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13. ABSTRACT (Maximum 200 Words)

In the present work we demonstrate that estradiol and its metabolites mainly 4-OH estradiol are able to induce transformation phenotypes in the human breast epithelial cells (HBEC) MCF-10F. MCF10F cells is ERa negative, although, they ER-bpositive that could indicate that the response of the cells to growth and form colonies in agar methocel could be mediated by this receptor. However, the Invasion phenotype is not modified when the cells are treated in presence of tamoxifen or ICI, suggesting that other pathways may be involved.. With the data presently available the direct effect of 4-OH-E2 support the concept that metabolic activation of estrogens mediated by various cytochrome P450 complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects leading to transformation. This assumption was confirmed when we found that all the transformation phenotypes induced by 4-OH-E2 were not abrogated when this compound was used in presence of the pure antiestrogenic ICI. We have detected loss of heterozygosity (LOH) in ch13q12.2-12.3 (D13S893) and in ch17q21.1 that has been reported in primary breast cancer, that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.

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Estrogens and Breast Cancer
PI: Jose Russo, MD
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A-INTRODUCTION

Estradiol-17β is biologically the most active estrogen in breast tissue. Circulating estrogens are mainly originated from ovarian steroidogenesis in premenopausal women and peripheral aromatization of ovarian and adrenal androgens in postmenopausal women (1). The importance of ovarian steroidogenesis in the genesis of breast cancer is highlighted by the fact that occurring naturally or induced early menopause prior to age 40 significantly reduces the risk of developing breast cancer (1). However, the uptake of estradiol-17β from the circulation does not appear to contribute significantly to the total content of estrogen in breast tumors, since the majority of estrogen present in the tumor tissues is derived from *de novo* biosynthesis (1). In fact, the concentrations of estradiol-17β in breast cancer tissues do not differ between premenopausal and postmenopausal women, even though plasma levels of estradiol-17β decrease by 90% following menopause (2). This phenomenon might be explained by the observation that enzymatic transformations of circulating precursors in peripheral tissues contribute 75% of estrogens in premenopausal women and almost 100% in postmenopausal women (3,4), the data that highlight the importance of *in situ* metabolism of estrogens. Three main enzyme complexes that are involved in the synthesis of biologically active estrogen (i.e. estradiol-17β) in the breast are: 1) aromatase that converts androstenedione to estrone, 2) estrone sulfatase that hydrolyses the estrogen sulfate to estrone, and 3) estradiol-17β hydroxysteroid dehydrogenase that preferentially reduces estrone to estradiol-17β in tumor tissues (5, 6).

Although 67% of breast cancers are manifested during the postmenopausal period, a vast majority, 95%, is initially hormone-dependent (1). This indicates that estrogens play a crucial role in their development and evolution (7-9). However, it is still unclear whether estrogens are carcinogenic to the human breast. There are three mechanisms that have been considered to be responsible for the carcinogenicity of estrogens: receptor-mediated hormonal activity, which has generally been related to stimulation of cellular proliferation, resulting in more opportunities for accumulation of genetic damages leading to carcinogenesis (10), a cytochrome P450-mediated metabolic activation, which elicits direct genotoxic effects by increasing mutation rates (11,2), and the induction of aneuploidy by estrogen (13-20). There is also evidence that estrogen compromises the DNA repair system and allows accumulation of lesions in the genome essential to estrogen-induced tumorigenesis (21).

A-i- Receptor mediated pathway.

The receptor-mediated activity of estrogen is generally related to induction of expression of the genes involved in the control of cell cycle progression and growth of human breast epithelium. The biological response to estrogen depends upon the local concentrations of the active hormone and its receptors. The level of ER expression is higher in breast cancer patients than in control subjects and is related to breast cancer risk in postmenopausal women (22). It has been suggested that overexpression of ER in normal human breast epithelium may augment estrogen responsiveness and hence the risk of breast cancer (22). The proliferative activity and the percentage of ERα-positive cells are highest in Lob 1 in comparison with the various lobular structures composing the normal breast. These findings provide a mechanistic explanation for the higher susceptibility of these structures to be transformed by chemical carcinogens *in vitro* (23,24), supporting as well the observations that Lob 1 are the site of origin of ductal carcinomas (25).

The presence of ERα-positive and ERα-negative cells with different proliferative activity in the normal human breast may help to elucidate the genesis of ERα-positive and ERα-negative breast cancers (26,

27). It has been suggested that either ERα-negative breast cancers result from the loss of the ability of the cells to synthesize ERα during clinical evolution of ERα-positive cancers, or that ERα-positive and ERα-negative cancers are different entities (27,28). Based on these observations, it is postulated that Lob 1 contain at least three cell types, ERα-positive cells that do not proliferate, ERα-negative cells that are capable of proliferating (Figure 1), and a small proportion of ERα-positive cells that can proliferate as well (29). Therefore, estrogen might stimulate ERα-positive cells to produce a growth factor that in turn stimulates neighboring ERα-negative cells capable of proliferating (29) (Figure 2). In the same fashion, the small proportion of cells that are ERα-positive and can proliferate could be the stem cell of ERα-positive tumors. The possibility exists, as well, that the ERα-negative cells convert to ERα-positive cells (29) or that they express ER-β.

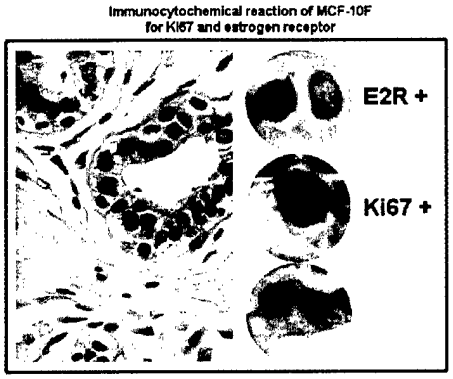

Figure 1: Ductal epithelium of the human breast. Single-layered epithelium of Lob 1 ductules contains Ki67 positive cells (brown nuclei), and ER positive cells (red-purple nuclei) (DAB-Hematoxylin) (x40).

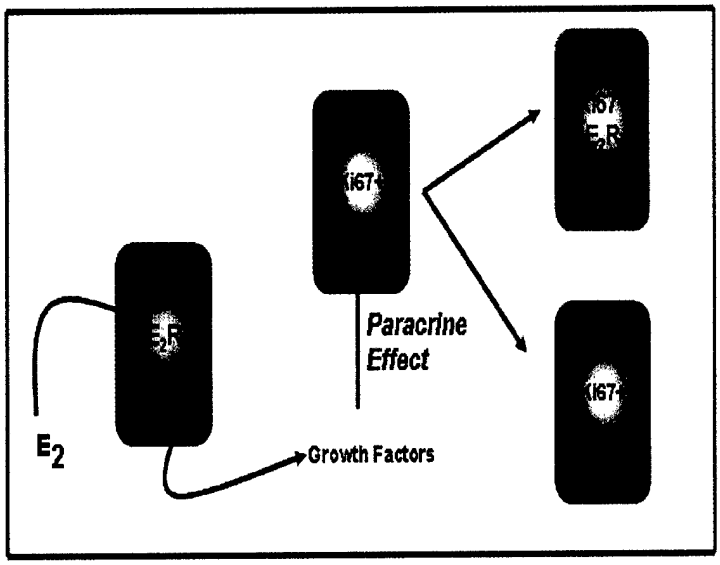

Figure 2: ***Schematic representation of the postulated pathways of estrogen actions on breast epithelial cells. Three different types of cells can be considered to be present in the mammary epithelium: Estrogen receptor negative (ER-) proliferating cells (Ki67 positive), ER positive (ER+) cells that do not proliferate (Ki67 negative), and a small proportion of ER+ and Ki67+ cells (Not shown). Estrogen might stimulate ER+ cells to produce a growth factor that in turn stimulates neighboring ER⁻ cells*** **capable of proliferating. ER+Ki67+ cells can proliferate and could be stimulated by estrogen to originate ER+ daughter cells or probably tumors. ER- cells may convert to ER+ cells during neoplastic transformation.**

The newly discovered ERβ opens another possibility that those cells traditionally considered negative for ERα might be positive for ERβ (30-32). It has recently been found that ERβ is expressed during the immortalization and transformation of ER-negative human breast epithelial cells (33), supporting the hypothesis of conversion from a negative to a positive receptor cell. The functional role of ERβ-mediated estrogen signaling pathways in the pathogenesis of malignant diseases is essentially unknown. In the rats, ERβ-mediated mechanisms have been implicated in the upregulation of PgR expression in the dysplastic acini of the dorsolateral prostate in response to treatment of testosterone and estradiol-17β (34). In the human, ERβ has been detected in both normal and cancerous breast tissues and cell lines, and is the predominant ER type in normal breast tissue. Expression of ERβ in breast tumors is inversely correlated with the PgR status and variant transcripts of ERβ have been observed in some breast tumors (1). ERβ and ERα are co-expressed in some breast tumors and a few breast cell lines, suggesting an interesting possibility that ERα and ERβ proteins may interact with each other and discriminate between target sequences leading to differential responsiveness to estrogens. In addition, estrogen responses mediated by ERα and ERβ may vary with different composition of their co-activators that transmit the effect of ER-ligand complex to the transcription complex at the promotor of target genes (35). Recently, it has been shown that an increase in the expression of ERα with a concomitant reduction in ERβ expression occurs during tumorigenesis of the breast (36) and ovary (37), but breast tumors expressing both ERα and ERβ are lymph node-positive and tend to be of higher histopathological grade (1). These data suggest a change in the interplay of ERα- and ERβ-mediated signal transduction pathways during breast tumorigenesis.

Even though it is now generally believed that alterations in the ER-mediated signal transduction pathways contribute to breast cancer progression toward hormonal independence and more aggressive phenotypes, there is also mounting evidence that a membrane receptor coupled to alternative second messenger signaling mechanisms (38, 39) are operational, and may stimulate the cascade of events leading to cell proliferation. This knowledge suggests that ERα-negative cells found in the human breast may respond to estrogens through this or other pathways. The biological responses elicited by estrogens are mediated, at least in part, by the production of autocrine and paracrine growth factors from the epithelium and the stroma in the breast (40). In addition, evidence has accumulated over the last decade supporting the existence of ER variants, mainly a truncated ER and an exon deleted ER (41). It has been suggested that expression of ER variants may contribute to breast cancer progression toward hormone independence (41). Although more studies need to be done in this direction, it is clear that the findings that in the normal breast the proliferating and steroid hormone receptor positive cells are different open new possibilities for clarifying the mechanisms through which estrogens might act on the proliferating cells to initiate the cascade of events leading to cancer.

A-ii- Oxidative metabolism of estrogen.

There is evidence that oxidative catabolism of estrogens mediated by various cytochrome P450 (CYP) complexes constitutes a pathway of their metabolic activation and generates reactive free radicals and intermediate metabolites reactive intermediates that can cause oxidative stress and genomic damage directly (11, 12). Estradiol-17β and estrone, which are continuously interconverted by estradiol-17β hydroxysteroid dehydrogenase (or 17β-oxidoreductase), are the two major endogenous estrogens (Figure 3). They are generally metabolized via two major pathways: hydroxylation at C-16α position and at the C-2 or C-4 positions (42, 43). The carbon position of the estrogen molecules to be hydroxylated differs among various tissues and each reaction is probably catalyzed by various CYP isoforms. For example, in MCF-7 human breast cancer cells, which produce catechol estrogens in culture, CYP 1A1 catalyzes hydroxylation of estradiol-17β at C-2, C-15α and C-16α, CYP 1A2 predominantly at C-2 (1, 44), and a

member of the CYP 1B subfamily is responsible for the C-4 hydroxylation of estradiol-17β. CYP3A4 and CYP3A5 have also been shown to play a role in the 16α-hydroxylation of estrogens in human (1).

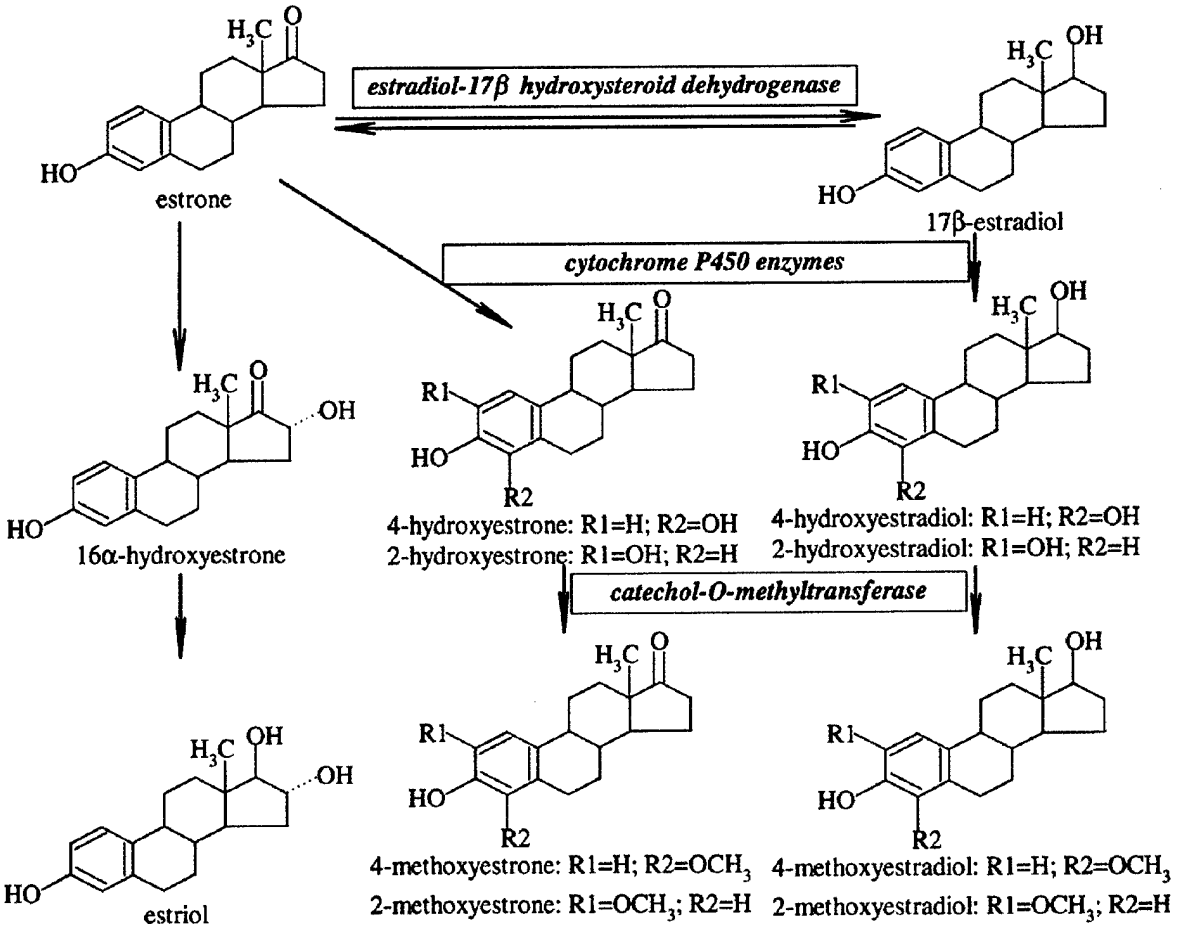

Figure 3: Biosynthesis and steady-state control of catechol estrogens in human breast tissues.

The hydroxylated estrogens are catechol estrogens that will easily be autooxidated to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA via a Michael addition and, thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidatic activation of catechol estrogens. In addition, a redox cycle consisting of the reversible formation of the semiquinones and quinones of catechol estrogens catalyzed by microsomal P450 and cytochrome P450-reductase can locally generate superoxide and hydroxyl radicals to produce additional DNA damage (Figure 4). Furthermore, catechol estrogens have been shown to interact synergistically with nitric oxide present in human breast generating a potent oxidant that induces DNA strand breakage (1). Steady state concentrations of catechol estrogens are determined by the cytochrome P450-mediated hydroxylations of estrogens and monomethylation of catechols catalyzed by blood-borne catechol *o*-methyltransferase (45, 46). Increased formation of catechol estrogens as a result of elevated hydroxylations of estradiol-17β at C-4 and C-16α (1, 47) positions occurs in human breast cancer patients and in women at a higher risk of developing this disease. There is also evidence that lactoperoxidase,

present in milk, saliva, tears and mammary glands, catalyzes the metabolism of estradiol-17β to its phenoxyl radical intermediates, with subsequent formation of superoxide and hydrogen peroxide that might be involved in estrogen-mediated oxidative stress (48). A substantial increase in base lesions observed in the DNA of invasive ductal carcinoma of the breast (49) has been postulated to result from the oxidative stress associated with metabolism of estradiol-17β (48).

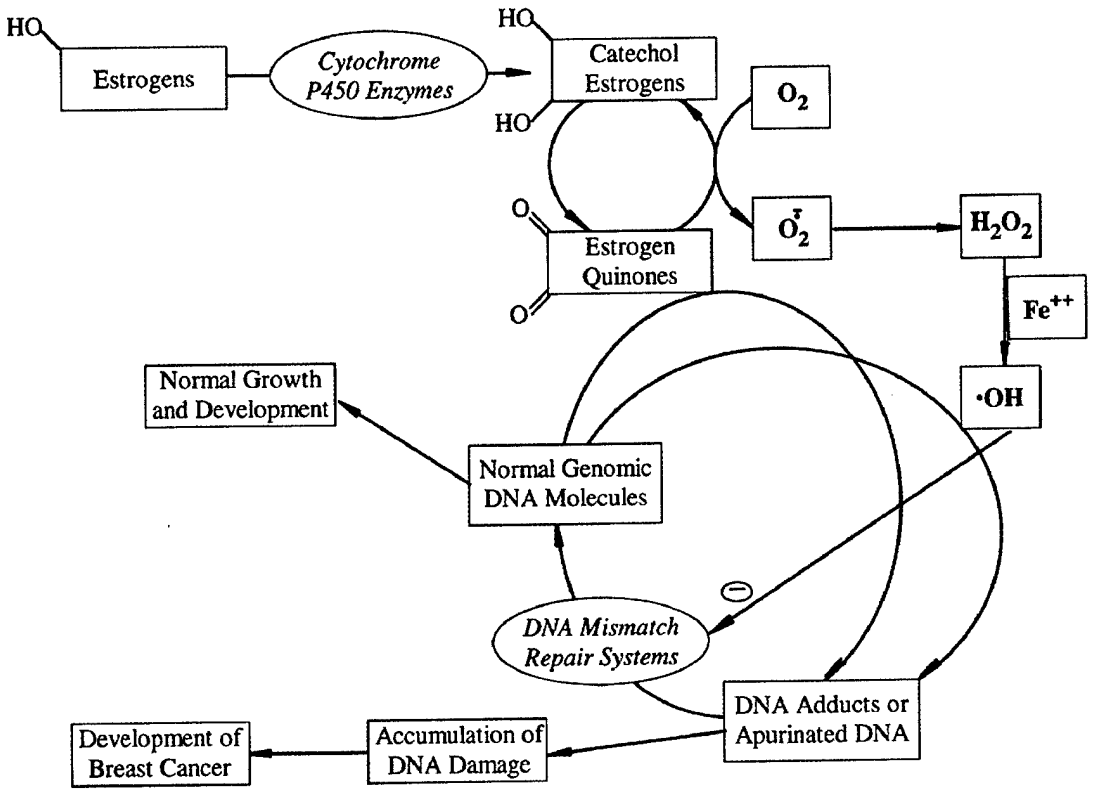

Figure 4:Carcinogenic effects associated with the metabolisms of catechol estrogens in human breast tissues

The detection of various types of DNA damage induced by estrogen metabolites in cell-free systems or in cells in culture and by parent hormones *in vivo* (50-54) has led to the hypothesis of an additional role of estrogen as mutagen and tumor initiator (55,56). The induction of mutations by estrogens or their metabolites has been demonstrated (57, 58) supporting the hypothesis that estrogens are mutagenic and that metabolic conversion of E_2 to cathecol estrogen is required for the induction of such mutations. In addition to mutations, E2 also induces microsatellite instability. Changes in DNA fragments containing microsatellite repeat sequences have been detected in E_2-induced hamster kidney tumors, in surrounding kidney tissue (59) and in MCF-10F HBEC transformed by E_2 (60). Microsatellite instability is a relatively common genetic modification (61-63), induced by the natural hormone E_2 in cells in culture (109), in Syrian hamster kidney tumors, and in surrounding tissues (59). It has also been detected with high frequency in human vaginal tumors in daughters of women treated with diethylstilbestrol (DES) (64). Microsatellite instability has also been detected in human breast tumors (65-72).

