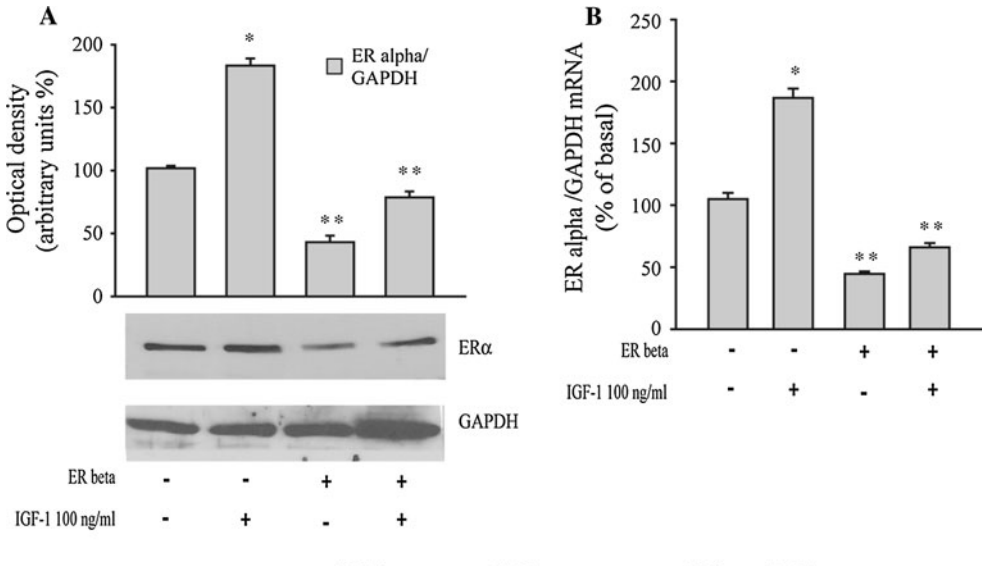
Fig. 4 Effects of NCoR silencing on ER beta-mediated down-regulation of ER alpha expression, Sp-1 recruitment to ER alpha promoter and cell proliferation. **a** Western blot analysis for NCoR in MCF-7 cells transfected with non-specific siRNA (C) or targeted against human NCoR (100 nM) for 16 and 32 h. GAPDH was used as a loading control. **b** RT-PCR for NCoR or GAPDH in MCF-7 cells transfected as above described. NC: negative control, RNA sample without the addition of reverse transcriptase. The histograms represent the mean ± S.D. of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. *$P < 0.01$ NCoR siRNA-transfected cells compared to non-specific siRNA (C)-transfected cells. **c** Western blot analysis for ER alpha in MCF-7 cells transfected with either empty vector(-) or ER beta expression plasmid in presence of non-specific or NCoR siRNA. GAPDH was used as loading control. The histograms represent the mean ± S.D. of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **d** RNA was extracted from MCF-7 cells transfected with either empty vector(-) or ER beta expression plasmid in presence of non-specific or NCoR siRNA, reverse transcribed and cDNA was subjected to qRT-PCR for analyzing ER alpha mRNA levels. Data represent the mean ± S.D. of values from three separate RNA samples expressed as percentage of control (-) assumed to be 100 %. Each sample was normalized to GAPDH mRNA content. **e, f** MCF-7 cells transfected with either empty vector(-) or ER beta expression plasmid in presence of non-specific or NCoR siRNA, were crosslinked with formaldehyde, and lysed. The precleared chromatin was immunoprecipitated with anti-Sp-1 **(e)** or anti-RNA-polymerase II (Pol II, **f**) antibodies. A 5 µl volume of each sample and input was analyzed by qRT-PCR using specific primers to amplify ER alpha promoter sequence, including the Sp-1 site. Similar results were obtained in two independent experiments. **g, h** MTT assays in MCF-7 and ZR75 cells transfected as indicated. Results are expressed as fold change ± S.D relative to empty vector-transfected cells and are representative of three different experiments each performed in triplicate. *$P < 0.01$ ER beta-transfected cells compared to empty vector-transfected cells. **$P < 0.01$ NCoR siRNA-transfected cells compared to ER beta-transfected cells