Chemical carcinogens covalently bind to DNA to form two types of adducts: stable ones that remain in DNA unless removed by repair and depurinating ones that are lost from DNA by destabilization of the glycosyl bond (73-74). Evidence that depurinating polycyclic aromatic hydrocarbon-DNA adducts play a major role in tumor initiation (73-75) and that estrogen metabolites form depurinating DNA adducts strongly indicates that estrogen is an endogenous initiators of cancer (50). Catechol estrogens (CE) are among the major metabolites of estrone (E_1) and estradiol (E_2). If these metabolites are oxidized to the electrophilic CE quinones (CE-Q), they may react with DNA. Specifically, the carcinogenic 4-CE (51, 76) are oxidized to CE-3,4-Q, which react with DNA to form depurinating adducts (50, 77). These adducts generate apurinic sites that may lead to oncogenic mutations (75, 77-79), thereby initiating cancer.

The breast is an endocrine organ and can synthesize E_2 *in situ* from precursor androgens via the enzyme aromatase (1). Breast tissue contains aromatase and produces amounts of E_2 that exert biologic effects on proliferation. The effects of local production exceed those exerted in a classical endocrine fashion by uptake of E_2 from plasma. One critical factor is excessive synthesis of E_2 by overexpression of CYP19 in target tissues (80-84) and/or the presence of excess sulfatase that converts stored E_1 sulfate to E_1 (85). The observation that breast tissue can synthesize E_2 *in situ* suggests that much more E_2 is present in some locations of target tissues than would be predicted from plasma concentration (84). A second critical factor might be high levels of 4-CE due to overexpression of CYP1B1, which converts E_2 predominantly to 4-OHE_2 (86-88). This could result in relatively large amounts of 4-CE and, subsequently, more extensive oxidation to their CE-3, 4-Q. A third factor could be a lack or low level of COMT activity. If this enzyme is insufficient, either through a low level of expression or its low activity allele, 4-CE will not be effectively methylated, but will be oxidized to the ultimate carcinogenic metabolite, CE-3, 4-Q. Fourth, a low level of GSH and/or low levels of quinone reductase and/or CYP reductase can leave available a higher level of CE-Q that may react with DNA.

The effects of some of these factors have already been observed in analyses of breast tissue samples from women with and without breast cancer (89). The levels of E_1 (E_2) in women with carcinoma were higher. In women without breast cancer, a larger amount of 2-CE than 4-CE was observed. In women with breast carcinoma, the 4-CE were 3.5 times more abundant than the 2-CE and were 4 times higher than in the women without breast cancer. Furthermore, a statistically lower level of methylation was observed for 2-CE and 4-CE in cancer cases *vs* controls. Finally, the level of CE-Q conjugates in women with cancer was 3 times that in the controls, suggesting a larger probability for the CE-Q to react with DNA in the breast tissue of women with carcinoma. The levels of $E_1(E_2)$ ($p<0.02$) and quinone conjugates ($p<0.01$) are highly significant predictors of breast cancer, and the levels of methylated CE ($p<0.02$) are significant predictors of protection against breast cancer. Altogether, these data are supporting the concept that estrogen and its metabolites can be found at high concentration in the breast tissue indicating a direct carcinogenic effect in the breast epithelial cells (89).

A-iii- Estrogens as inducers of aneuploidy.

Breast cancer is considered the result of sequential changes that accumulate over time. DNA content changes, i.e., loss of heterozygosity (LOH) and aneuploidy, can be detected at early stages of morphological atypia, supporting the hypothesis that aneuploidy is a critical event driving neoplastic development and progression (90, 91). Aneuploidy is defined as the gain or loss of chromosomes; it is a dynamic, progressive, and accumulative event that is almost universal in solid tumors (92, 93). The extensive array of altered gene expression observed in tumors and the numerous altered chromosomes detected by CGH (19, 94) provide striking evidence that aneuploidy can totally disrupt cell homeostatic

control. The main question is whether aneuploidy is a consequence of neoplastic development or a cause of neoplastic development (19, 20, 94). One of the several mechanisms proposed for the development of aneuploidy is the failure to appropriately segregate chromosomes (20, 21, 95). For example interference with mitotic spindle dynamics, abnormal centrosome duplication, altered chromosome condensation and cohesion, defective centromeres, and loss of mitotic checkpoints (95). Functional consequences of centrosome defects may play a role during neoplastic transformation and tumor progression, increasing the incidence of multipolar mitoses that lead to chromosomal segregation abnormalities and aneuploidy. In considering estrogen as a carcinogenic agents there is evidence that they affect microtubules (96) and a recently report indicates that progesterone may facilitate aneuploidy (97). The importance of these findings is magnified with the recent publications that demonstrate women on hormone replacement treatments that include progesterone have increased mammographic breast density and increased breast cancer risk than women taking only estrogen (98-100).

In the center stage of the research endeavor on aneuploidy are the centrosomes that are organelles that nucleate microtubule growth and organize the mitotic spindle for segregating chromosomes into daughter cells, establishing cell shape and cell polarity, processes essential for epithelial gland organization (19,95). Centrosomes also coordinate numerous intracellular activities, in part by providing a site enriched for regulatory molecules, including those that control cell cycle progression, centrosome and spindle function, and cell cycle checkpoints (20, 101). Although the underlying mechanisms for the formation of abnormal centrosomes are not clear, several possibilities have been proposed and implicated in the development of cancer such as alterations of checkpoint controls initiating multiple rounds of centrosome replication within a single cell cycle and failure of cytokinesis, cell fusion, and cell cycle arrest in S-phase uncoupling DNA replication from centrosome duplication (102).

To fully demonstrate that estrogens are carcinogenic in the human breast through one or more of the mechanisms explained above it will require an experimental system in which, estrogens by itself or one of the metabolites would induce transformation phenotypes indicative of neoplasia in HBEC *in vitro* and also induce genomic alterations similar to those observed in spontaneous malignancies, such as DNA amplification and loss of genetic material that may represent tumor suppressor genes (103-118).

B-BODY

The information described below represents all the data obtained under this grant award and constitutes the final report of our work.

B-i-The in vitro model of cell transformation

The transforming potential of estrogens on human breast epithelial cells (HBEC) *in vitro,* have being evaluated by utilizing the spontaneously immortalized HBEC MCF-10F (119,120) (Figure 5). The spontaneously immortalized MCF-10F cells, treated cells and derived clones were maintained in DMEM:F-12 [1:1] medium with a 1.05 mM Ca^{2+} concentration. All cell lines were regularly tested for correct identity using a fingerprint cocktail of three minisatellite plasmid probes (ATCC, Rockville, MD). Culture media were prepared by the Central Center Tissue Culture Facility at the Fox Chase Cancer (Philadelphia, PA). In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were first treated with 0, 0.007nM, 70nM and 1μM of E_2, DES, BP, Progesterone, 2-OH-E2, 4-OH-E2 and 16-α-OH E2 at 72 hrs and 120 hours post plating. Treatments were repeated during the second week, and cells were collected at the 14^{th} day for phenotypic and genotypic analysis. At the end of each treatment period, the culture medium was replaced with fresh medium. At the end of the second week of

treatment, the cells were assayed for determination of, survival efficiency (SE), colony efficiency (CE), colony size (CS), ductulogenic capacity and invasiveness in a reconstituted basement membrane [21, 22].

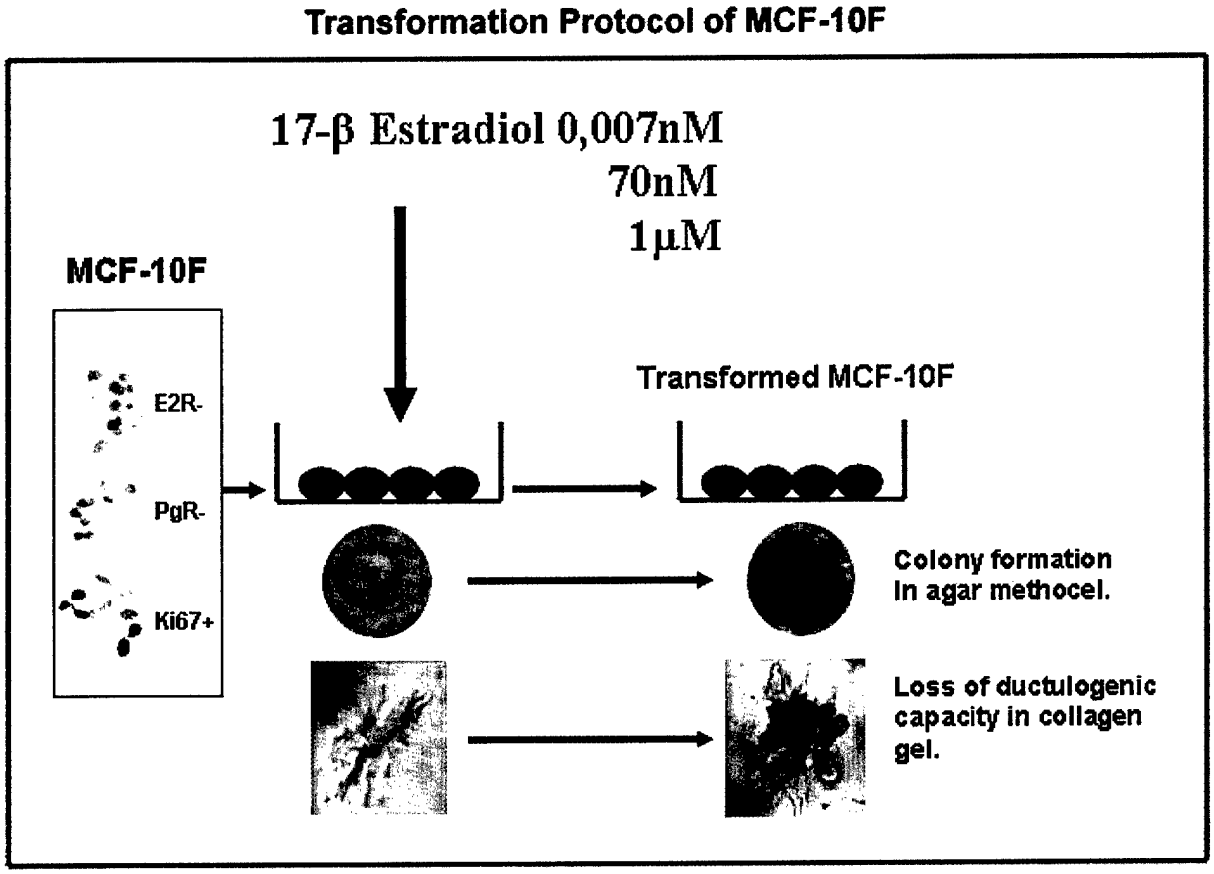

Figure 5: MCF-10F cells are $E_2R\alpha$ and progesterone receptor (PgR) negative but they proliferate and are positive for Ki67. MCF10F treated with estrogen or its metabolites at different concentration are transformed and selected in agar-methocel for colony assay. Control cells do not form colonies. Colonies are formed in E_2., 2-OH-E_2, 4-OH-E_2, 16-α-OH-E_2, and BP-treated MCF-10F cells. MCF-10F cells treated with different doses of E_2 or its metabolites induce the loss of the ductulogenic capacity in collagen gel. This in vitro technique evaluates the capacity of cells to differentiate by providing evidence of whether-treated cells form three-dimensional structures when grown in a collagen matrix.

B-ii-Tranformation effect of estrogens and its metabolites in MCF-10F cells

We have determined the optimal doses for the expression of the cell transformation phenotype by treating the immortalized human breast epithelial cells (HBEC) MCF-10F with 17β-estradiol (E_2) with 0.0, 0.07 nM, 70 nM, or 1 μM of E_2 twice a week for two weeks. The survival efficiency (SE) was increased with 0.007nM and 70 nM of 17 β estradiol and decrease with 1 μM and the proliferative activity of these E2 transformed cells, measured by the percentage of cells in the S phase of the cell cycle, was also increased in a dose dependent fashion. The cells treated with either doses of E2 formed colonies in agar methocel

and the size was not different among them, however, the CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses (Figure 6).

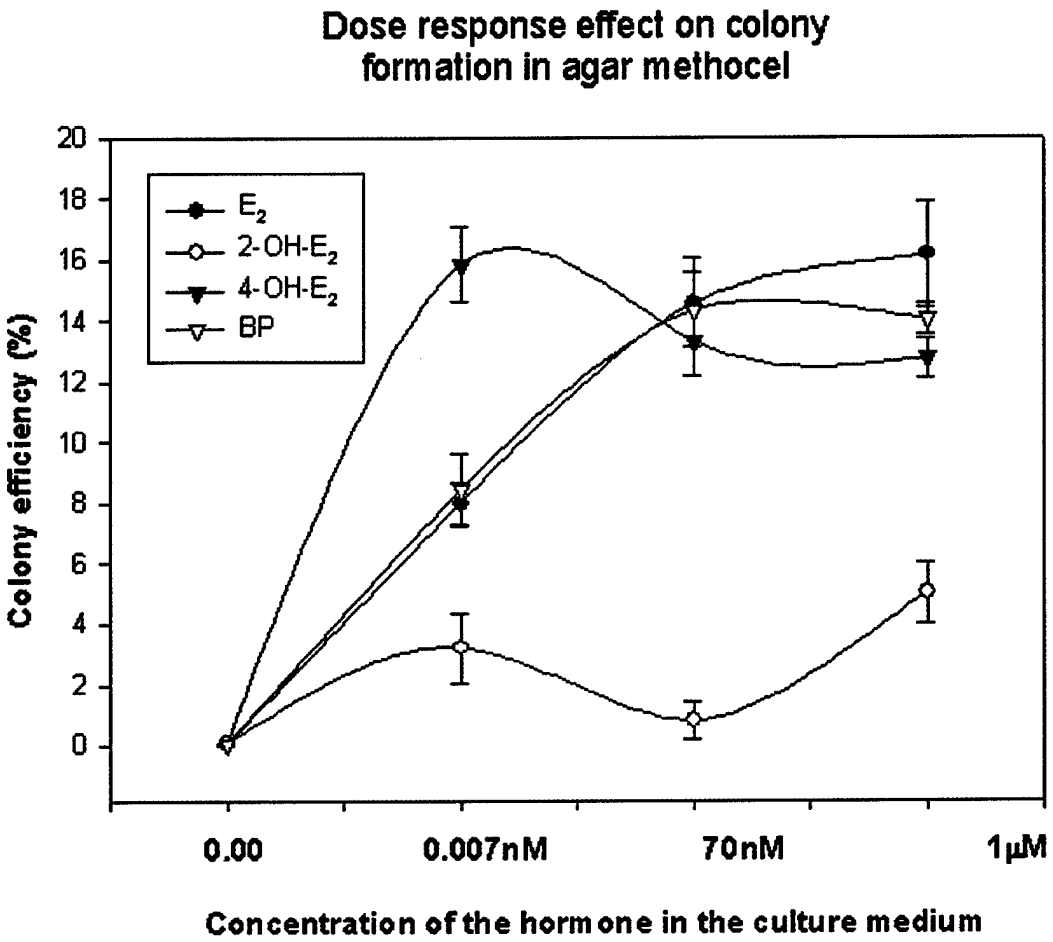

Figure 6: Curves showing the dose response effect of MCF-10F cells to the transforming effect of 17-β estradiol or its metabolites. The left ordinate depicts the percentage of colonies or colony efficiency (CE) of MCF-10F cells. The CE was determined by a count of the number of colonies greater than 100 µm in diameter, and expressed a percentage of the original number of cells plated per well.

Ductulogenesis was quantitatively evaluated by estimating the ability of the cell plated in collagen to form tubules or spherical masses (SM). Non-transformed cells produce ductules like structure and transformed cells produce spherical or solid masses of cells. Cells treated with DMSO, cholesterol or progesterone at different concentrations was unable to alter the ductular pattern. E2, BP and DES treated cells induces the loss of MCF10F cells to produce ductules in a dose dependent fashion and the number of solid masses paralleled the formation of colonies in agar methocel. Histological analysis shows that MCF10-F cells form ductules in collagen matrix that are lined by a single layer of cuboidal epithelial cells, this pattern was not disturbed by cholesterol or progesterone treatment. Most of the cells growing in the collagen matrix are actively proliferating as detected by immunostaining with Ki67.

2-OH-E_2, 4-OH-E_2, and 16α-OH-E_2 induce the formation of colonies in agar methocel. Cells treated with cholesterol were unable to produce colonies. The size of the colonies was significantly smaller in those cells treated with 2-OH-E2 or progesterone. Whereas the number of colonies was dose dependent reaching its maximum efficiency at the concentration of 70nM for most of the compounds, 4-OH-E2 was

the most efficient in inducing larger colonies and number at a doses of 0.007nM. E2, and BP behave very similar and are more transforming agents than DES and 2-OH-E2 (Figure 6).

The metabolites of estrogen significantly impair the formation of ductules replacing them by structures filled by large cuboidal cells. Some of the cells present cytoplasmic vacuolization and piknosis. Cells treated with 2-OH E2 or 16-α-OH-E2 is less efficient in altering the ductulogenic capacity. Importantly 4-OH-E2 at a dose of 0.007nM induces significant changes in the ductulogenic capacity with a maximal number of solid masses. These structures also have a high proliferative index.