Finally, the anti-proliferative effects exerted by ER beta were completely reversed in the presence of NCoR siRNA knockdown in MCF-7 and ZR75 breast cancer cells (Fig. 4g, h), suggesting a crucial role for NCoR in mediating the ER beta-induced inhibitory effects on breast cancer cell proliferation.

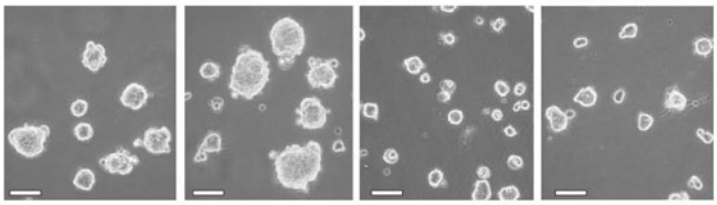
ER beta antagonizes IGF-1 mediated up-regulatory effects on ER alpha expression and three-dimensional cell growth

It has been previously demonstrated that IGF-1 and insulin can increase ER alpha expression and stimulate proliferation in breast cancer cells [45–48]. Thus, we investigated the ability of ER beta to reverse IGF-1 effects on ER alpha expression in MCF-7 cells, by western blotting and real time PCR analysis (Fig. 5a, b). As expected, IGF-1


A
Optical density (arbitrary units %)
NCoR/ GAPDH
NCoR
GAPDH
C 16h 32h
siRNA NCoR
B
Optical density (arbitrary units %)
NCoR/ GAPDH
NCoR
GAPDH
C 16h 32h
siRNA NCoR
C
Optical density (arbitrary units %)
ER alpha/ GAPDH
ERα
GAPDH
ER beta
siRNA NCoR
Non specific
D
ER alpha / GAPDH mRNA (% of basal)
ER beta
siRNA NCoR
Non specific
E
IP: Sp-1/Input (Fold over basal)
ER beta
siRNA NCoR
Non specific
F
IP: Pol II/Input (Fold over basal)
ER beta
siRNA NCoR
Non specific
G
MCF-7
MTT assay (Fold Change)
ER beta
siRNA NCoR
Non specific
H
ZR75
MTT assay (Fold Change)
ER beta
siRNA NCoR
Non specific



C



	25 μm ≤ 50 μm	50 μm ≤ 100 μm	≥100 μm
-	30 ± 1.0	2.8 ± 1.0	0.0 ± 0.0
IGF-1	5 ± 2.5	34 ± 1.5	85.0 ± 3.1
ER beta	90 ± 3.2	0.6 ± 0.5	0.0 ± 0.0
ER beta + IGF-1	65 ± 1.5	0.8 ± 1.6	0.0 ± 0.0

Fig. 5 Overexpressed ER beta reverses IGF-1 enhanced ER alpha expression and cell–cell adhesion. **a** *Bottom panel*, MCF-7 cells transiently transfected with either empty vector(-) or ER beta expression plasmid were treated with vehicle or IGF1 (100 ng/ml) for 48 h. Total proteins were extracted and western blot analysis was performed to evaluate the expression of ER alpha. GAPDH was used as loading control. *Upper panel*, the histograms represent the mean ± S.D. of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100 %. **b** qRT-PCR for analyzing ER alpha mRNA levels in cells transfected and treated as indicated. Data represent the mean ± S.D. of values from three separate RNA samples expressed as percentage of control assumed to be 100 %. Each sample was normalized to GAPDH RNA content. $*P < 0.01$ IGF-1 treatment compared to vehicle treated-cells. $**P < 0.01$ ER beta overexpressing cells compared to empty vector-transfected cells. **c** MCF-7 cells were transiently transfected with either empty vector (-) or ER beta expression plasmid and growth as three-dimensional cultures in the presence or absence of IGF-1 (100 ng/ml, 48 h). Scale *bar* = 25 μm. Images are representative of three independent experiments. The extent of aggregation was scored by measuring the spheroid diameters. The values represent the sum of spheroids in 10 optical fields under ×10 magnification