The invasiveness capacity of E2, DES, 4OH-E2 and BP transformed cells measured in the Boyden Chamber, was very high when compared with the control or those treated with DMSO, P, or 2OH-E2 (Figure 7)

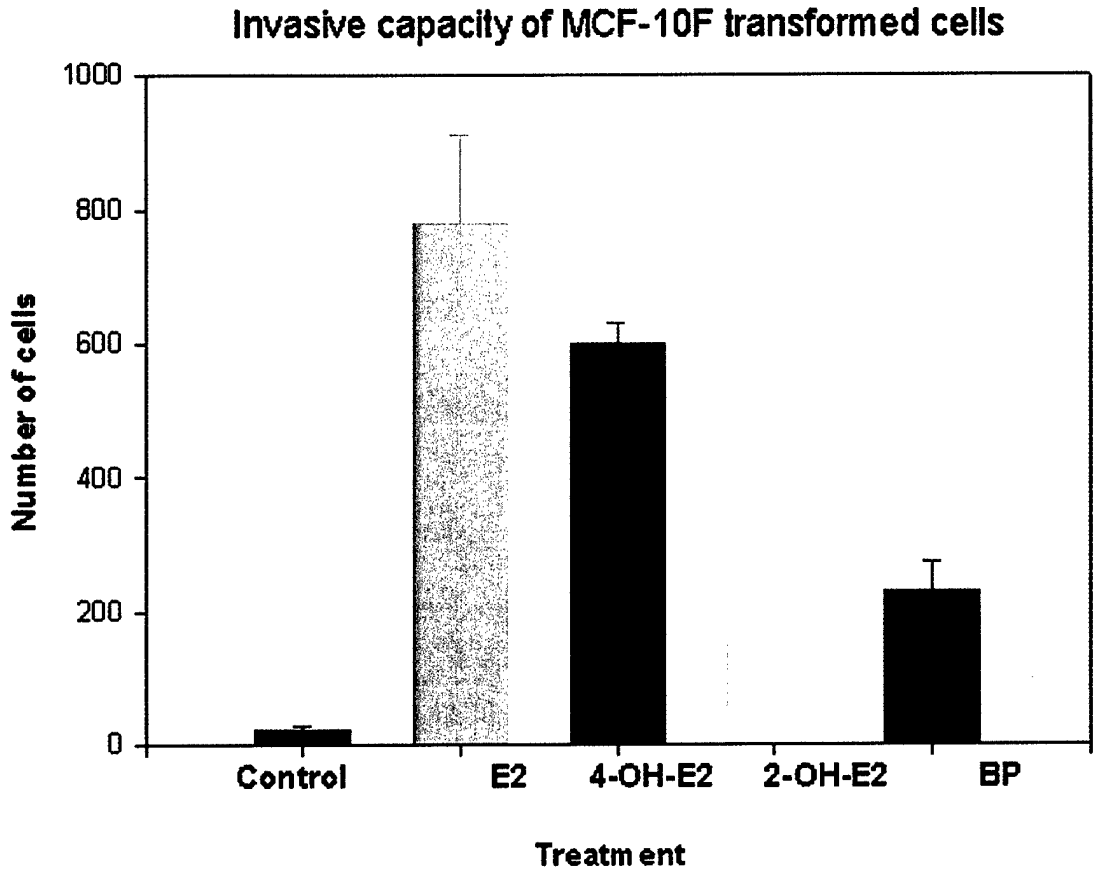

Figure 7: Histogram depicting the invasive capacity of MCF-10F cells treated with different compounds (abscise) as indicated in figure 5. The ordinate shows the numbers of cells that have crossed the matrigel membrane.

B-iii-Antiestrogens in the expression of the transformation phenotype

The proliferative activity of the MCF-10F cells that has been treated with Tamoxifen alone or ICI-182,780 was not modified when compared with the control. Instead those cells that were treated with 17-β-estradiol in presence of Tamoxifen or ICI-182,780 showed no increment of the proliferative activity neither in monolayer nor collagen matrix. The colony formation in agar methocel was abrogated and the

ductulogenic capacity was maintained. The proliferative activity of these cells in collagen matrix was also abrogated. 4-OH-E2 transforming efficiency was not abrogated by ICI neither in the colony efficiency assay nor in the loss of ductulogenic capacity. The histology of the solid masses induced by 4-OH estradiol in collagen matrix were not modified by ICI, even the number of cells was significantly higher. ICI-182,780 was unable to abrogate the invasive phenotype induced by estrogen and tamoxifen even exacerbate the invasive phenotype.

B-IV-Detection of estrogen receptors in MCF 10F cells

The ER alpha was not detected in the MCF-10Fcells or in those transformed by estrogens or its metabolites. The positive control MCF-7 cells was positive for ER alpha showing by Western blot the specific band corresponding to a 67 kDa, instead the band was absent in the negative control MDA-MB-235 cell line. The ER beta protein expression analysis showed two bands 68 and 53 kDa of molecular weight corresponding to ER beta long and short form, respectively. Both bands were present in the MCF-10F cells and in the transformed cells. Those cells transformed by 17β estradiol as well as those treated with progesterone significantly overexpressed the long form of ER beta. Instead, MCF-7 cells showed the short form of the ER beta.

The progesterone receptor (PR) expression was negative in the MCF-10F cells when compared with MCF-7 cells that was used a positive control presenting the 186 and 82 kDa PR long and short form respectively. The estrogen-transformed cells also expressed PR.

B-v-Genomic changes induced by estrogen and its metabolites in the transformation of human breast epithelial cells.

In order to determine if the gene expression profile induced by E2, 4-OH estradiol and BP were the same or whether they are divergent in their pattern of expression, mRNA from these transformed cells was extracted and hybridized to cDNA array membranes that contained 1,176 human genes (Clontech Human Cancer 1,2 array).

Table 1
Common up-regulated genes in MCF-10F cells transformed by Bp, E2 and 4OH using cDNA array

Gene Description	Swissprot #	Function	Bp/10F	E2/10F	4OH/10F
c-myc oncogene	P01106	Oncogene	3.24	3.66	6.21
fos-related antigen	P15407	Oncogene	10.25	2.31	15.04
HER3	P21860	Oncogene	2.09	3.32	7.95
SRF accessory protein 2	P41970	Transcription	3.61	2.46	9.11
hEGR1	P18146	Transcription	3.2	6.49	2.91
Splicing factor 9G8	Q16629	mRNA processing	2.23	2.93	4.42
antigen KI-67	P46013	Cell proliferation	3.2	2.7	5.97
HMG-I	P17096	Chromatin	2.36	3.26	7.95
nm23-H4	O00746	Kinase	2.02	2	2.24
cytokeratin 2E	P35908	Keratin	43.09	2.38	4.37

Table 2

. Common down-regulated genes in MCF-10F cells transformed by Bp, E2 and 4OH using cDNA array

Array Location	Gene Description	Swissprot #	Function	Bp/10F	E2/10 F	4OH/10 F
A11g	PIG7	Q99732	Tumor suppressor	0.02	0.04	0.19
A14h	CD82 antigen	P27701	Tumor suppressor	0	0.18	0
B06k	rho GDP dissociation inihibitor 2	P52566	Tumor suppressor	0	0	0.21
A02g	neurogenic locus notch protein	Q04721	Transcription	0.29	0.47	0.38
A13h	active breakpoint cluster region-related protein	Q12979	Transcription	0.13	0.25	0.46
A14c	ets-related protein tel	P41212	Transcription	0	0.08	0.08
C06m	B4-2 protein	Q12796	Transcription	0	0	0
B03n	T3 receptor-associating cofactor 1	O00613	Intracellular transducers	0.48	0.41	0.22
E04b	HDGF	P51858	Growth factor	0.34	0.1	0.24
F07i	HNRNPK	Q07244	mRNA processing	0	0	0.17
B02j	RalB GTP-binding protein	P11234	G protein	0	0.24	0
B04j	rhoC	P08134	G protein	0.09	0.06	0.48
B12j	p21-rac2	P15153	G protein	0.12	0.2	0.49
B13i	p21-rac1	P15154	G protein	0	0	0.33
A06j	CDK5	Q00535	Kinase	0.18	0	0.41
B05h	NDR protein kinase	Q15208	Kinase	0	0	0
B08c	tissue-specific extinguisher 1	P10644	Kinase	0	0	0.19
A09l	CDKN1A	P38936	Kinase inhibitor	0.09	0.03	0.08
A10d	HGF-SF receptor	P08581	Kinase inhibitor	0	0	0.31
B02m	hint protein	P49773	Kinase inhibitor	0	0	0.37
B07l	calvasculin	P26447	Calcium-binding	0	0.11	0.46
B09n	CD27 ligand	P32970	Death receptor ligand	0.37	0	0
C02c	BAG-1	Q99933	BCL family protein	0	0	0.19
C09m	AH receptor	P35869	Nuclear receptor	0.06	0.12	0
F04i	lipocalin 2	P80188	Trafficking	0	0	0
F09h	TRAM protein	Q15629	Trafficking	0	0	0.29
F10h	dual-specificty A-kinase anchoring protein 1	Q92667	Targeting	0	0.19	0.24
D01d	cadherin 3)	P22223	Cell adhesion	0.32	0.14	0.08
D02e	integrin beta 6 precursor	P18564	Cell adhesion	0.16	0.11	0.22
E02f	IGF-binding protein 3	P17936	Hormone	0	0	0
E02m	HLA-C	Q30182	Immune	0.19	0.17	0
E02n	GRP 78	P11021	Immune	0	0	0
F03b	fibronectin precursor	P02751	Extracellular matrix	0.32	0.13	0.09
F13n	insulin-induced protein 1	O15503	Unclassified	0.13	0.33	0.35
F08m	PM5 protein	Q15155	Unclassified	0.17	0.34	0

Table 3

Specific up-regulated genes in BP-transformed cells by cDNA array

Array Location	Gene Description	Swissprot #	Function	BP/10F
C05l	RAR-gamma 1	P13631	Transcription	3.77
B04k	caveolin-1	Q03135	Signaling	3.35
A03b	ezrin	P15311	Oncogene	2.01
C04h	HHR23A	P54725	Stress response	2.04
C08g	mutL protein homolog	P40692	Stress response	4.31
E07h	glycosylation-inhibiting factor	P14174	Cell communication	4.44
D06e	integrin beta 4	P16144	Cell adhesion	4.24
D08e	integrin alpha 7B precursor	Q13683	Cell adhesion	3.06
D05e	integrin alpha 6 precursor	P23229	Cell adhesion	2.24
D07e	integrin alpha 1	P56199	Cell adhesion	2.31
F05d	LDHA	P00338	Carbohydrate metabolism	6.25
F08f	cytokeratin 18	P05783	Cytokeratin	3.04
F14e	BIGH3	Q15582	Microfilament	6.73

The genomic signature of the three transformed cells present a cluster of genes that are commonly unregulated (Table 1), indicating that a similar mechanism is involved in the transformation pathway. Interestingly there are genes that are upregulated in the E_2 and 4-OH-E_2 transformed cells such as the CENP-E (Table 2) that are not modified in the BP transformed cells. The same occurs for several genes that are downregulated differentially in the three transformed cells (Table 3).

B-vi-Chromosomal alterations induced by estrogens and its metabolites.

During the process of cell transformation induced by estrogen and its metabolites there is an increase in the number of multinucleated cells and abnormal mitoses that is associated with the overexpression of one component of the centromere-kinetochore complex CENP-E (Figure 8).

Figure 8: (a) Multinucleated cell, (b)abnormal mitosis observed in MCF10F cells transformed with estrogen.

It is important to emphasize that the percentage of these abnormal mitoses is lees than 1%. The movements that chromosomes undergo during mitosis are facilitated by the mitotic spindle, an apparatus composed principally of microtubule fibers that attach to a pair of kinetochores located on opposite sides of the centromere region of chromosomes. The microtubule-kinetochore interaction is essential for chromosome segregation. Disruptions of this interaction will lead to unequal distribution of chromosomes in daughter cells (123). We have found that the CENP-E, a *ca.* 300 kDa protein that have been recently identified to be a novel member of the kinesin superfamily of microtubule-based motor proteins (123) is overexpressed in MCF-10F transformed cells by estrogens and its metabolites but not in the BP transformed cells. CENP-E staining appeared only in mitotic cells (123), suggesting that it is a mitosis-specific motor. Its association with kinetochores suggests that it functions to translocate chromosomes along the spindle microtubules. This phenomena, however, was not observed in the BP transformed cells indicating that whereas aneuploidy is part of the neoplastic transformation process is depending of the carcinogenic insult and probably not the main driving force to cause genomic instability. This concept was further confirmed by the lack of significant karyotipic changes detected in these transformed cells and by the fact that the same cluster of genes were overexpressed in cells transformed with E2, 4-OH-E2 and BP (Table 1), indicating that there is a common pathway of transformation and that may be responsible for driving the normal cell to neoplasia. The data also point toward the concept that certain compounds like steroid hormones or its metabolites may affect certain genes more readily than other exerting the expression of genes that are altering the mitotic spindle and therefore making the cell aneuploidy. However, does not support the concept that aneuploidy is the driving force of transformation but a consequence of it.

B-vii-LOH in HBEC treated with estrogen and its metabolites.

Genomic DNA was analyzed for the detection of micro-satellite DNA polymorphism using 64 markers covering chromosomes (chr) 3, 11, 13 and 17. We have detected loss of heterozygosity (LOH) in ch13q12.2-12.3 (D13S893) and in ch17q21.1 (D17S800) in E_2, 2-OH-E_2, 4-OH-E_2-, E_2+ICI, E_2+Tamoxifen and BP treated cells (Figures 9 and 10).

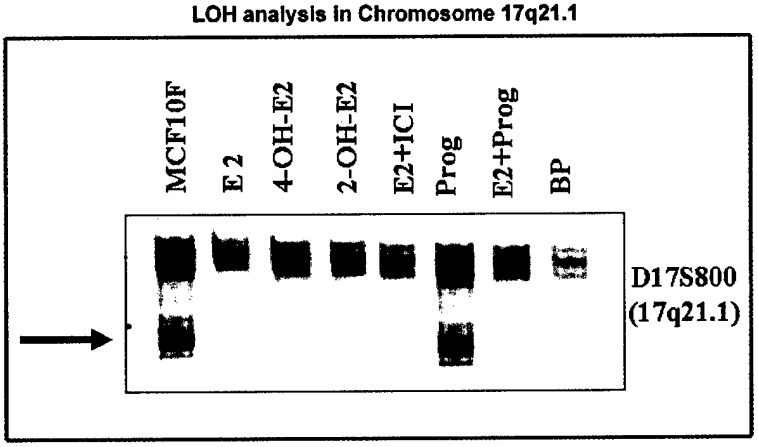

Figure 9:LOH analysis of MCF-10F, and the E_2, 4-OH-E_2, 2-OH-E_2. E_2+ICI, Prog, E_2+Prog and BP treated cells. Arrows indicate the loss of alleles in chromosome 17q21.1 using marker D17S800.

LOH in ch17q21.1-21.2 (D17S806) was also observed in E_2, 4-OH-E_2, E_2+ICI,E_2+Tamoxifen and BP-treated cells. MCF-10Fcells treated with P or P+E_2 did not show LOH in the any of the markers studied. LOH was strongly associated with the invasion phenotype. Altogether our data indicate that E_2 and its metabolites induce in HBEC LOH in loci of chromosomes 13 and 17, that has been reported in primary breast cancer, that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.

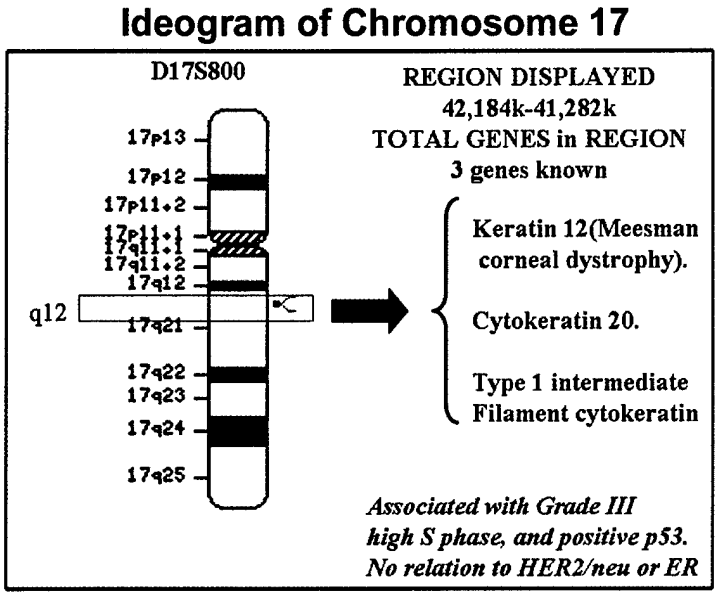

Figure 10: Ideogram of chromosome 17 depicting the locus studied and showed in figure 9.

C-KEY RESEARCH ACCOMPLISHMENTS

C-i-Short term treatment of HBEC with physiological doses of 17-β estradiol induces anchorage independent growth, colony formation in agar methocel, and reduced ductulogenic capacity in collagen gel, all phenotypes whose expression is indicative of neoplastic transformation, and that are induced by BP under the same culture conditions.

C-ii-Progesterone was unable to induce significant increase in colony formation, although small colonies less than 50 μm in diameter were observed, whereas none were found in the MCF10F cells treated with DMSO or cholesterol. The ductulogenic pattern was not impaired by progesterone but the luminal size was smaller that those found in the MCF10F cells treated with DMSO or cholesterol.

C-iii-The fact that the MCF10F cells are ERα negative, indicate that this receptor pathway is not involved in the carcinogenic process. Although the presence of ER-β may indicate that the response of the cells to growth and form colonies in agar methocel could be mediated by this receptor. This is supported by the fact that either tamoxifen or a pure antiestrogen like ICI abrogated these phenotypes.

C-iv-The Invasion phenotype, an important marker of tumorigenesis is not modified when the cells are treated in presence of tamoxifen or ICI, suggesting that other pathways may be involved. Although we cannot rule out the possibility, that 4-OH-E_2 may interact with other receptors still not identified, with the data presently available the direct effect of 4-OH-E2 at so low doses support the concept that metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects leading to transformation. An increase in cathecol estrogen (4-OH-E2) due to either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA. Through this pathway estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis. This assumption was confirmed when we found that all the transformation phenotypes induced by 4-OH-E2 were not abrogated when this compound was used in presence of the pure antiestrogenic ICI. The novelty of this observation lays in that the ER-β pathway in transformation can successfully bypassed by the estrogen metabolite 4-OH E2.

C-v- Genomic DNA was analyzed for the detection of micro-satellite DNA polymorphism using 64 markers covering chromosomes (chr) 3, 11, 13 and 17. We have detected loss of heterozygosity (LOH) in ch13q12.2-12.3 (D13S893) and in ch17q21.1 (D17S800) in E_2, 2-OH-E_2, 4-OH-E_2-, E_2+ICI, E_2+Tamoxifen and BP treated cells. LOH in ch17q21.1-21.2 (D17S806) was also observed in E_2, 4-OH-E_2, E_2+ICI,E_2+Tamoxifen and BP-treated cells. MCF-10Fcells treated with P or P+E_2 did not show LOH in the any of the markers studied.

C-vi-LOH was strongly associated with the invasion phenotype. Altogether our data indicate that E_2 and its metabolites induce in HBEC LOH in loci of chromosomes 13 and 17, that has been reported in primary breast cancer, that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.

D-REPORTABLE OUTCOMES

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E-CONCLUSIONS

In the present work we demonstrate that estradiol and its metabolites mainly 4-OH estradiol are able to induce transformation phenotypes in the human breast epithelial cells (HBEC) MCF-10F. The fact that the MCF10F cells are ERα negative, indicate that this receptor pathway is not involved in the carcinogenic process. Although the presence of ER-β may indicate that the response of the cells to growth and form colonies in agar methocel could be mediated by this receptor, the Invasion phenotype, an important marker of tumorigenesis, is not modified when the cells are treated in presence of tamoxifen or ICI. We cannot rule out the possibility, that 4-OH-E_2 may interact with other receptors still not identified, with the data presently available the direct effect of 4-OH-E2 at so low doses support the concept that metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects leading to transformation. An increase in catechol estrogen (4-OH-E2) due to either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA. Through this pathway estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis. This assumption was confirmed when we found that all the transformation phenotypes induced by 4-OH-E2 were not abrogated when this compound was used in presence of the pure antiestrogenic ICI. We have detected loss of heterozygosity (LOH) in ch13q12.2-12.3 (D13S893) and in ch17q21.1 (D17S800) in E_2, 2-OH-E_2, 4-OH-E_2-, E_2+ICI, E_2+Tamoxifen and BP treated cells. LOH in ch17q21.1-21.2 (D17S806) was also observed in E_2, 4-OH-E_2, E_2+ICI,E_2+Tamoxifen and BP-treated cells. MCF-10Fcells treated with P or P+E_2 did not show LOH in the any of the markers studied. LOH was strongly associated with the invasion phenotype. Altogether our data indicate that E_2 and its metabolites induce in HBEC LOH in loci of chromosomes 13 and 17, that has been reported in primary breast cancer, that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.

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APPENDIX

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PI: Jose Russo, MD
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A- Pages containing Color photographs

B-Publications:

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A- Pages containing Color photographs

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Review

Estrogen and its metabolites are carcinogenic agents in human breast epithelial cells

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Abstract

Estrogens play a crucial role in the development and evolution of human breast cancer. However, it is still unclear whether estrogens are carcinogenic to the human breast. There are three mechanisms that have been considered to be responsible for the carcinogenicity of estrogens: receptor-mediated hormonal activity, a cytochrome P450 (CYP)-mediated metabolic activation, which elicits direct genotoxic effects by increasing mutation rates, and the induction of aneuploidy by estrogen. To fully demonstrate that estrogens are carcinogenic in the human breast through one or more of the mechanisms explained above it will require an experimental system in which, estrogens by itself or one of the metabolites would induce transformation phenotypes indicative of neoplasia in HBEC in vitro and also induce genomic alterations similar to those observed in spontaneous malignancies. In order to mimic the intermittent exposure of HBEC to endogenous estrogens, MCF-10F cells that are ERα negative and ERβ positive were first treated with 0, 0.007, 70 nM and 1 μM of 17β-estradiol (E_2), diethylstilbestrol (DES), benz(*a*)pyrene (BP), progesterone (P), 2-OH-E_2, 4-hydoxy estradiol (4-OH-E_2) and 16-α-OH-E_2 at 72 h and 120 h post-plating. Treatment of HBEC with physiological doses of E_2, 2-OH-E_2, 4-OH-E_2 induce anchorage independent growth, colony formation in agar methocel, and reduced ductulogenic capacity in collagen gel, all phenotypes whose expression are indicative of neoplastic transformation, and that are induced by BP under the same culture conditions. The presence of ERβ is the pathway used by E_2 to induce colony formation in agar methocel and loss of ductulogenic in collagen gel. This is supported by the fact that either tamoxifen or the pure antiestrogen ICI-182,780 (ICI) abrogated these phenotypes. However, the invasion phenotype, an important marker of tumorigenesis is not modified when the cells are treated in presence of tamoxifen or ICI, suggesting that other pathways may be involved. Although we cannot rule out the possibility, that 4-OH-E_2 may interact with other receptors still not identified, with the data presently available the direct effect of 4-OH-E_2 support the concept that metabolic activation of estrogens mediated by various cytochrome P450 complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects leading to transformation. This assumption was confirmed when we found that all the transformation phenotypes induced by 4-OH-E_2 were not abrogated when this compound was used in presence of the pure antiestrogen ICI. The novelty of these observations lies in the role of ERβ in transformation and that this pathway can successfully bypassed by the estrogen metabolite 4-OH-E_2. Genomic DNA was analyzed for the detection of micro-satellite DNA polymorphism using 64 markers covering chromosomes (chr) 3, 11, 13 and 17. We have detected loss of heterozygosity (LOH) in ch13q12.2–12.3 (D13S893) and in ch17q21.1 (D17S800) in E_2, 2-OH-E_2, 4-OH-E_2, E_2 + ICI, E_2 + tamoxifen and BP-treated cells. LOH in ch17q21.1–21.2 (D17S806) was also observed in E_2, 4-OH-E_2, E_2 + ICI, E_2 + tamoxifen and BP-treated cells. MCF-10F cells treated with P or P + E_2 did not show LOH in the any of the markers studied. LOH was strongly associated with the invasion phenotype. Altogether our data indicate that E_2 and its metabolites induce in HBEC LOH in loci of chromosomes 13 and 17, that has been reported in primary breast cancer, that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.

Keywords: Breast; Cancer; Aneuploidy; Oxidative metabolism; Estrogen receptors; Human breast epithelial cells

Contents

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1. Introduction

Intensive epidemiological studies have identified a number of genetic risk factors associated with breast cancer [1]. An increased risk has also been associated with early onset of menstruation, nulliparity or delayed first childbirth, short duration of breast feeding, late menopause, use of hormone replacement therapy and increased bone density [2–4]. A principal culprit common for all these endocrine-related risk factors is the prolonged exposure to female sex hormones [5–8]. The hormonal influences have been mainly attributed to unopposed exposure to elevated levels of estrogens [5], as has been indicated for a variety of female cancers, namely, vaginal, hepatic and cervical carcinomas [9–11]. Exposure to estrogens, particularly during the critical developmental periods (e.g. in utero, puberty, pregnancy, menopause), also affects affective behaviors (e.g. depression, aggression, alcohol intake) and increases breast cancer risk [12]. In addition, both environmental and genetic factors are believed to exert their influence by a hormonal mechanism [13–18].

It is generally accepted that the biological activities of estrogens are mediated by nuclear estrogen receptors (ER) which, upon activation by cognate ligands, form homodimers with another ER–ligand complex and activate transcription of specific genes containing the estrogen response elements [19]. According to this classical model, the biological responses to estrogens are mediated by the ER universally identified until recently, which has been termed as ERα after the discovery of a second type of ER (ERβ). The presence of ERα in target tissues or cells is essential to their responsiveness to estrogen action. In fact, the expression levels of ERα in a particular tissue have been used as an index of the degree of estrogen responsiveness [20]. ERβ and ERα share high sequence homology, especially in the regions or domains responsible for specific binding to DNA and the ligands. ERβ can be activated by estrogen stimulation, and blocked with antiestrogens [21,22]. Upon activation, ERβ can form homodimers as well as heterodimers with ERα [22,23]. The existence of two ER subtypes and their ability to form DNA-binding heterodimers suggests three potential pathways of estrogen signaling: via the ERα or ERβ subtype in tissues exclusively expressing each subtype and via the formation of heterodimers in tissues expressing both ERα and ERβ. The pathways of the ER-mediated signal transduction have become even more complicated by the recent discovery of other types of ER [24,25]. In addition, estrogens and antiestrogens can induce differential activation of ERα and ERβ to control transcription of genes that are under the control of an AP-1 element [23].

The most biologically active estrogen in breast tissue is 17β-estradiol (E_2). Circulating estrogens are mainly originated from ovarian steroidogenesis in premenopausal women and peripheral aromatization of ovarian and adrenal androgens in postmenopausal women [26]. The importance of ovarian steroidogenesis in the genesis of breast cancer is highlighted by the fact that occurring naturally or induced early menopause prior to age 40 significantly reduces the risk of developing breast cancer [26]. However, the uptake of 17β-estradiol from the circulation does not appear to contribute significantly to the total content of estrogen in breast tumors, since the majority of estrogen present in the tumor tissues is derived from de novo biosynthesis [26]. In fact, the concentrations of 17β-estradiol in breast cancer tissues do not differ between premenopausal and postmenopausal women, even though plasma levels of 17β-estradiol decrease by 90% following menopause [27]. This phenomenon might be explained by the observation that enzymatic transformations of circulating precursors in peripheral tissues contribute 75% of estrogens in premenopausal women

and almost 100% in postmenopausal women [28,29], the data that highlight the importance of in situ metabolism of estrogens [26,30–48].

Even though the breast is influenced by a myriad of hormones and growth factors [49–52], estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium [49,50]. Estradiol acts locally in the mammary gland, stimulating DNA synthesis and promoting bud formation, probably through an ER-mediated mechanism [49]. It is also known that the prevailing metabolic condition of an individual animal or human may significantly influence mammary gland responses to hormones. In addition, the mammary gland responds selectively to given hormonal stimuli for either cell proliferation or differentiation, depending upon specific topographic differences in gland development. In either case, the response of the mammary gland to these complex hormonal and metabolic interactions results in developmental changes that permanently modify both the architecture and the biological characteristics of the gland [49,51].

The fact that the normal epithelium contains receptors for both estrogen and progesterone lends support to the receptor-mediated mechanism as a major player in the hormonal regulation of breast development. The role of these hormones on the proliferative activity of the breast, which is indispensable for its normal growth and development, has been for a long time, and still is, the subject of heated controversies [26]. There is little doubt, however, that the proliferative activity of the mammary epithelium in both rodents and humans varies with the degree of differentiation of the mammary parenchyma [49–55]. In humans, the highest level of cell proliferation is observed in the undifferentiated lobules type 1 (Lob 1) present in the breast of young nulliparous females [49–52]. The progressive differentiation of Lob 1 into lobules types 2 (Lob 2) and 3 (Lob 3), occurring under the hormonal influences of the menstrual cycle, and the full differentiation into lobules type 4 (Lob 4), as a result of pregnancy, leads to a concomitant reduction in the proliferative activity of the mammary epithelium [49–55]. The content of ERα and progesterone receptor (PgR) in the lobular structures of the breast is directly proportional to the rate of cell proliferation, being also maximal in the undifferentiated Lob 1, and decreasing progressively in Lob 2, Lob 3, and Lob 4 [51,56]. The findings that proliferating cells are different from those that are ERα- and PgR-positive support data that indicate that estrogen controls cell proliferation by an indirect mechanism. This phenomenon has been demonstrated using supernatant of estrogen-treated ERα-positive cells that stimulates the growth of ERα-negative cell lines in culture. The same phenomenon has been shown in vivo in nude mice bearing ER-negative breast tumor xenografts [57]. ERα-positive cells treated with antiestrogens secrete transforming growth factor-β that inhibits the proliferation of ERα-negative cells [58]. The findings that proliferating cells in the human breast are different from those that contain steroid hormone receptors explain many of the in vitro data [59,60]. Of interest are the observations that while the ERα-positive MCF-7 cells respond to estrogen treatment with increased cell proliferation, and that the enhanced expression of the ERα by transfection also increases the proliferative response to estrogen [59–61], ERα-negative cells, such as MDA-MB-468 and others, when transfected with ERα, exhibit inhibition of cell growth under the same type of treatment [60]. Although the negative effect of estrogen on those ERα-negative cells transfected with the ERα has been interpreted as an interference of the transcription factor used to maintain estrogen independent growth [61], there is no definitive explanation for their lack of survival. However, it can be explained by the finding that proliferating and ERα-positive cells are two separate populations. Further support is the finding that when Lob 1 of normal breast tissue are placed in culture, they lose the ERα-positive cells, indicating that only proliferating cells that are also ERα-negative can survive and constitute the stem cells [62,63].

Although 67% of breast cancers are manifested during the postmenopausal period, a vast majority, 95%, is initially hormone-dependent [26]. This indicates that estrogens play a crucial role in their development and evolution. It has been established that in situ metabolism of estrogens through aromatase-mediated pathway is correlated with the risk of developing breast cancer [37,38]. A recent finding that expression of estrone sulfatase is inversely correlated with relapse-free survival of human breast cancer patients [42] reiterates the importance of estrone sulfatase-mediated local production of estrogen in the development and progression of human breast cancer. However, it is still unclear whether estrogens are carcinogenic to the human breast. Most of the current understanding of carcinogenicity of estrogens is based on studies in experimental animal systems and clinical observations of a greater risk of endometrial hyperplasia and neoplasia associated with estrogen supplementation or polycystic ovarian syndrome [26].

There are three mechanisms [62–148] that have been considered to be responsible for the carcinogenicity of estrogens: receptor-mediated hormonal activity, which has generally been related to stimulation of cellular proliferation, resulting in more opportunities for accumulation of genetic damages leading to carcinogenesis [56,63,75–86], a cytochrome P450 (CYP)-mediated metabolic activation, which elicits direct genotoxic effects by increasing mutation rates [26,64,65,87–133], and the induction of aneuploidy by estrogen [65–73,134–148]. There is also evidence that estrogen compromises the DNA repair system and allows accumulation of lesions in the genome essential to estrogen-induced tumorigenesis [74].

To fully demonstrate that estrogens are carcinogenic in the human breast through one or more of the mechanisms explained above it will require an experimental system in which, estrogens by itself or one of the metabolites would induce transformation phenotypes indicative of neoplasia in HBEC in vitro and also induce genomic alterations similar to those observed in spontaneous malignancies, such as

DNA amplification and loss of genetic material that may represent tumor suppressor genes [149–164]. For this purpose, we have develop an in vitro system in which we have demonstrated that estrogens are transforming agents on human breast epithelial cells (HBEC), by utilizing the spontaneously immortalized HBEC MCF-10F [165,166]. In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were treated repetitively with different concentrations of 17β-estradiol inducing phenotypic and genotypic changes indicative of cell transformation [167,168]. In the present work, we further demonstrate that metabolites of estrogens are also able to induce phenotypic and genotypic changes in human breast epithelial cells furthering our understanding of the complex role of estrogen in breast carcinogenesis.

2. Materials and methods

2.1. The in vitro model of cell transformation

The transforming potential of estrogens on human breast epithelial cells in vitro, have being evaluated by utilizing the spontaneously immortalized HBEC MCF-10F cells [167,168]. The spontaneously immortalized MCF-10F cells, treated cells and derived clones were maintained in DMEM:F-12 (1:1) medium with a 1.05 mM Ca^{2+} concentration. All cell lines were regularly tested for correct identity using a fingerprint cocktail of three minisatellite plasmid probes (ATCC, Rockville, MD). Culture media were prepared by the Central Center Tissue Culture Facility at the Fox Chase Cancer Center (Philadelphia, PA). In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were first treated with 0, 0.007, 70 nM and 1 μM of E_2, DES, BP, progesterone, 2-OH-E_2, 4-hydoxy estradiol (4-OH-E_2) and 16-α-OH-E_2 (Aldrich, St. Louis, MO) at 72 and 120 h post-plating. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis (Fig. 1). At the end of each treatment period, the culture medium was replaced with fresh medium. At the end of the second week of treatment, the cells were assayed for determination of, survival efficiency (SE), colony efficiency (CE), colony size (CS), ductulogenic capacity and invasiveness in a reconstituted basement membrane [94,169].

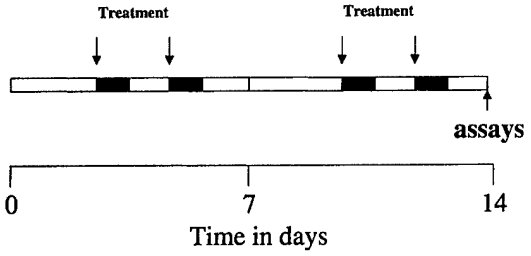

Fig. 1. Scheme of treatment. MCF-10-F cells were treated with E_2, DES, BP, 2-OH-E_2, 4-OH-E_2, 16-α-OH-E_2, progesterone or cholesterol, at 72 and 120 h post-plating. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis.

2.2. Colony formation in agar methocel assay

This technique was utilized as an in vitro assay for anchorage independent growth, a parameter indicative of transformation. Parental, control, and treated cells were suspended at a density of 2×10^4 cells/ml in 2 ml of 0.8% methocel (Sigma, St. Louis, MO) dissolved in DMEM:F-12 (1:1) medium containing 20% horse serum. Cells from each treatment group and time point were plated in four 24-well chambers pre-coated with 0.5 ml of 0.8% agar base in DMEM:F-12 medium, which was replaced with fresh feeding medium containing 0.8% methocel twice a week. The actual number of cells plated was calculated as the average of cells counted at 10× magnification in five individual fields, and multiplied by a factor of 83. CE and CS were measured 21 days after plating. CE was determined by a count of the number of colonies greater than 100 μm in diameter, and expressed as a percentage of the original number of cells plated per well.

2.3. Ductulogenesis in collagen matrix

This in vitro technique evaluates the capacity of cells to differentiate by providing evidence of whether treated cells form three-dimensional structures when grown in a collagen matrix. Parental, control, and treated cells were suspended at a final density of 2×10^3 cells/ml in 89.3% Vitrogen^{100} collagen matrix (Collagen Co., Palo Alto, CA) and plated into four 24-well chambers pre-coated with agar base. The cells were fed fresh feeding medium containing 20% horse serum twice a week. The cells were examined under an inverted microscope for a period of 21 days or longer for determining whether they formed ductule-like structures or whether they grew as unorganized clumps. The final structures were photographed, and then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin for histological examination. Immunohistochemical techniques were utilized for detecting the proliferative index.

2.4. Invasion assay

The invasion assay is performed by using the Boyden-type chambers (Transwell, Coastar Cambridge, MA) separated by a porous polycarbonate filter (8 μm pore size) (Nucleopore, Pleasanton, CA), coated with reconstituted basement membrane material (Matrigel; Collaborative Research, Bedford, MA). For the chemoinvasion assay, filters were coated with Matrigel, which was prepared by reconstituting Matrigel with 100 μm of MEM with 0.1% BSA. The filters

were coated and dried overnight. Fibronectin (Collaborative Research, Bedford, MA) at a concentration of 1 μg/ml in 0.5 ml of MEM with 0.1% BSA was used as chemoattractant and placed in the lower chamber. Trypsinized cells (3×10^5) were seeded in the upper chamber and incubated for 12 h at 37 °C in a carbon dioxide incubator. Then the filters were fixed, stained by Diff Quick (Sigma, St. Louis, MO), cut out and mounted onto glass slides. The total number of cells that crossed the membrane was counted under a light microscope. The values were expressed as chemoinvasion index. Values of chemoinvasion were expressed as the number of cells that migrated to the lower chamber. The experiments were repeated three times and results expressed as the mean ± S.E. of the three experiments.

2.5. Detection of cell proliferation (Ki67 index)

Paraffin tissue sections of 5 μm thickness were mounted on positively charged slides. They were incubated in two changes of Target Retrieval Solution at 98 °C for 5 min each and then incubated in diluted normal blocking serum for 20 min. The sections were incubated with mouse monoclonal anti-human Ki67 antibody, clone M1B-1 (Dako A/S, Glostrup, Denmark) at a dilution of 1:400 overnight at 4 °C in a humidity chamber. After washing the sections in buffer they were incubated with horse anti-mouse biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA) at room temperature for 30 min, rinsed in buffer and incubated with Vectastain Elite ABC kit for mouse (Vector Laboratories, Inc., Burlingame, CA) for 30 min. After a wash in PBS buffer sections were incubated in peroxidase substrate solution containing hydrogen peroxide and 3,3′-diaminobenzidine–HCl for 2 min. Sections incubated with non-immune serum were used as negative controls. All sections were lightly counterstained with hematoxylin. Immunostaining was evaluated by examination of slides under a bright field microscope. Cell proliferation was determined by counting the number of labeled nuclei per total number of epithelial cells. The Ki67 index was expressed as the number of labeled nuclei per 100 epithelial cells.

2.6. Western blots of ERα, ERβ and progesterone receptors

Proteins were isolated from MCF-10F cells transformed with 70 nM, ICI + 4-OH-E_2, 4-OH-E_2, 17β-estradiol, ICI + 17β-estradiol, progesterone and progesterone + 17β-estradiol as indicated in Fig. 1. MCF-7 and MDA-MB-235 cell lines were used as control. The medium was removed and the cells were rinsed with PBS at room temperature. The cells were lysed using a syringe with a 21-gauge needle followed by microcentrifugation of the cell lysate at 2000 × *g* for 10 min at 4 °C. The proteins were electrophoretically separated in a SDS-PAGE polyacrylamide gel 10% running at 90 V during 8 h at room temperature. The proteins were transferred to nitrocellulose membranes (Amersham Arlington Heights, IL). Membranes were blocked using 5% of non-fat dried milk during 1 h at room temperature and hybridized to anti-ER monoclonal antibody against the full length α form of the estrogen receptor (San Cruz Biotech, Santa Cruz, CA) at a concentration of 1/50, anti-ERβ (Clone ER-7G5) polyclonal antibody against 19aac synthetic peptide derived from human ERβ protein (Zymed Lab, Inc., San Francisco, CA), at a concentration 1/50 (60 μg/ml), anti-PR Clone PR-2C5) monoclonal antibody against peptide representing N terminal of human PR conjugates to carrier protein (Zymed Lab., Inc., San Francisco, CA), at a concentration 1/50 (20 μg/ml) overnight at 4 °C. Horseradish peroxidase-conjugated anti-mouse, anti-rabbit (Amersham, Arlington Heights, IL) were used as secondary antibodies in a concentration 1/2500 and incubated during 1 h at room temperature. Enhanced chemiluninescence system (Amersham, Arlington Heights, IL) was used for final immunoblot detection.