enhanced ER alpha protein and mRNA levels and ER beta overexpression significantly abrogated this increase. Then, the effects of ER beta on IGF-1-induced growth were assessed using three-dimensional MCF-7 cell culture, that simulate "in vivo" the biological features of tumors. Our results showed that ER beta overexpression blocked the IGF-1 induced cell growth, as evidenced by the extent of aggregation scored by measuring the spheroid diameters (Fig. 5c).

Discussion

In this study, we show for the first time that ER beta overexpression down-regulates ER alpha gene expression in a ligand-independent manner in ER alpha-positive breast cancer cells. This occurs through ER beta interactions with Sp-1 and an enhanced NCoR corepressor recruitment within the human ER alpha promoter region, up-stream of the initiating transcription site.

ER alpha and ER beta have both overlapping and distinct expression patterns, and mammary gland development in animal models requires ER alpha signaling. It has been hypothesized that dysregulated ER isoform expression may induce abnormal cell proliferation and survival, thus impacting mammary tumorogenesis. It is also well known that ER alpha expression is increased and ER beta expression is decreased in early breast cancers, whereas expression of both receptors declines in more invasive cancers [49–51]. Expression of ER beta is lost in other early tumor types in respect to normal tissue [52–54], leading to the hypothesis that ER beta may function as a tumor suppressor [26, 27]. Data coming from cell studies have suggested that ER beta negatively interferes with ER alpha signaling in breast cancer cells, and mediates antiproliferative effects [27, 33]. ER beta over-expression inhibits tumor establishment and growth as well as E2-induced tumor formation "in vivo" in mouse xenografts of ER alpha-positive MCF-7 and T47D breast cancer cells [25, 29, 55]. Indeed, ER beta induces inhibition of classical estrogen-regulated genes, such as VEGF and PDGFβ [31]. Recently, Song and Pan [56] demonstrated that ER alpha-mediated estrogenic activity in the mammary gland can be opposed by ER beta, and it has been suggest that selective agonists such as DPN should be explored for the development of better hormone replacement therapy regimens to reduce or eradicate the risk for breast cancer. In the majority of clinical studies, ER beta expression indicates a favorable response to adjuvant tamoxifen (Tam) therapy, and patients with ER alpha +/ER beta + tumors appear to respond at least as well as or better to endocrine therapy than patients with ER alpha +/ER beta- tumors. In addition, in Tam-treated patients, high ER beta expression correlates with increased overall, and disease-free survival, no disease progression, or no relapse within 5 years [24, 57–59]. Thus, ER beta has emerged as potential marker for predicting response to endocrine therapy.

These findings led us to investigate the molecular mechanism through which ER subtypes are regulated in breast cancer cells. Here, we have demonstrated that ER beta overexpression in a ligand-independent manner resulted in inhibition of ER alpha in terms of mRNA and protein content in breast cancer cells. Similar inhibitory effects were also obtained for the expression of estrogen-dependent genes such as IRS-1, pS2, and cyclin D1. These data underline how ER beta-induced ER alpha down-regulation might arise via transcriptional mechanisms. Therefore, we focused on the molecular mechanisms by which ER beta mediates repression of ER alpha gene expression and on the biological consequences of ER beta overexpression on growth of breast cancer cells.