2.7. cDNA array

The RNA was extracted from BP, E_2 and 4-OH-E_2 transformed cells as well as the untreated MCF-10F cells. The cells were homogenized in TRIzol Reagent (Gibco BRL, Gaithersburg, MD). The RNA was isolated and stored in RNase-free water at −70 °C. The integrity of total RNA was determined by analyzing on agar gel. For cDNA probe synthesis, 5 μg of total RNA together with 1 μl of CDS primer mix (Clontech Laboratories, Palo Alto, CA) in a total volume of 6 μl were heated to 70 °C for 10 min and then cooled on ice. A mixture consisting of 4 μl of five times first-strand cDNA buffer, 1 μl of 100 mM DTT, 2 μl of 100 mM dNTPs (Clontech Laboratories, Palo Alto, CA), and 5 μl of [α-32P]dATP (3000 Ci/μl; ICN) was added into the tube and heated at 42 °C for 2 min. One microliter of SuperScrit II RNase H reverse transcriptase was then added, and the reaction was continued at the same temperature for 50 min, followed by heating to 70 °C for 15 min for enzyme inactivation. The cDNA probe was purified with a CHROMA SPIN-200DEPC-H_2O column (Clontech Laboratories, Palo Alto, CA). Incorporation of ^{32}P into the probe was determined by counting in a liquid scintillation counter. The first two fractions showing the highest counts were collected and used for hybridization. The Atlas Human Cancer 1.2 Arrays containing cDNA fragments of 1176 cancer-associated human genes/clones were purchased from Clontech. Array membranes were prehybridized with 5 ml of ExpressHyb solution at 68 °C with continuous rotation in a glass hybridization roller. After prehybridization for 30 min, purified α-^{32}P-labeled cDNA probes made from MCF-10F and transformed cells RNAs were added into different rollers, and hybridization was continued overnight at the same temperature. Arrays were subsequently washed twice in 200 ml of wash solution 1 (2 × SSC, 1% SDS) at 68 °C for 20 min

with agitation and then washed once in 200 ml of wash solution 2 (0.1 × SSC, 0.5% SDS) at 68 °C for 20 min with agitation. After a final wash with 200 ml of 2 × SSC for 5 min at room temperature, the damp membranes were sealed in plastic wrap and exposed to Kodak Biomax MS X-ray film with an intensifying screen at −80 °C for 3 days. Array images on the X-ray film were scanned at 400 dpi by using an image scanner and then analyzed using the ArrayExplorer in VisualBasic (Microsoft, Inc.). We first eliminated by visual inspection false positive signals due to apparent artifacts; the intensity of each spot on the array was then calculated after background subtraction. Putative functions of the genes identified were obtained by use of the AtlasInfo database (http://www.atlasinfo.clontech.com).

2.8. Genomic analysis of treated cells

To obtain DNA, treated and control cells were lysed in 5 ml of TNE (0.5 M Tris pH 8.9, 10 mM NaCl, 15 mM EDTA) with 500 μg/ml proteinase K and 1% sodium dodecyl sulfate (SDS), and incubated at 48 °C for 24 h. Following two extractions with phenol (equilibrated with 0.1 M Tris pH 8.0), the DNA was spooled from two volumes of 100% ethanol, air dried and resuspended in 20 mM EDTA. The DNA was then treated sequentially with RNase A (100 μg/ml) for 1 h at 37 °C and 100 μg/ml proteinase K, 1% SDS, at 48 °C for 3 h, followed by two extractions with saturated phenol. The DNA was again retrieved from the aqueous phase by ethanol precipitation, washed extensively in 70% ethanol, and after air-drying suspended in TE (10 mM Tris, pH8.0), 1 mM EDTA.

2.9. Detection of allelic loss

We evaluated for allelic losses the regions of chromosomes (chr) 1–3, 6–9, 11–13, 16, 17, and 18 most frequently reported to exhibit loss of heterozygosity (LOH) in spontaneous breast tumors [170]. DNA amplification of microsatellite length polymorphisms was utilized for detecting allelic losses present in the transformed clones. Microsatellites are polymorphic markers used primarily for gene mapping which can be broadly defined as relatively short (<100 bp) runs of tandem repeated di- to tetranucleotide sequence motifs. The origin and nature of these polymorphism sequences is not well established, but they may result from errors of the polymerase during replication and/or from slightly unequal recombination between homologous chromatids during meiosis. These microsatellites have proven to be useful markers for investigating LOH and could be applicable to allelotyping as well as regional mapping of deletions in specific chromosomal regions. They are highly polymorphic, very common (between 10^5 and 10^6 per genome), and are flanked by unique sequences that can serve as primers for polymerase chain reaction (PCR) amplification.

2.10. PCR analysis of microsatellites

Primers used for the analysis of microsatellite polymorphisms are given elsewhere [170]. Conditions for PCR amplification were as follows: 30 ng of genomic DNA, 100 pmoles of each oligonucleotide primer, 1 × PCR buffer (Perkin-Elmer, Cetus), 5 μM each of TTP, dCTP, dGTP, and dATP, 1 μCi [^{32}P] dATP (300 mCi/mmol) (Dupont, NEN, Boston, MA), and 0.5 units of Amplitaq DNA polymerase (Perkin-Elmer, Cetus) in 50 μl volumes. The reactions were processed through 27 cycles of 1 min at 94 °C, 1 min at the appropriate annealing temperatures determined for each set of primers, and 1 min at 72 °C; with a final extension of 7 min at 72 °C. Reaction products were diluted 1:2 in loading buffer (90% formamide, 10 mM EDTA, 0.3% bromophenol blue, and 0.3% xylene cyanol), heated at 90 °C for 5 min and loaded (4 μl) onto 5–6% denaturing polyacrylamide gels. After electrophoresis, gels were dried at 70 °C and exposed to XAR-5 film with a Lightning Plus intensifying screen at −80 °C for 12–24 h. Allele sizes were determined by comparison to M13mp18 sequencing ladders.

2.11. Detection of allelic loss

LOH was defined as a total loss of, or a 50%, or more reduction in density in one of the heterozygous alleles. All experiments were repeated at least three times to avoid false positive or false negative results. To control for possible DNA degradation, the same blots used to assess allelic loss were analyzed with additional DNA gene probes that detect large fragments. The bands were quantitated using a UltraScan XL laser densitometry (Pharmacia LKB Biotechnology, Inc.) within the linear range of the film.

3. Results

3.1. Transformation effect of estrogens and its metabolites in MCF-10F cells

We have determined the optimal doses for the expression of the cell transformation phenotype by treating the immortalized human breast epithelial cells MCF-10F with 17β-estradiol (E_2) with 0.0, 0.07, 70 nM, or 1 μM of E_2 twice a week for 2 weeks. The survival efficiency was increased with 0.007 and 70 nM of 17β-estradiol and decrease with 1 μM. The cells treated with either doses of E_2 formed colonies in agar methocel (Fig. 2) and the size was not different among them, however, the CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses (Fig. 2).

Ductulogenesis was quantitatively evaluated by estimating the ability of the cell plated in collagen to form tubules or spherical masses (SM) (Fig. 2). Non-transformed cells produce ductules like structure and transformed cells produce spherical or solid masses of cells. Cells treated with DMSO, cholesterol or progesterone at different concentrations was

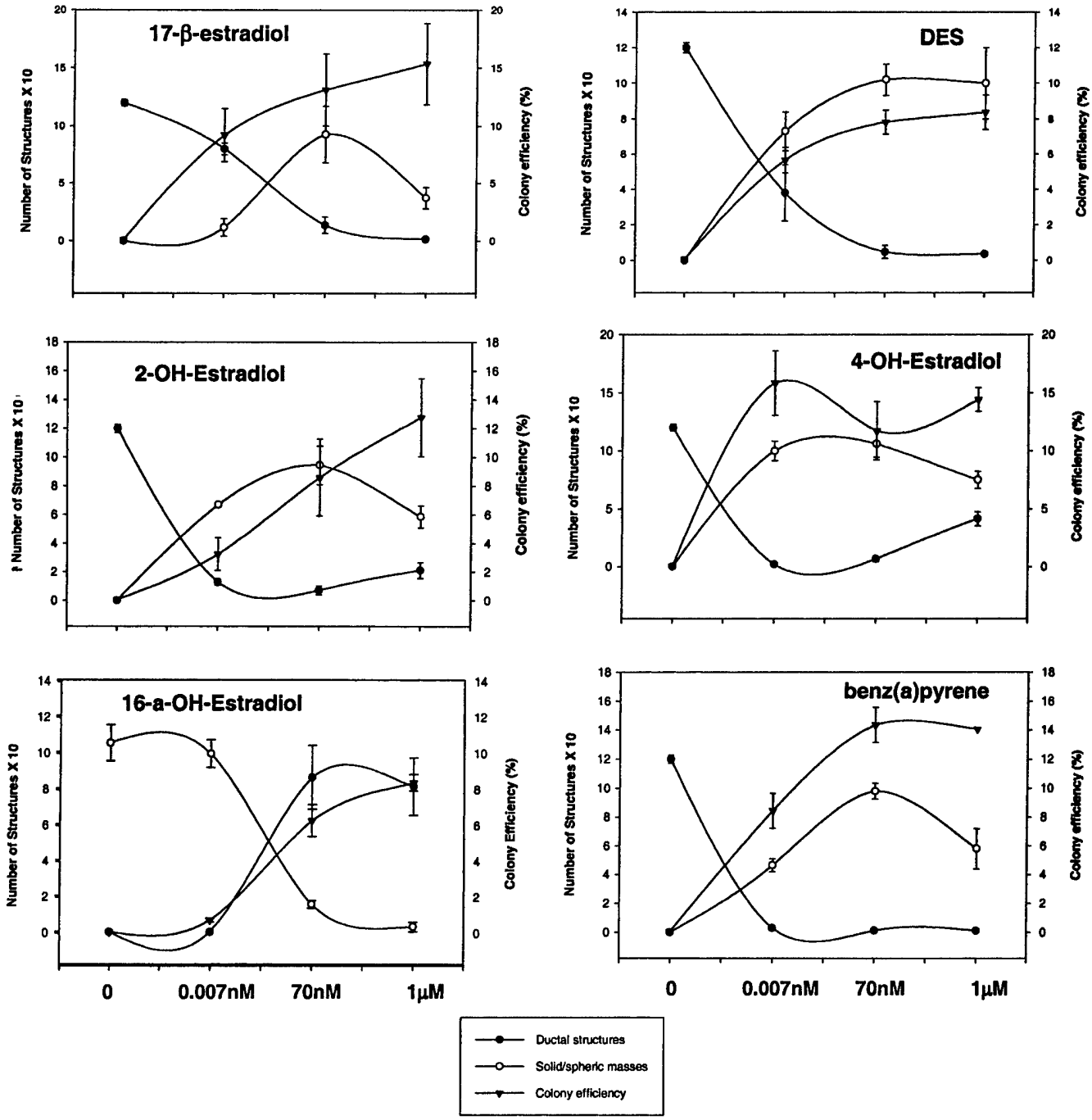

Fig. 2. Curves showing the dose response effect of MCF-10F cells to the transforming effect of 17β-estradiol, DES, 2-OH-estradiol, 4-OH-estradiol, 16-α-OH-estradiol and benz(*a*)pyrene. The left ordinate expresses the number of structures (ductules and solid masses) detected by 10,000 cells plated in collagen matrix. The right ordinate depicts the percentage of colonies or colony efficiency (CE) of MCF-10F cells. The CE was determined by a count of the number of colonies greater than 100 μm in diameter, and expressed a percentage of the original number of cells plated per well.

unable to alter the ductular pattern. E_2, BP and DES treated cells induces the loss of MCF-10F cells to produce ductules in a dose dependent fashion and the number of solid masses paralleled the formation of colonies in agar methocel (Fig. 2). Histological analysis shows that MCF10-F cells form ductules in collagen matrix that are lined by a single layer of cuboidal epithelial cells (Fig. 3a), this pattern was not disturbed by cholesterol or progesterone treatment. Most of the cells growing in the collagen matrix are actively proliferating as detected by immunostaining with Ki67 (Fig. 4).

2-OH-E_2, 4-OH-E_2, and 16α-OH-E_2 (Fig. 2) induce the formation of colonies in agar methocel. Cells treated with

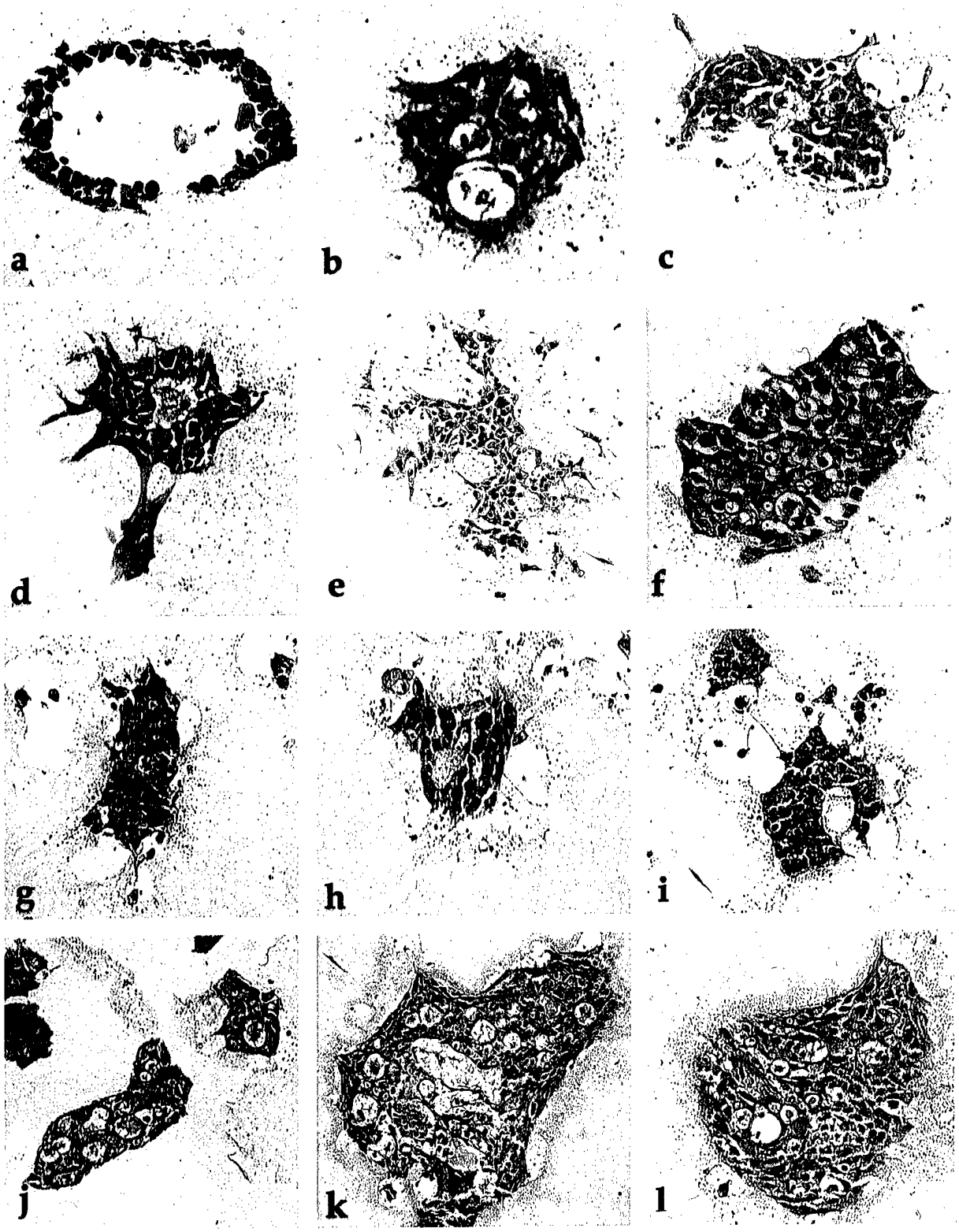

Fig. 3. Histological sections of cells growing in collagen matrix, fixed in 10% buffered formalin and embedded in paraffin and stained with hematoxylin and eosin. Photographs taken at 40× magnification. (a) MCF-10F cells in collagen matrix showing a well-organized ductular pattern; (b and c) MCF-10F cells transformed with 70 nM and 1 μM of E_2, respectively; (d, e and f) MCF-10F transformed cells with 0.007, 70 nM and 1 μM of BP, respectively; (g, h and i) MCF-10F cells transformed with 0.007 nM, 70 nM and 1 μM of 2-OH-E_2, respectively; (j, k and l) MCF-10F cells transformed with 0.007 nM, 70 and 1 μM of 4-OH-E_2, respectively.

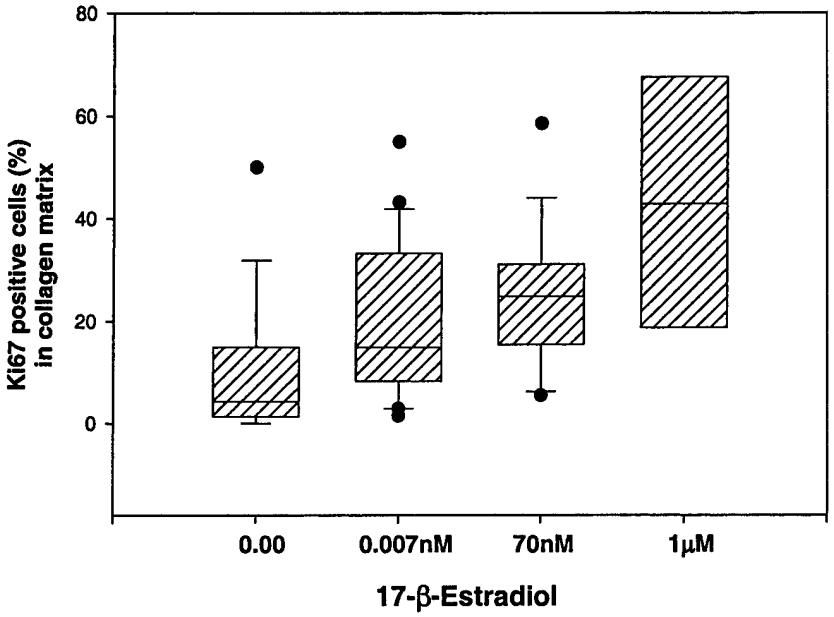

Fig. 4. Dose response effect of 17β-estradiol-transformed cells growing in collagen matrix. The proliferative activity was determined by counting the number Ki67 positive cells in histological sections of paraffin embedded cells growing in collagen.

cholesterol were unable to produce colonies. The size of the colonies was significantly smaller in those cells treated with 2-OH-E_2 or progesterone. Whereas the number of colonies was dose dependent reaching its maximum efficiency at the concentration of 70 nM for most of the compounds, 4-OH-E_2 was the most efficient in inducing larger colonies and number at a doses of 0.007 nM (Fig. 2). E_2, and BP behave very similar and are more transforming agents than DES and 2-OH-E_2 (Fig. 2).