ER alpha and ER beta are transcriptional factors that can regulate gene expression through several different modes including direct DNA-binding (acting as homodimers or as heterodimers) or through tethering to other transcription factors such as activating protein-1 (AP-1) and stimulating protein-1(Sp-1) [8, 60]. This has been most extensively investigated in relationship to protein complexes involving Sp-1 and ER alpha at GC boxes, which are classic binding sites for members of the Sp-1 family of transcription factors [61–63]. Many studies have observed that ER alpha is able to enhance binding of Sp1 to its site in several promoter regions [13–15]. The analysis of different functional motifs present within the ER alpha proximal promoter [37] has identified two half-ERE and one Sp-1 responsive elements, as potential targets of ER beta. Functional experiments using ER alpha promoter-deleted or mutated constructs have shown that Sp-1 sequence is an important prerequisite for the down-regulatory effects of ER beta on ER alpha promoter activity. These results were well supported by electrophoretic mobility shift assays, which revealed a marked increase in a specific DNA-binding complex in nuclear extracts from MCF-7 cells over-expressing ER beta. This complex was immune-depleted by anti-Sp-1 and anti-ER beta antibodies, suggesting the presence of these proteins in the complex. Furthermore, we observed an enhanced recruitment of Sp-1 and ER beta to the ER alpha promoter, that was concomitant with a decrease in RNA-polymerase II and acetyl histone H4 recruitment, further supporting a negative role for ER beta in modulating ER alpha gene transcriptional machinery.

A recent study reported that the ZFDBD (Zinc Finger DNA-Binding Domain) and ID (Inhibitory Domain) domains of Sp-1 are involved in protein–protein interactions with other transcription regulatory molecules, such as the corepressors SMRT, NCoR, and BCoR (BCL6 corepressor) [41–43]. These corepressors interact with unliganded nuclear receptors, through an elongated helix of sequence LXXI/HIXXXI/L, alternatively referred to as the CoRNR-box [64–66]. It has been recently documented that NCoR and SMRT are also recruited by both ER and PR in the presence of ligands to regulate transcription of different genes [67, 68]. Our results demonstrate that NCoR was the corepressor crucially recruited on the Sp-1 site of the ER alpha gene promoter together with Sp-1 and ER beta. In general, NCoR and SMRT share the same molecular architecture, interact with many of the same transcription factors, and assemble into similar corepressor complexes [69]. We also detected a slight recruitment of SMRT under the same experimental conditions. Finally, the contribution of the NCoR corepressor factor in ER beta-mediated effects emerges from experiments showing that silencing of NCoR gene expression was able to reverse the inhibitory effects of ER beta on ER alpha mRNA and protein content, Sp-1 recruitment to the ER alpha promoter gene and cell growth proliferation.

Previous "in vitro" studies have shown that insulin and IGF-1 up-regulate the ER alpha expression as well as its DNA-binding capacity [45–48]. We demonstrated how ER beta reduced the stimulatory effects induced by IGF1 on ER alpha expression and three-dimensional cell growth, and became a negative modulator of the well known cross-talk between ER alpha and IGF1-R signaling pathways.

In conclusion, we suggest that inhibition of ER alpha by ER beta is a critical regulatory pathway occurring in ER-positive cells, addressing prospectively that therapeutic tools which potentiate ER beta action and thereby deplete intratumoral ER alpha content may be useful to inhibit breast cancer cell growth and progression.

Acknowledgments This study was supported by PRIN 2009 and AIRC grants 2011. Reintegration AIRC/Marie Curie International Fellowship in Cancer Research to IB. NIH/NCI R01 CA72038 to SAWF.

Conflict of interest The authors declare no conflict of interest.