The metabolites of estrogen significantly impair the formation of ductules replacing them by structures filled by large cuboidal cells. Some of the cells present cytoplasmic vacuolization and pyknosis (Fig. 3). Cells treated with 2-OH-E_2 or 16-α-OH-E_2 is less efficient in altering the

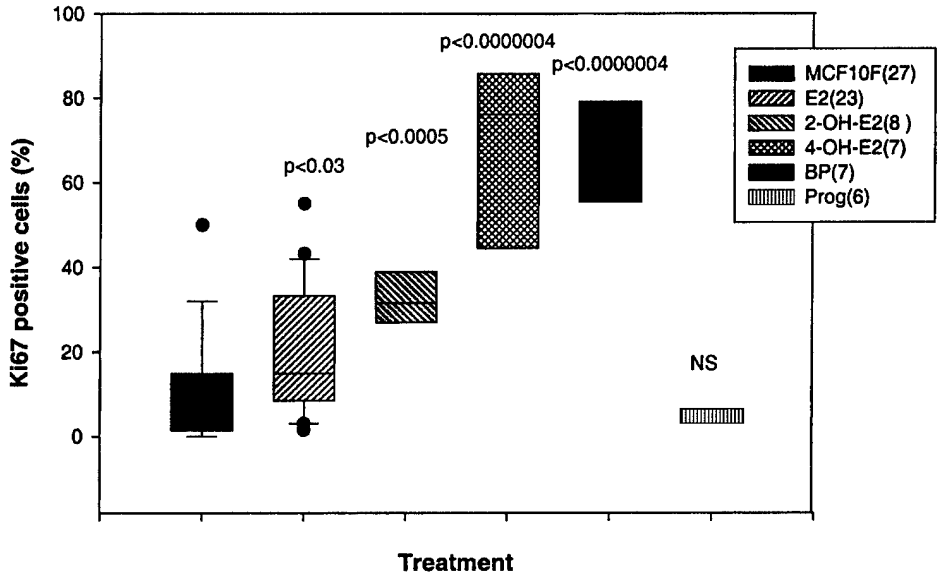

Fig. 5. Histogram depicting the proliferative activity of MCF-10F cells treated as indicated in Fig. 1 with different compounds at 70 nM concentration and growing in a collagen matrix. The values are expressing the percentage of positive cells immunoreacted with antibody Ki67. 4-OH-E_2 transformed cells are the ones with the highest number of proliferating cells. Progesterone treated cells do not stimulate the proliferation of MCF-10F cells.

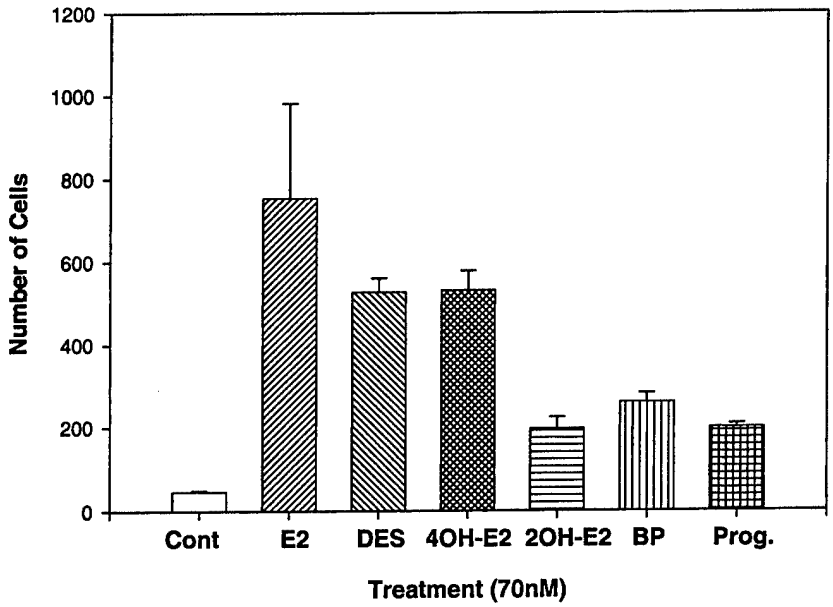

Fig. 6. Histogram depicting the invasive capacity of MCF-10F cells treated with different compounds (abscise) as indicated in Fig. 1. The ordinate shows the numbers of cells that have crossed the matrigel membrane.

ductulogenic capacity (Fig. 2). Importantly, 4-OH-E_2 at a dose of 0.007 nM induce significant changes in the ductulogenic capacity with a maximal number of solid masses (Fig. 3). These structures also have a high proliferative index (Fig. 5).

The invasiveness capacity of E_2, DES, 4-OH-E_2 and BP-transformed cells measured in the Boyden Chamber, was very high when compared with the control or those treated with DMSO, P, or 2OH-E_2 (Fig. 6).

3.2. Antiestrogens in the expression of the transformation phenotype

The proliferative activity of the MCF-10F cells that has been treated with tamoxifen alone or ICI-182,780 was not modified when compared with the control. Instead those cells that were treated with 17β-estradiol in presence of tamoxifen or ICI-182,780 (Fig. 7) showed no increment of the proliferative activity neither in monolayer nor collagen

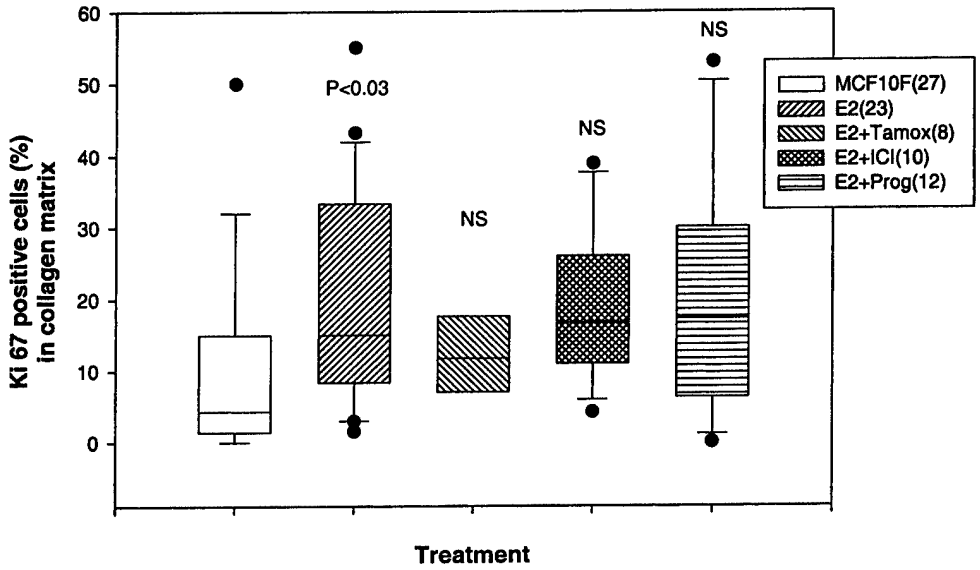

Fig. 7. Histogram depicting the proliferative activity of MCF-10F cells treated as indicated in Fig. 1 with combination of 17β-estradiol + tamoxifen (E_2 + Tamox) or plus ICI (E_2 + ICI) or plus progesterone (E_2 + Prog). The number in parenthesis indicates the number of ductules or structures counted.

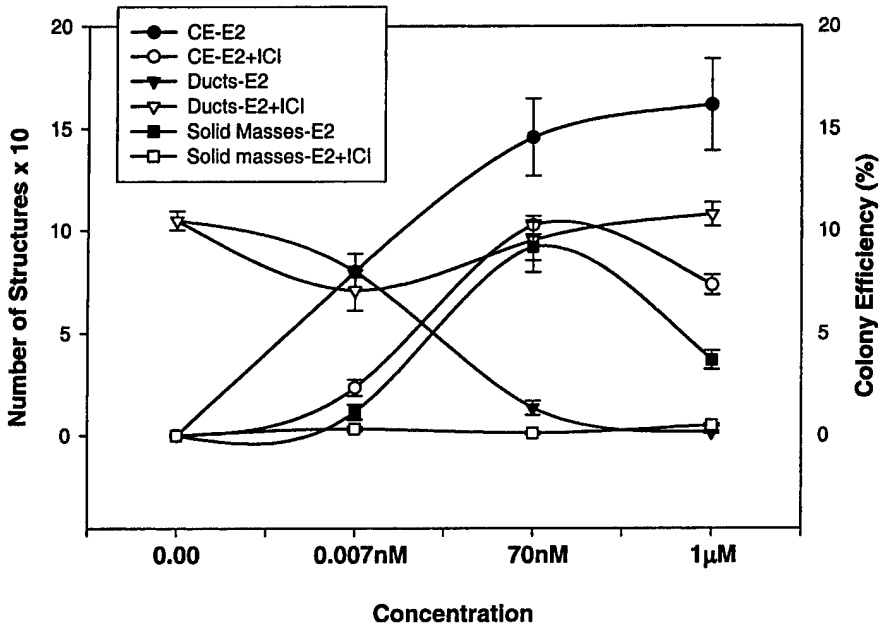

Fig. 8. Curves depicting the transforming affect of 17β-E_2 alone or in combination with ICI (nomenclature as described in Fig. 2).

matrix. The colony formation in agar methocel was abrogated and the ductulogenic capacity was maintained (Fig. 8). The proliferative activity of these cells in collagen matrix was also abrogated (Fig. 7). 4-OH-E_2 transforming efficiency was not abrogated by ICI neither in the colony efficiency assay nor in the loss of ductulogenic capacity (Fig. 9). The histology of the solid masses induced by 4-OH estradiol in collagen matrix were not modified by ICI, even the number of cells was significantly higher (Fig. 8). ICI-182,780 was unable to abrogate the invasive phenotype induced by estrogen and tamoxifen even exacerbate the invasive phenotype (Fig. 10).

3.3. Detection of estrogen receptors in MCF-10F cells

The ERα was not detected in the MCF-10F cells or in those transformed by estrogens or its metabolites (Fig. 11). The positive control MCF-7 cells was positive for ERα

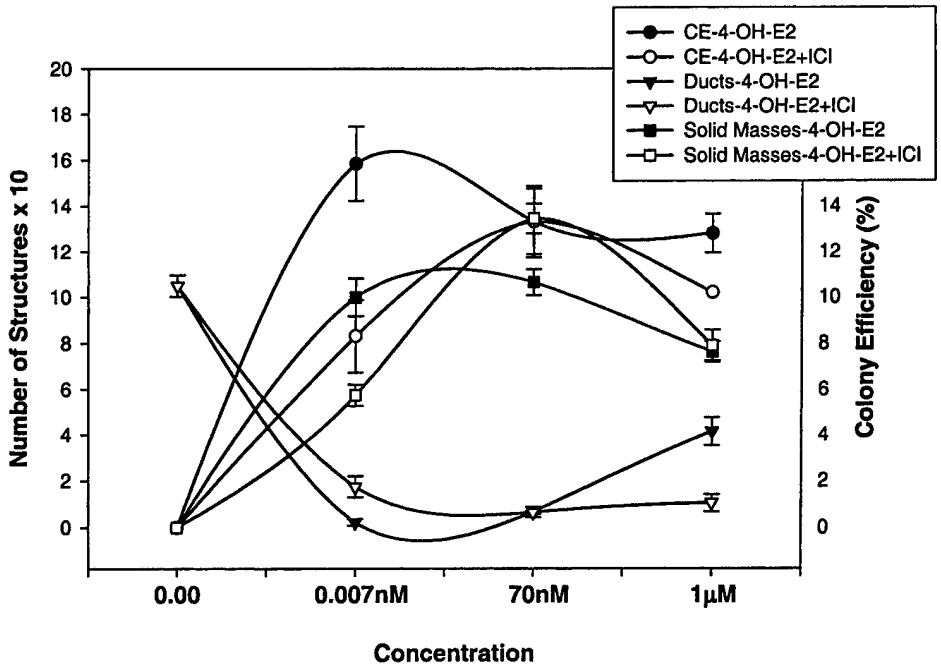

Fig. 9. Curves depicting the transforming effect of 4-OH-E_2 alone and in combination with ICI (4-OH-E_2 + ICI) (nomenclature as described in Fig. 2).

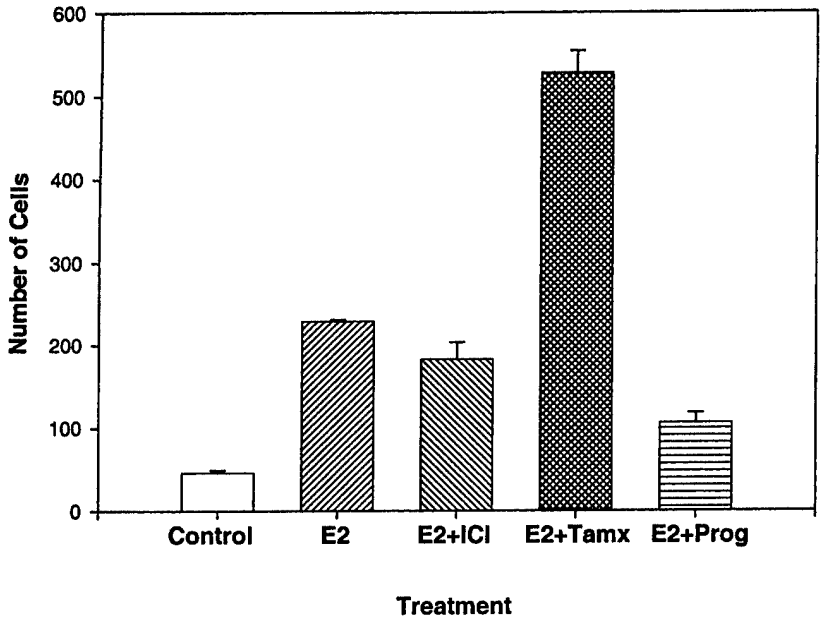

Fig. 10. Invasiveness phenotype in MCF-10F cells treated with 17β-estradiol (E_2) and in combination with ICI (E_2 + ICI), tamoxifen (E_2 + Tamx) and with progesterone (E_2 + Prog).

showing by Western blot the specific band corresponding to a 67 kDa, instead the band was absent in the negative control MDA-MB-235 cell line (Fig. 11).

The ERβ protein expression analysis showed two bands 68 and 53 kDa of molecular weight corresponding to ERβ long and short form, respectively. Both bands were present in the MCF-10F cells and in the transformed cells. Those cells transformed by 17β-estradiol as well as those treated with progesterone significantly overexpressed the long form of ERβ. Instead, MCF-7 cells showed the short form of the ERβ (Fig. 11).

The progesterone receptor (PR) expression was negative in the MCF-10F cells (Fig. 11) when compared with MCF-7 cells that was used a positive control presenting the 186 and 82 kDa PR long and short form, respectively. The estrogen-transformed cells also expressed PR (Fig. 11).

3.4. Genomic changes induced by estrogen and its metabolites in the transformation of human breast epithelial cells

In order to determine if the gene expression profile induced by E_2, 4-OH estradiol and BP were the same or whether they are divergent in their pattern of expression, mRNA from these transformed cells was extracted and hybridized to cDNA array membranes that contained 1176 human genes (Clontech Human Cancer 1.2 Array). The genomic signature of the three transformed cells present a

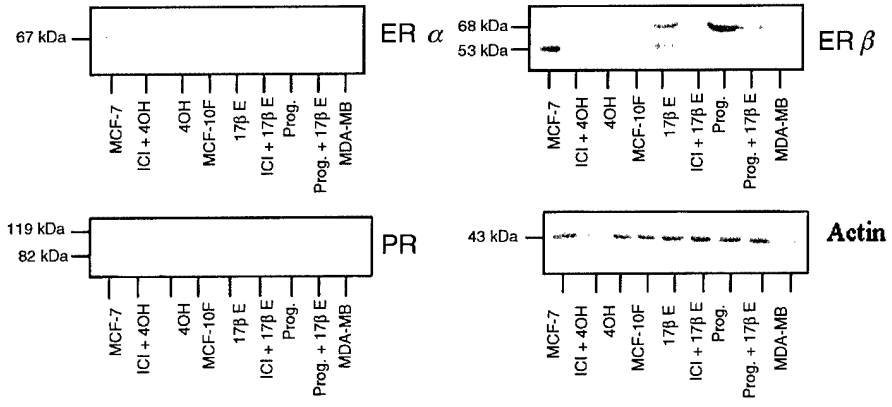

Fig. 11. Western blots of ERα, ERβ and progesterone receptors. Proteins were isolated from MCF-10F cells transformed with 70 nM, ICI + 4-OH-E_2, 4-OH-E_2, 17β-estradiol, ICI + 17β-estradiol, progesterone and progesterone + 17β estradiol as indicated in Fig. 1. MCF-7 and MDA-MB-235 cell lines were used as control. The medium was removed and the cells were rinsed with PBS at room temperature.

Table 1
Common up-regulated genes in MCF-10F cells transformed by BP, E_2 and 4-OH-E_2 using cDNA array

Gene description	Swissprot #	Function	Ratio BP/10F cells	Ratio E_2/10F cells	Ratio 4-OH-E_2/10F cells
c-myc oncogene	P01106	Oncogene	3.24	3.66	6.21
fos-related antigen	P15407	Oncogene	10.25	2.31	15.04
HER3	P21860	Oncogene	2.09	3.32	7.95
SRF accessory protein 2	P41970	Transcription	3.61	2.46	9.11
hEGR1	P18146	Transcription	3.2	6.49	2.91
Splicing factor 9G8	Q16629	mRNA processing	2.23	2.93	4.42
Antigen Ki67	P46013	Cell proliferation	3.2	2.7	5.97
HMG1	P17096	Chromatin	2.36	3.26	7.95
nm23-H4	O00746	Kinase	2.02	2	2.24
Cytokeratin 2E	P35908	Keratin	43.09	2.38	4.37

cluster of genes that are commonly up-regulated (Table 1), indicating that a similar mechanism is involved in the transformation pathway. Interestingly, there are genes that are up-regulated in the E_2 and 4-OH-E_2 transformed cells such as the CENP-E (Tables 2 and 3) that are not modified in the BP-transformed cells (Table 4). The same occurs for several genes that are down-regulated differentially in the three transformed cells (Table 5).

3.5. Chromosomal alterations induced by estrogens and its metabolites

During the process of cell transformation induced by estrogen and its metabolites there is an increase in the number of multinucleated cells and abnormal mitoses (Figs. 12 and 13) that is associated with the overexpression of one component of the centromere–kinetochore complex CENP-E. It is important to emphasize that the percentage of these abnormal mitoses is lees than 1% (Figs. 14 and 15).