References

1. Hall JM, Couse JF, Korach KS (2001) The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 276:36869–36872
2. 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
3. Osborne CK, Schiff R, Fuqua SA et al (2001) Estrogen receptor: current understanding of its activation and modulation. Clin Cancer Res 7:4338s–4342s (discussion 4411s–4412s)
4. Barone I, Brusco L, Fuqua SA (2010) Estrogen receptor mutations and changes in downstream gene expression and signaling. Clin Cancer Res 16:2702–2708 Review
5. Cato AC, Nestl A, Mink S (2002) Rapid actions of steroid receptors in cellular signaling pathways. Science STKE 138:re9
6. Nemere I, Pietras RJ, Blackmore PF (2003) Membrane receptors for steroid hormones: signal transduction and physiological significance. J Cell Biochem 88:438–445
7. Ordonez-Moran P, Munoz A (2009) Nuclear receptors: genomic and nongenomic effects converge. Cell Cycle 8:1675–1680
8. Paech K, Webb P, Kuiper GG et al (1997) Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 277:1508–1510
9. Webb P, Nguyen P, Valentine C et al (1999) The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol 13:1672–1685
10. Kushner PJ, Agard DA, Greene GL et al (2000) Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74:311–317
11. Salvatori L, Pallante P, Ravenna L et al (2003) Oestrogens and selective oestrogen receptor (ER) modulators regulate EGF receptor gene expression through human ER α and β subtypes via an Sp1 site. Oncogene 22:4875–4881
12. Safe S, Kim K (2004) Nuclear receptor-mediated transactivation through interaction with Sp proteins. Nucleic Acid Res Mol Biol 77:1–36
13. Panno ML, Mauro L, Marsico S et al (2006) Evidence that mouse IRS-1 belongs to the family gene which promoter is activated by ERα through its interaction with Sp-1. J Mol Endocrinol 36:91–105
14. Catalano S, Rizza P, Gu G et al (2007) Fas ligand expression in TM4 Sertoli cells is enhanced by estradiol in situ production. J Cell Physiol 211:448–456
15. Mauro L, Catalano S, Bossi G et al (2007) Evidences that leptin upregulates E-cadherin expression in breast cancer effects on tumor growth and progression. Cancer Res 67:3412–3421
16. Higgins KJ, Shengxi L, Abdelrahim M et al (2008) Vascular endothelial growth factor receptor-2 expression is down-regulated by 17 beta estradiol in MCF-7 breast cancer cells by estrogen receptor alpha/Sp-1 proteins. Mol Endocrinol 22:388–402
17. Varlakhanova N, Snyder C, Jose S et al (2010) Estrogen receptors recruit SMRT and N-CoR corepressors through newly recognized contacts between the corepressor N terminus and the receptor DNA binding domain. Mol Cell Biol 30:1434–1445
18. Allegra JC, Lippman ME, Green L et al (1979) Estrogen receptor values in patients with benign breast disease. Cancer 44:228–231
19. Petersen OW, Hoyer PE, van Deurs B (1987) Frequency and distribution of estrogen receptor-positive cells in normal, non-lactating human breast tissue. Cancer Res 47:5748–5751
20. Ricketts D, Turnbull L, Ryall G et al (1991) Estrogen and progesterone receptors in the normal female breast. Cancer Res 51:1817–1822
21. Yager JD, Davidson NE (2006) Estrogen carcinogenesis in breast cancer. N Engl J Med 354:270–282
22. Zhao C, Dahlman-Wright K et al (2008) Estrogen receptor beta: an overview and update. Nucl Recept Signal 6:e003
23. Skliris GP, Munot K, Bell SM et al (2003) Reduced expression of estrogen receptor beta in invasive breast cancer and its re-expression using DNA methyl transferase inhibitors in a cell line model. J Pathol 201:213–220
24. Hopp TA, Weiss HL, Parra IS et al (2004) Low levels of estrogen receptor beta protein predict resistance to tamoxifen therapy in breast cancer. Clin Cancer Res 10:7490–7499
25. Paruthiyil S, Parmar H, Kerekatte V et al (2004) Estrogen receptor β inhibits human breast cancer cell proliferation and tumor formation by causing a G2 Cell Cycle Arrest. Cancer Res 64:423–428
26. Chang EC, Frasor J, Komm B et al (2006) Impact of estrogen receptor β on gene networks regulated by estrogen receptor α in breast cancer cells. Endocrinology 147:4831–4842
27. Williams C, Edvardsson K, Lewandowski SA et al (2008) A genome-wide study of repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. Oncogene 27:1019–1032
28. Ström A, Hartman J, Foster JS et al (2004) Estrogen receptor β inhibits 17β-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci 101:1566–1571
29. Hartman J, Lindberg K, Morani A et al (2006) Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts. Cancer Res 66:11207–11213
30. 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
31. Buteau-Lozano H, Ancelin M, Lardeux B et al (2002) Transcriptional regulation of vascular endothelial growth factor by estradiol and tamoxifen in breast cancer cells: a complex interplay between estrogen receptors α and β. Cancer Res 62:4977–4984
32. Omoto Y, Eguchi H, Yamamoto-Yamaguchi Y et al (2003) Estrogen receptor (ER) β1 and ERβcx/β ERα function differently in breast cancer cell line MCF7. Oncogene 22:5011–5020
33. Pettersson K, Delaunay F, Gustafsson JA (2000) Estrogen receptor β acts as a dominant regulator of estrogen signaling. Oncogene 19:4970–4978

34. Nilsson S, Makela S, Treuter E et al (2001) Mechanisms of estrogen action. Physiol Rev 81:1535–1565
35. Liu MM, Albanese C, Anderson CM et al (2002) Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem 277:24353–24360
36. Acconcia F, Totta P, Ogawa S et al (2005) Survival versus apoptotic 17beta-estradiol effect: role of ER alpha and ER beta activated non-genomic signaling. J Cell Physiol 203:193–201
37. deGraffenried LA, Hopp TA, Valente AJ et al (2004) Regulation of the estrogen receptor α minimal promoter by Sp1, USF-1 and ERα. Breast Cancer Res and Treat 85:111–120
38. Catalano S, Malivindi R, Giordano C et al (2010) Farnesoid X Receptor through its binding to Steroidogenic Factor 1 responsive element inhibits aromatase expression in tumor Leydig cells. J Biol Chem 285:5581–5593
39. Andrews NC, Faller DV (1991) A rapid microperation technique foe extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499
40. Barone I, Cui Y, Herynk MH et al (2009) Expression of the K303R estrogen receptor-alpha breast cancer mutation induces resistance to an aromatase inhibitor via addiction to the PI3 K/Akt kinase pathway. Cancer Res 69:4724–4732
41. Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457
42. Zamir I, Dawson J, Lavinsky RM et al (1997) Cloning and characterization of a corepressor and potential component of the nuclear hormone receptor repression complex. Proc Natl Acad Sci USA 94:14400–14405
43. Huynh KD, Fischle W, Verdin E et al (2000) BCoR, a novel corepressor involved in BCL-6 repression. Genes Dev 14:1810–1823
44. Lee JA, Suh DC, Kang JE et al (2005) Transcriptional activity of Sp1 is regulated by molecular interactions between the zinc finger DNA binding domain and the inhibitory domain with corepressors, and this interaction is modulated by MEK. J Biol Chem 280:28061–28071
45. Panno ML, Salerno M, Pezzi V et al (1996) Effect of estradiol and insulin on proliferative pattern and on estrogen and progesterone receptor contents in MCF-7 cells. J Cancer Res Clin Oncol 122:745–749
46. Lee AV, Weng C-N, Jackson JG et al (1997) Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. J Endocrinol 152:39–47
47. Andò S, Panno ML, Salerno M et al (1998) Role of IRS-1 signaling in insulin-induced modulation of estrogen receptors in breast cancer cells. Biochem Biophys Res Commun 253:315–319
48. Lannigan DA (2003) Estrogen receptor phosphorylation. Steroids 68:1–9
49. Leygue E, Dotzlaw H, Watson PH et al (1998) Altered estrogen receptor α and β messenger RNA expression during human breast tumorigenesis. Cancer Res 58:3197–3201
50. Jarvinen TA, Pelto-Huikko M, Holli K et al (2000) Estrogen receptor β is coexpressed with ERα and PR and associated with nodal status, grade, and proliferation rate in breast cancer. Am J Pathol 156:29–35
51. Roger P, Sahla ME, Makela S et al (2001) Decreased expression of estrogen receptor β protein in proliferative preinvasive mammary tumors. Cancer Res 61:2537–2541