3.6. LOH in HBEC treated with estrogen and its metabolites

Genomic DNA was analyzed for the detection of micro-satellite DNA polymorphism using 64 markers covering chromosomes 3, 11, 13 and 17. We have detected loss of heterozygosity in ch13q12.2–12.3 (D13S893) and in ch17q21.1 (D17S800) in E_2, 2-OH-E_2, 4-OH-E_2,

Table 2
Specific up-regulated genes in E_2-transformed cells by cDNA array

Array location	Gene description	Swissprot #	Function	Ratio E_2/10F cells
A02g	Neurogenic locus notch protein	Q04721	Oncogene	2.2
A03g	c-myc binding protein MM-1	Q99471	Oncogene	2.19
B14n	Retinoic acid receptor β	P28702	Transcription	4.76
C05l	Retinoic acid receptor γ 1	P13631	Transcription	4.59
C10m	TAX1-binding protein 151	Q13311	Transcription	4.57
D14a	**CENP-F kinetochore protein**	**P49454**	**Transcription**	**2.33**
C04b	TRAP1	Q12931	Signaling	3.06
E13j	GDNPF	none	Signaling	3.16
B04n	hBAP	Q99623	Transducer	2.15
C05d	GADD153	P35638	Apoptosis	6.34
B06e	KIAA0175	Q14680	Kinase	2.24
B09d	Casein kinase I γ 2	P78368	Kinase	2.88
A11k	CKS2	P33552	Kinase	2.24
B02d	PCTK1	Q00536	Kinase	2.51
B14k	51C protein	Q13577	Phospholipase	4.14
E10j	TIMP1	P01033	Protease inhibitor	2.8
D02d	Cadherin 5	P33151	Cell adhesion	2.14
F14c	Adenylosuccinate lyase	P30566	Nucleotide metabolism	2.13
C04h	HHR23A	P54725	Stress response	2.37
F06d	LDHB	P07195	Carbohydrate metabolism	3.16
D06c	Mesothelin precursor	Q13421	Surface antigen	6.46
D06e	Integrin β 4	P16144	Cell adhesion	3.23
D08e	Integrin α 7B precursor	Q13683	Cell adhesion	2.59
E07f	Interleukin-1β precursor	P01584	Interleukin	2.09
F08f	Cytokeratin 18	P05783	Keratin	2.34
F13l	RI58	Q13325	Unclassfied	2.48

Table 3
Specific up-regulated genes in 4-OH-E_2 transformed cells by cDNA array

Array location	Gene description	Swissprot #	Function	Ratio 4-OH-E_2/10F cells
A01i	Leukemia-associated gene 1	O43261	Oncogene	2.74
A02b	EB1 protein	Q15691	Oncogene	5.45
A03b	Ezrin	P15311	Oncogene	3.5
A04e	Tyrosine-protein kinase receptor tyro3	Q06418	Oncogene	2.84
A02g	Neurogenic locus notch protein	Q04721	Oncogene	2.75
A03e	VEGFR1	P17948	Oncogene	2.64
A03g	c-myc binding protein MM-1	Q99471	Oncogene	4.06
B03m	14-3-3 protein sigma	P31947	Oncogene	2.96
A08n	HG4-1	O43846	Cell cycle	8.92
A10m	CDC10 protein homolog	Q16181	Cell cycle	3.89
A12n	GTP-binding protein GST1-HS	P15170	Cell cycle	8.66
C05f	KIAA0030	P49736	Cell cycle	3.88
C06f	MCM4 DNA replication licensing factor	P33991	Cell cycle	14.74
C07h	KIAA0078	O60216	Cell cycle	3.54
C13e	Proliferating cyclic nuclear antigen	P12004	Cyclin	10.1
A05l	G2/mitotic-specific cyclin B1	P14635	Cyclin	3.69
D03b	DNA-binding protein CPBP	Q99612	Transcription	4.19
A01c	AP-1	P05412	Transcription	11.47
E04e	Interferon γ antagonist	None	Growth factor	2.65
E12b	Heparin-binding EGF-like growth factor	Q99075	Growth factor	3.26
E14d	Fibroblast growth factor 8	P55075	Growth factor	2.79
B12a	GRB3-3	P29354	Signaling	2.74
B14j	rho GDP dissociation inihibitor 1	P52565	Signaling	2.66
C04b	TRAP1	Q12931	Signaling	4.05
B04k	Caveolin-1	Q03135	Signaling	2.54
C02I	TDG	Q13569	DNA repair	7.74
A13b	p78 putative serine/threonine-protein kinase	P27448	Kinase	3.67
B06e	KIAA0175	Q14680	Kinase	15.24
A05j	Cell division protein kinase 6	Q00534	Kinase	3.19
D09m	Glutathione-S-transferase (GST) homolog	P78417	Stress response	6.55
D07b	High mobility group protein HMG2	P26583	Chromatin	9.7
D11a	Heterochromatin protein homolog 1	P45973	Chromatin	3.47
D08a	High mobility group protein I&Y	P17096	Chromatin	7.95
D14a	**CENP-F kinetochore protein**	**P49454**	**Chromatin**	**4.13**
D08b	Histone H4	none	Histone	11.06
F03d	Thymidylate synthase	P04818	Nucleotide metabolism	2.83
F04d	Purine nucleoside phosphorylase	P00491	Nucleotide metabolism	2.63
F07e	Ribonucleotide reductase	P31350	Nucleotide metabolism	5.19
F08b	UMK	Q92528	Nucleotide metabolism	3.65
F09c	Uridine phosphorylase	Q16831	Nucleotide metabolism	3.82
F12d	Uridine 5′-monophosphate synthase	P11172	Nucleotide metabolism	6.34
F05e	Ornithine decarboxylase	P11926	Metabolism	12.9
F06d	L-Lactate dehydrogenase H subunit	P07195	Metabolism	5.93
B05l	Calmodulin 1	P02593	Calcium-binding	5.41
D02d	Cadherin 5 (CDH5)	P33151	Cell adhesion	2.77
D03e	Integrin α 3 (ITGA3)	P26006	Cell adhesion	3.89
E04k	PRSM1 metallopeptidase	Q15779	Metalloproteinase	2.58
E10j	TIMP1	P01033	Protease inhibitor	3.07
F06f	Cytokeratin 14	P02533	Keratin	4.29
F08j	HSC70-interacting protein	P50502	Chaperone	3.37
F03n	KIAA0204	Q92603	Unclassified	3.69

E_2 + ICI, E_2 + tamoxifen and BP-treated cells. LOH in ch17q21.1–21.2 (D17S806) was also observed in E_2, 4-OH-E_2, E_2 + ICI, E_2 + tamoxifen and BP-treated cells. MCF-10F cells treated with P or P + E_2 did not show LOH in the any of the markers studied. LOH was strongly associated with the invasion phenotype. These genomic changes were not abrogated by antiestrogens.

4. Discussion

We have demonstrated that 17β-estradiol induces cell transformation of the human breast epithelial cells MCF-10F. The cells treated with either doses of E_2 formed colonies in agar methocel a phenotype indicative of neoplastic transformation [61,75,76,167]. Non-transformed

Table 4
Specific up-regulated genes in BP-transformed cells by cDNA array

Array location	Gene description	Swissprot #	Function	Ratio BP/10F cells
C05l	RAR-γ 1	P13631	Transcription	3.77
B04k	Caveolin-1	Q03135	Signaling	3.35
A03b	Ezrin	P15311	Oncogene	2.01
C04h	HHR23A	P54725	Stress response	2.04
C08g	mutL protein homolog	P40692	Stress response	4.31
E07h	Glycosylation-inhibiting factor	P14174	Cell communication	4.44
D06e	Integrin β 4	P16144	Cell adhesion	4.24
D08e	Integrin α 7B precursor	Q13683	Cell adhesion	3.06
D05e	Integrin α 6 precursor	P23229	Cell adhesion	2.24
D07e	Integrin α 1	P56199	Cell adhesion	2.31
F05d	LDHA	P00338	Carbohydrate metabolism	6.25
F08f	Cytokeratin 18	P05783	Cytokeratin	3.04
F14e	BIGH3	Q15582	Microfilament	6.73

Table 5
Common down-regulated genes in MCF-10F cells transformed by BP, E_2 and 4OH using cDNA array

Array location	Gene description	Swissprot #	Function	Ratio BP/ 10F cells	Ratio E_2/ 10F cells	Ratio 4-OH-E_2/ 10F cells
A11g	PIG7	Q99732	Tumor suppressor	0.02	0.04	0.19
A14h	CD82 antigen	P27701	Tumor suppressor	0	0.18	0
B06k	rho GDP dissociation inihibitor 2	P52566	Tumor suppressor	0	0	0.21
A02g	Neurogenic locus notch protein	Q04721	Transcription	0.29	0.47	0.38
A13h	Active breakpoint cluster region-related protein	Q12979	Transcription	0.13	0.25	0.46
A14c	ets-related protein tel	P41212	Transcription	0	0.08	0.08
C06m	B4-2 protein	Q12796	Transcription	0	0	0
B03n	T3 receptor-associating cofactor 1	O00613	Intracellular transducers	0.48	0.41	0.22
E04b	HDGF	P51858	Growth factor	0.34	0.1	0.24
F07l	HNRNPK	Q07244	mRNA processing	0	0	0.17
B02j	RalB GTP-binding protein	P11234	G protein	0	0.24	0
B04j	rhoC	P08134	G protein	0.09	0.06	0.48
B12j	p21-rac2	P15153	G protein	0.12	0.2	0.49
B13l	p21-rac1	P15154	G protein	0	0	0.33
A06j	CDK5	Q00535	Kinase	0.18	0	0.41
B05h	NDR protein kinase	Q15208	Kinase	0	0	0
B08c	Tissue-specific extinguisher 1	P10644	Kinase	0	0	0.19
A09l	CDKN1A	P38936	Kinase inhibitor	0.09	0.03	0.08
A10d	HGF-SF receptor	P08581	Kinase inhibitor	0	0	0.31
B02m	Hint protein	P49773	Kinase inhibitor	0	0	0.37
B07l	Calvasculin	P26447	Calcium binding	0	0.11	0.46
B09n	CD27 ligand	P32970	Death receptor ligand	0.37	0	0
C02c	BAG-1	Q99933	BCL family protein	0	0	0.19
C09m	AH receptor	P35869	Nuclear receptor	0.06	0.12	0
F04l	Lipocalin 2	P80188	Trafficking	0	0	0
F09h	TRAM protein	Q15629	Trafficking	0	0	0.29
F10h	Dual-specificty A-kinase anchoring protein 1	Q92667	Targeting	0	0.19	0.24
D01d	Cadherin 3	P22223	Cell adhesion	0.32	0.14	0.08
D02e	Integrin β 6 precursor	P18564	Cell adhesion	0.16	0.11	0.22
E02f	IGF-binding protein 3	P17936	Hornone	0	0	0
E02m	HLA-C	Q30182	Immune	0.19	0.17	0
E02n	GRP 78	P11021	Immune	0	0	0
F03b	Fibronectin precursor	P02751	Extracellular matrix	0.32	0.13	0.09
F13n	Insulin-induced protein 1	O15503	Unclassified	0.13	0.33	0.35
F08m	PM5 protein	Q15155	Unclassified	0.17	0.34	0

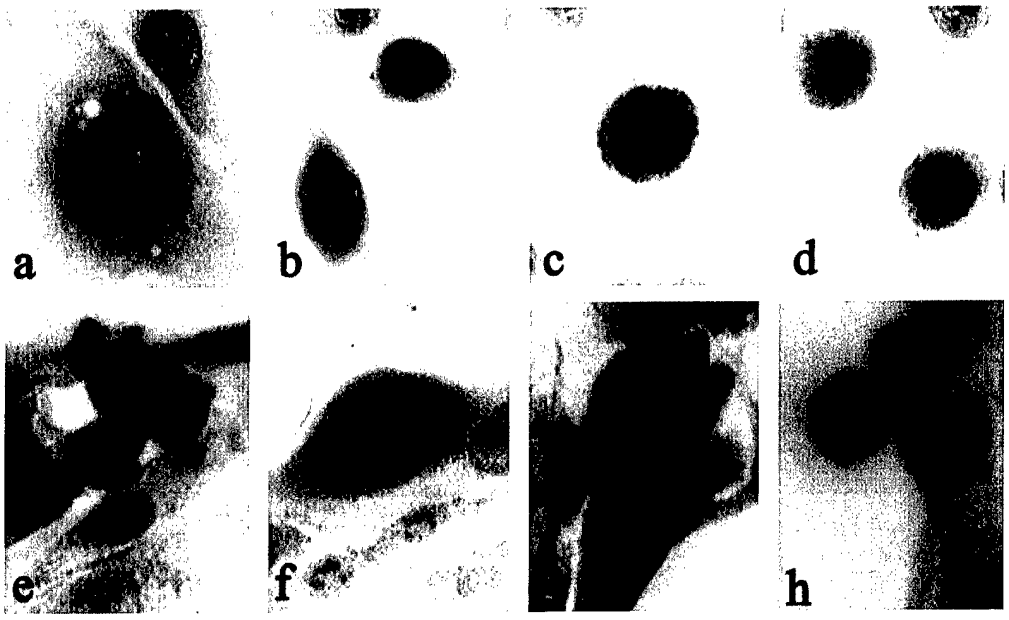

Fig. 12. (a–h) Cytospin preparation stained with H&E. (a) Multinucleated E_2-MCF-10F-transformed cells; (b) normal mitosis of MCF-10F cells; (c and d) abnormal mitosis of E_2-transformed cells; (e and f) 2-OH-E_2-transformed cells; (g and h) 4-OH-E_2-transformed cells (40×).

cells produce ductules like structure and transformed cells produce spherical or solid masses of cells [169,176]. Cells treated with DMSO, cholesterol or progesterone at different concentrations was unable to alter the ductular pattern. E_2, BP and DES treated cells induces the loss of MCF-10F cells to produce ductules in a dose dependent fashion and the number of solid masses paralleled the formation of colonies in agar methocel [169]. Most of the cells growing in the collagen matrix are actively proliferating as detected by immunostaining with Ki67.

The ERα was not detected in the MCF-10F cells or in those transformed by estrogens or its metabolites. Interestingly MCF-10F and the transformed cells are ERβ positive showing two bands 68 and 53 kDa of molecular weight corresponding to ERβ long and short form, respectively. Those cells transformed by 17β-estradiol as well as those treated with progesterone significantly overexpressed the long form of ERβ. Instead, MCF-7 cells showed the short form of the ERβ. These data explain why the proliferative activity of the MCF-10F cells that has been treated with tamoxifen alone or

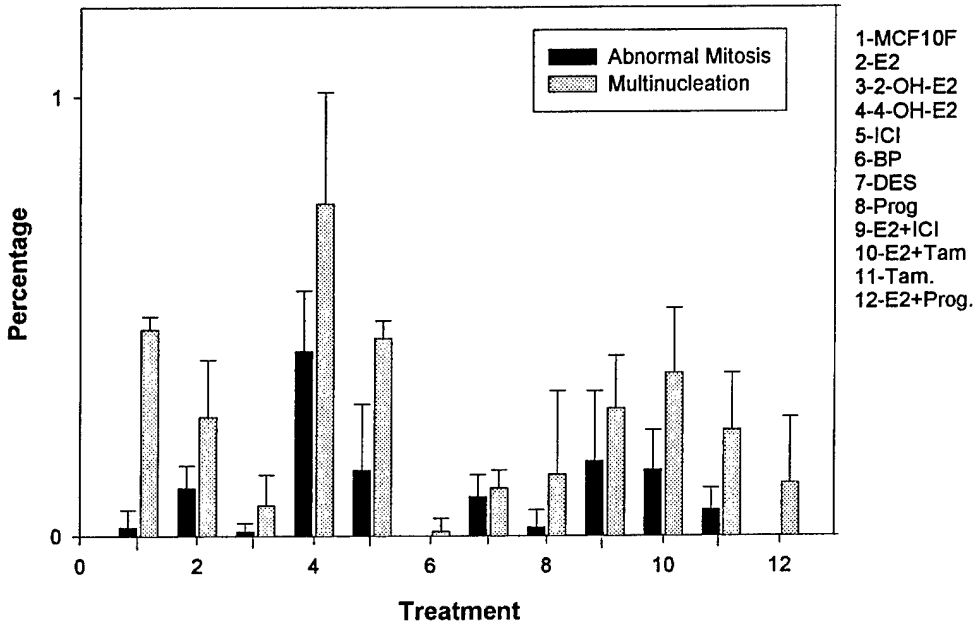

Fig. 13. Histogram showing the percentage of abnormal mitoses and multinucleated cells.

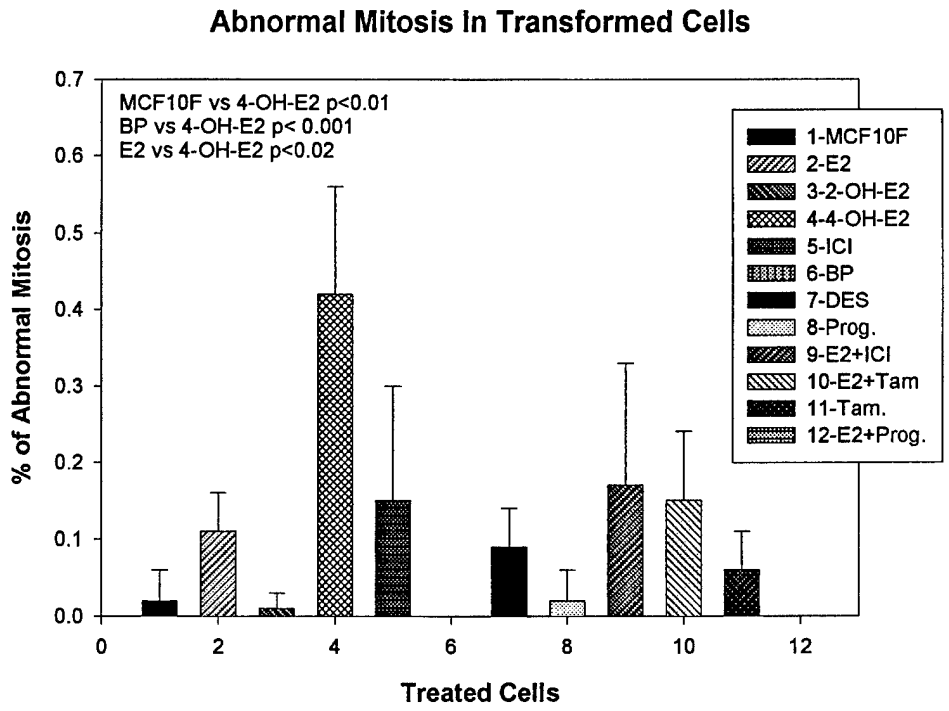

Fig. 14. Histogram showing the percentage of abnormal mitosis.

ICI-182,780 was not modified when compared with the control. Instead those cells that were treated with 17β-estradiol in presence of tamoxifen or ICI-182,780 showed no increment of the proliferative activity neither in monolayer nor collagen matrix. The colony formation in agar methocel was abrogated and the ductulogenic capacity was maintained. The proliferative activity of these cells in collagen matrix was also abrogated. Indicating that the response of MCF-10F to estrogen could be mediated by the presence of ERβ. The functional role of ERβ-mediated estrogen signaling pathways in the pathogenesis of malignant diseases is essentially unknown. In the rats, ERβ-mediated mechanisms have been implicated in the upregulation of PgR expression in the dysplastic acini of the dorsolateral prostate in response to

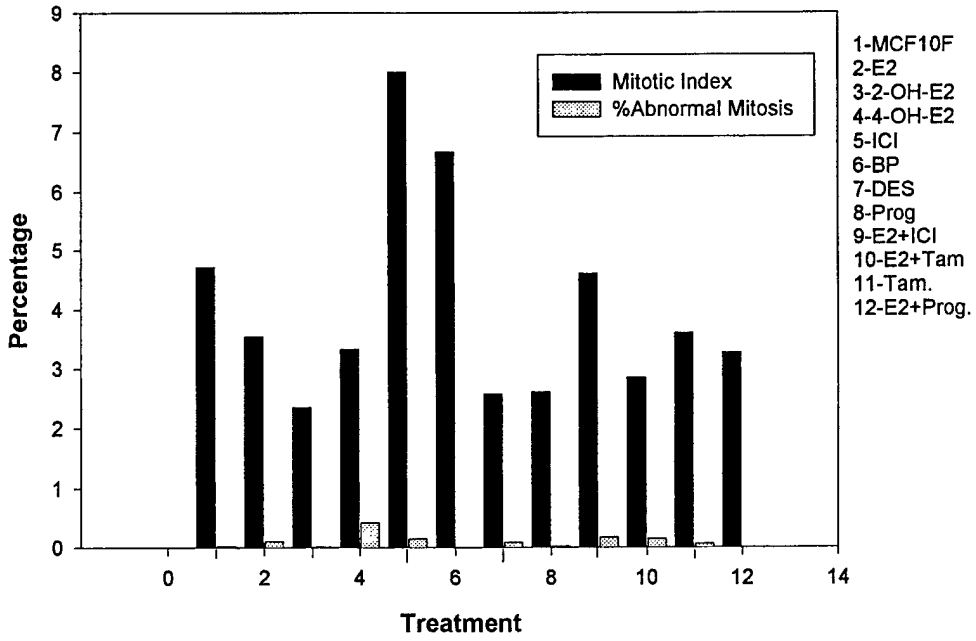

Fig. 15. Histogram showing a comparative value of the percentage of mitosis of mitotic index, and the percentage of abnormal mitosis.

treatment of testosterone and 17β-estradiol [82]. In the human, ERβ has been detected in both normal and cancerous breast tissues or cell lines, and is the predominant ER type in normal breast tissue. Expression of ERβ in breast tumors is inversely correlated with the PgR status and variant transcripts of ERβ have been observed in some breast tumors [26]. ERβ and ERα are co-expressed in some breast tumors and a few breast cell lines, suggesting an interesting possibility that ERα and ERβ proteins may interact with each other and discriminate between target sequences leading to differential responsiveness to estrogens. In addition, estrogen responses mediated by ERα and ERβ may vary with different composition of their co-activators that transmit the effect of ER–ligand complex to the transcription complex at the promotor of target genes. Recently, it has been shown that an increase in the expression of ERα with a concomitant reduction in ERβ expression occurs during tumorigenesis of the breast and ovary [83], but breast tumors expressing both ERα and ERβ are lymph node-positive and tend to be of higher histopathological grade. These data suggest a change in the interplay of ERα- and ERβ-mediated signal transduction pathways during breast tumorigenesis.