52. Fixemer T, Remberger K, Bonkhoff H (2003) Differential expression of the estrogen receptor beta in human prostate tissue, premalignant changes, and in primary, metastatic, and recurrent prostatic adenocarcinoma. Prostate 54:79–87
53. Foley EF, Jazaeri AA, Shupnik MA et al (2000) Selective loss of estrogen receptor β in malignant human colon. Cancer Res 60:245–248
54. Pujol P, Rey JM, Nirde P et al (1998) Differential expression of estrogen receptor-alpha and -beta messenger RNAs as a potential marker of ovarian carcinogenesis. Cancer Res 58:5367–5373
55. Behrens D, Gill JH, Fichtner I (2007) Loss of tumourigenicity of stably ERβ-transfected MCF-7 breast cancer cells. Mol Cell Endocrinol 274:19–29
56. Song K, Pan ZZ (2012) Estrogen receptor beta agonist diarylpropionitrile counteracts the estrogenic activity of estrogen receptor alpha agonist propylpyrazole-triol in the mammary gland of ovariectomized Sprague Dawley rats. J Steroid Biochem Mol Biol 130:26–35
57. Mann S, Laucirica R, Carlson N et al (2001) Estrogen receptor beta expression in invasive breast cancer. Hum Pathol 32:113–118
58. Murphy LC, Leygue E, Niu Y et al (2002) Relationship of coregulator and oestrogen receptor isoform expression to de novo tamoxifen resistance in human breast cancer. Br J Cancer 87:1411–1416
59. Esslimani-Sahla M, Simony-Lafontaine J, Kramar A et al (2004) Estrogen receptor beta (ER beta) level but not its ER beta cx variant helps to predict tamoxifen resistance in breast cancer. Clin Cancer Res 10:5769–5776
60. 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
61. Porter W, Saville B, Hoivik D et al (1997) Functional synergy between the transcription factor Sp1 and the estrogen receptor. Mol Endocrinol 11:1569–1580
62. Duan R, Porter W, Safe S (1998) Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. Endocrinology 139:1981–1990
63. Vyhlidal C, Samudio I, Kladde MP et al (2000) Transcriptional activation of transforming growth factor alpha by estradiol: requirement for both a GC-rich site and an estrogen response half-site. J Mol Endocrinol 24:329–338
64. Nagy L, Kao HY, Chakravarti D et al (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell 89:373–380
65. Hu X, Lazar MA (1999) The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature 402:93–96
66. Webb P, Anderson CM, Valentine C et al (2000) The nuclear receptor corepressor (N-CoR) contains three isoleucine motifs (I/LXXII) that serve as receptor interaction domains (IDs). Mol Endocrinol 14:1976–1985
67. Jiang S, Meyer R, Kang K et al (2006) Scaffold attachment factor SAFB1 suppresses estrogen receptor α-mediated transcription in part via interaction with nuclear receptor corepressor. Mol Endocrinol 20:311–320
68. De Amicis F, Zupo S, Panno ML et al (2009) Progesterone receptor B recruits a repressor complex to a half-TRE site of the estrogen receptor α gene promoter. Mol Endocrinol 23:454–465
69. Ghisletti S, Huang W, Jepsen K et al (2009) Cooperative NCoR/SMRT interactions establish a corepressor-based strategy for integration of inflammatory and anti-inflammatory signaling pathways. Gene Dev 23:681–693