Although the presence of ERβ may indicate that the response of the cells to growth and form colonies in agar methocel could be mediated by this receptor. The presence of estrogen receptor β does not explain the data obtained using the metabolite 4-OH-estradiol. The transforming efficiency of 4-OH-E_2 was not abrogated by ICI neither in the colony efficiency assay nor in the loss of ductulogenic capacity. More importantly, ICI-182,780 was unable to abrogate the invasive phenotype induced by estrogen and tamoxifen, even exacerbate the invasive phenotype. Therefore, the data indicate that the ERβ pathway is not involved in the carcinogenic process. The biological role of the ERβ has been in part explained by gene knockout studies, in which the presence of ERα but not ERβ was necessary for the development of the mouse mammary gland [177]. ERβ may be acting as an antagonist of ERα, thus, by removing ERβ the suppressive effect of the receptor is lost. If that were the case in our HBEC, the presence of ERβ will abrogate the emergence of transformation. Alternatively, the downstream signaling pathway may dictate the putative suppressive effects of ERβ. Both ER subtypes can signal via classic estrogen response elements or via AP-1 enhancers. The downstream effects of signaling through AP-1 are both receptor and ligand specific [178]. In the model described above, it seems that the presence of ERβ is the pathway used by estrogen to induce cell proliferation in MCF-10F cells. This is supported by the fact that either tamoxifen or a pure antiestrogen like ICI abrogated these phenotypes. However, the invasion phenotype, an important marker of tumorigenesis is not modified when the cells are treated in presence of tamoxifen or ICI, suggesting that other pathways may be involved.

Although we cannot rule out the possibility, that 4-OH-E_2 may interact with other receptors still not identified, with the data presently available the direct effect of 4-OH-E_2 supports the concept that metabolic activation of estrogens mediated by various cytochrome P450 complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects leading to transformation. An increase in cathecol estrogen (4-OH-E_2) due to either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA. Through this pathway estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis [64,65]. This assumption was confirmed when we found that all the transformation phenotypes induced by 4-OH-E_2 were not abrogated when this compound was used in presence of the pure antiestrogenic ICI. The novelty of this observation lies in the fact that this pathway can successfully bypass the ERβ pathway.

17β-Estradiol and estrone, which are continuously interconverted by 17β-estradiol hydroxysteroid dehydrogenase (or 17β-oxidoreductase), are the two major endogenous estrogens. They are generally metabolized via two major pathways: hydroxylation at C-16α position and at the C-2 or C-4 positions [87–89]. The carbon position of the estrogen molecules to be hydroxylated differs among various tissues and each reaction is probably catalyzed by various CYP isoforms. For example, in MCF-7 human breast cancer cells, which produce catechol estrogens (CE) in culture, CYP1A1 catalyzes hydroxylation of 17β-estradiol at C-2, C-15α and C-16α, CYP1A2 predominantly at C-2 [26,90], and a member of the CYP1B subfamily is responsible for the C-4 hydroxylation of 17β-estradiol. CYP3A4 and CYP3A5 have also been shown to play a role in the 16α-hydroxylation of estrogens in human [26].

The hydroxylated estrogens are catechol estrogens that will easily be auto-oxidated to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA via a Michael addition and, thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidatic activation of catechol estrogens. In addition, a redox cycle consisting of the reversible formation of the semiquinones and quinones of catechol estrogens catalyzed by microsomal P450 and cytochrome P450-reductase can locally generate superoxide and hydroxyl radicals to produce additional DNA damage. Furthermore, catechol estrogens have been shown to interact synergistically with nitric oxide present in human breast generating a potent oxidant that induces DNA strand breakage [26].

Steady state concentrations of catechol estrogens are determined by the cytochrome P450-mediated hydroxylations of estrogens and monomethylation of catechols catalyzed by blood-borne catechol *o*-methyltransferase [91]. Increased formation of catechol estrogens as a result of elevated hydroxylations of 17β-estradiol at C-4 and C-16α [26,92] positions occurs in human breast cancer patients and in women

at a higher risk of developing this disease. There is also evidence that lactoperoxidase, present in milk, saliva, tears and mammary glands, catalyzes the metabolism of 17β-estradiol to its phenoxyl radical intermediates, with subsequent formation of superoxide and hydrogen peroxide that might be involved in estrogen-mediated oxidative stress [93]. A substantial increase in base lesions observed in the DNA of invasive ductal carcinoma of the breast [94] has been postulated to result from the oxidative stress associated with metabolism of 17β-estradiol [93].

During the process of cell transformation induced by estrogen and its metabolites there is an increase in the number of multinucleated cells and abnormal mitoses that is associated with the overexpression of one component of the centromere–kinetochore complex CENP-E. It is important to emphasize that the percentage of these abnormal mitoses is lees than 1%. The movements that chromosomes undergo during mitosis are facilitated by the mitotic spindle, an apparatus composed principally of microtubule fibers that attach to a pair of kinetochores located on opposite sides of the centromere region of chromosomes. The microtubule–kinetochore interaction is essential for chromosome segregation. Disruptions of this interaction will lead to unequal distribution of chromosomes in daughter cells [171]. We have found that the CENP-E, a ca. 300 kDa protein that have been recently identified to be a novel member of the kinesin superfamily of microtubule-based motor proteins [171] is overexpressed in MCF-10F transformed cells by estrogens and its metabolites but not in the BP-transformed cells. CENP-E staining appeared only in mitotic cells [171], suggesting that it is a mitosis-specific motor. Its association with kinetochores suggests that it functions to translocate chromosomes along the spindle microtubules. This phenomena, however, was not observed in the BP-transformed cells indicating that whereas aneuploidy is part of the neoplastic transformation process is depending of the carcinogenic insult and probably not the main driving force to cause genomic instability. This concept was further confirmed by the lack of significant karyotipic changes detected in these transformed cells [179] and by the fact that the same cluster of genes were overexpressed in cells transformed with E_2, 4-OH-E_2 and BP, indicating that there is a common pathway of transformation and that may be responsible for driving the normal cell to neoplasia. The data also point toward the concept that certain compounds like steroid hormones or its metabolites may affect certain genes more readily than other exerting the expression of genes that are altering the mitotic spindle and therefore making the cell aneuploidy.

Breast cancer is considered the result of sequential changes that accumulate over time. DNA content changes, i.e. loss of heterozygosity and aneuploidy, can be detected at early stages of morphological atypia, supporting the hypothesis that aneuploidy is a critical event driving neoplastic development and progression [134,135]. Aneuploidy is defined as the gain or loss of chromosomes; it is a dynamic, progressive, and accumulative event that is almost universal in solid tumors [136,137]. The extensive array of altered gene expression observed in tumors and the numerous altered chromosomes detected by CGH [72,138] provide striking evidence that aneuploidy can totally disrupt cell homeostatic control. The main question is whether aneuploidy is a consequence of neoplastic development or a cause of neoplastic development [72,73,138]. One of the several mechanisms proposed for the development of aneuploidy is the failure to appropriately segregate chromosomes [73,74,139]. For example, interference with mitotic spindle dynamics, abnormal centrosome duplication, altered chromosome condensation and cohesion, defective centromeres, and loss of mitotic checkpoints [139]. Functional consequences of centrosome defects may play a role during neoplastic transformation and tumor progression, increasing the incidence of multipolar mitoses that lead to chromosomal segregation abnormalities and aneuploidy. In considering estrogen as a carcinogenic agents there is evidence that they affect microtubules [140] and a recently report indicates that progesterone may facilitate aneuploidy [141]. The importance of these findings is magnified with the recent publications that demonstrate women on hormone replacement treatments that include progesterone have increased mammographic breast density and increased breast cancer risk than women taking only estrogen [142–144].

In the center stage of the research endeavor on aneuploidy are the centrosomes that are organelles that nucleate microtubule growth and organize the mitotic spindle for segregating chromosomes into daughter cells, establishing cell shape and cell polarity, processes essential for epithelial gland organization [72,139]. Centrosomes also coordinate numerous intracellular activities, in part by providing a site enriched for regulatory molecules, including those that control cell cycle progression, centrosome and spindle function, and cell cycle checkpoints [73,145–148]. Although the underlying mechanisms for the formation of abnormal centrosomes are not clear, several possibilities have been proposed and implicated in the development of cancer such as alterations of checkpoint controls initiating multiple rounds of centrosome replication within a single cell cycle and failure of cytokinesis, cell fusion, and cell cycle arrest in S-phase uncoupling DNA replication from centrosome duplication [146].

The genomic signature of the three transformed cells present a cluster of genes that are commonly unregulated, indicating that a similar mechanism is involved in the transformation pathway. Interestingly, there are genes that are up-regulated in the E_2 and 4-OH-E_2 transformed cells such as the CENP-E that are not modified in the BP-transformed cells. The same occurs for several genes that are down-regulated differentially in the three transformed cells.

A more striking change induced by estrogen and its metabolites in MCF-10F cells is the loss of heterozygosity in ch13q12.2–12.3 (D13S893) and in ch17q21.1 (D17S800) in E_2, 2-OH-E_2, 4-OH-E_2, E_2 + ICI, E_2 + tamoxifen and

BP-treated cells. LOH in ch17q21.1–21.2 (D17S806) was also observed in E_2, 4-OH-E_2, E_2 + ICI, E_2 + tamoxifen and BP-treated cells. MCF-10F cells treated with P or P + E_2 did not show LOH in the any of the markers studied. LOH was strongly associated with the invasion phenotype. Altogether our data indicate that E_2 and its metabolites induce in HBEC LOH in loci of chromosomes 13 and 17, that has been reported in primary breast cancer [172–175], that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.

The detection of LOH in HBEC transformed with estrogen and its metabolites are supported by various types of DNA damage induced by estrogen metabolites in cell-free systems or in cells in culture and by parent hormones in vivo [95–99], leading to the hypothesis of estrogen as mutagen and tumor initiator [100–103]. Estrogens induces microsatellite instability, changes in DNA fragments containing microsatellite repeat sequences in E_2-induced hamster kidney tumors, in surrounding kidney tissue [104] and in MCF-10F HBEC transformed by E_2 [105]. Microsatellite instability is a relatively common genetic modification [106–108], induced by the natural hormone E_2 in cells in culture [105], in Syrian hamster kidney tumors, and in surrounding tissues [104]. It has also been detected with high frequency in human vaginal tumors in daughters of women treated with diethylstilbestrol (DES) [109]. Microsatellite instability has also been detected in human breast tumors [110–117].

Chemical carcinogens covalently bind to DNA to form two types of adducts: stable ones that remain in DNA unless removed by repair and depurinating ones that are lost from DNA by destabilization of the glycosyl bond [118,119]. Evidence that depurinating polycyclic aromatic hydrocarbon–DNA adducts play a major role in tumor initiation [118–120] and that estrogen metabolites form depurinating DNA adducts strongly indicates that estrogen is an endogenous initiators of cancer [95]. Catechol estrogens are among the major metabolites of estrone (E_1) and estradiol (E_2). If these metabolites are oxidized to the electrophilic CE quinones (CE-Q), they may react with DNA. Specifically, the carcinogenic 4-CE [96,121] are oxidized to CE-3,4-Q, which react with DNA to form depurinating adducts [95,122]. These adducts generate apurinic sites that may lead to oncogenic mutations [74,120,122,123], thereby initiating cancer. The effects of some of these factors have already been observed in analyses of breast tissue samples from women with and without breast cancer [133]. The levels of E_1 (E_2) in women with carcinoma were higher. In women without breast cancer, a larger amount of 2-CE than 4-CE was observed. In women with breast carcinoma, the 4-CE were 3.5 times more abundant than the 2-CE and were 4 times higher than in the women without breast cancer [133], supporting the finding that E_2 and its metabolites mainly 4-OH-E_2 are carcinogenic agents in breast epithelial cells.

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Glossary

Definition of key terms

ATCC: American Tissue Culture Collection
BP: Benz(*a*)pyrene
BSA: Bovine serum albumin
CE-Q: Catechol estrogen-quinone
CENP-E: Centromere–kinetochore complex
CE: Colony efficiency
CGH: Comparative genomic hybridization
CS: Colony size
CYP: Cytochrome P450
DES: Diethylstilbestrol
DTT: Dithiothreitol
EDTA: Ethylene-diamino-tetraacetic-acid
E_1: Estrone
E_2: Estradiol
ER: Estrogen receptors
ERα: Estrogen receptor α
ERβ: Estrogen receptor β
4-OH-E_2: 4-Hydoxy estradiol
HBEC: Human breast epithelial cells
HCl: Hydrochloric acid
Lob 1: Lobule type 1
Lob 2: Lobule type 2
Lob 3: Lobule type 3
Lob 4: Lobule type 4
LOH: Loss of heterozygosity
MEM: Minimal essential medium
PAGE: Polyacrylamide gel electrophoresis
PCR: Polymerase chain reaction
PR: Progesterone receptor
PgR: Progesterone receptor
16-α-OH-E_2: 16-α-Hydroxy-estradiol
2-OH-E_2: 2-Hydroxy-estradiol
SE: Survival efficiency
SM: Spherical masses

Genotoxicity of steroidal estrogens

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Key words: breast cancer, cell transformation, MCF10F, estradiol, 4-OH-Estradiol, antiestrogens, loss of heterozygosity.

Teaser: Estrogen and its metabolites, mainly 4-OH-estradiol, are carcinogenic agents in the human breast using a non-receptor mediated pathway.

The molecular mechanisms underlying the development of breast cancer in general, and estrogen-associate breast carcinogenesis in particular, are not completely understood. There are three mechanisms considered responsible for the carcinogenicity of estrogens in the human breast: (i) receptor-mediated hormonal activity, which stimulates cellular proliferation, resulting in more opportunities for accumulation of genetic damages leading to carcinogenesis; (ii) a cytochrome P450-mediated metabolic activation, which elicits direct genotoxic effects by increasing mutation rates; and (iii) the induction of aneuploidy by estrogen. In this article we will concentrate in discussing the role of estrogen receptors and the metabolic activation of E_2 as mechanisms of breast cancer initiation.

Even though the breast is influenced by many hormones and growth factors [1-4], estrogens play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium [1,2]. There are three mechanisms considered responsible for the carcinogenicity of estrogens: (i) receptor-mediated hormonal activity, which stimulates cellular proliferation, resulting in more opportunities for accumulation of genetic damages leading to carcinogenesis; (ii) a cytochrome P450-mediated metabolic activation, which elicits direct genotoxic effects by increasing mutation rates; and (iii) the induction of aneuploidy by estrogen [5-11]. Estrogen also compromises the DNA repair system and enables accumulation of lesions in the genome essential to estrogen-induced tumorigenesis [12]. In this article we will concentrate in discussing the role of estrogen receptors and the metabolic activation of E_2 as mechanisms of breast cancer initiation.

Receptor mediated mechanism of cancer initiation.

The receptor-mediated activity of estrogen is generally related to induction of expression of the genes involved in the control of cell-cycle progression and growth of human breast epithelium. The biological response to estrogen depends upon the local concentrations of the active hormone and its receptors. The level of estrogen receptor alpha (ERα) expression is higher in breast cancer patients than in control subjects and is related to breast cancer risk in postmenopausal women. Overexpression of ERα in normal human breast epithelium might augment estrogen responsiveness and, hence, the risk for breast cancer. The proliferative activity and the percentage of ERα -positive cells are highest in the terminal ductal lobular unit or lobule type 1 in comparison with the more differentiated lobular structures of the normal breast [2,4]. These

findings provide a mechanistic explanation for the higher susceptibility of these structures to transformation by chemical carcinogens *in vitro*, also supporting as well the observations that lobules type 1 are the site of origin of ductal carcinomas [13].

The presence of $ER\alpha^+$ and $ER\alpha^-$ cells with different proliferative activity in the normal human breast may help to elucidate the genesis of $ER\alpha^+$ and $ER\alpha^-$ breast cancers. It has been suggested that either $ER\alpha^-$ breast cancers result from the loss of the ability of the cells to synthesize $ER\alpha$ during clinical evolution of $E_2R\alpha^+$ cancers, or that $ER\alpha^+$ and $ER\alpha^-$ cancers are different entities [14]. Based on these observations, it is postulated that lobules type 1 contain at least three cell types, (i) $ER\alpha^+$ cells that do not proliferate, (ii) $ER\alpha^-$ cells that are capable of proliferating, and (iii) a few $ER\alpha^+$ cells that can also proliferate [14]. Therefore, estrogen might stimulate $ER\alpha^+$ cells to produce a growth factor, which in turn, stimulates neighboring $ER\alpha^-$ cells that can proliferate (Ki67+) [14]. In the same fashion, the few cells that are $ER\alpha^+$ and can proliferate (Ki67+) could be the stem cells of $ER\alpha^+$ tumors. $ER\alpha^-$ cells might also convert to $ER\alpha^+$ cells [14] or they might express $ER\beta$.

The newly discovered $ER\beta$ [15, 16] opens another possibility that those cells traditionally considered negative for $ER\alpha$ might be positive for $ER\beta$. $ER\beta$ is expressed during the immortalization and transformation of $ER\alpha^-$ human breast epithelial cells [17], supporting the hypothesis of conversion from a negative to a positive receptor cell. In the human, $ER\beta$ has been detected in both normal and cancerous breast tissues and cell lines, and is the predominant ER type in normal breast tissue. Expression of $ER\beta$ in breast tumors is inversely correlated with the

progesterone receptor (PgR) status, and variant transcripts of ERβ have been observed in some breast tumors [18]. ERβ and ERα are co-expressed in some breast tumors and a few breast cell lines, suggesting that ERα and ERβ proteins interact with each other and discriminate between target sequences, leading to differential responsiveness to estrogens. In addition, estrogen responses mediated by ERα and ERβ might vary with the different composition of their co-activators that transmit the effect of ER-ligand complex to the transcription complex at the promotor of target genes. An increase in the expression of ERα with a concomitant reduction in ERβ expression occurs during tumorigenesis of the breast and ovary [19], but breast tumors expressing both ERα and ERβ have metastasized to lymph nodes and tend to be of higher histopathological grade. These data suggest a change in the interplay of ERα and ERβ-mediated signal transduction pathways during breast tumorigenesis.

Even though it is now generally believed that alterations in the ER-mediated signal transduction pathways contribute to breast cancer progression toward hormonal independence and more aggressive phenotypes, there is also increasing evidence that a membrane receptor coupled to alternative second messenger signaling mechanisms is operational, and might stimulate the cascade of events leading to cell proliferation. This suggests that ERα$^-$ cells in the human breast respond to estrogens through this or other pathways [20]. The biological responses elicited by estrogens are mediated, at least in part, by the production of autocrine and paracrine growth factors from the epithelium and the stroma in the breast [21]. Evidence has also accumulated to support the existence of ER variants, mainly a truncated ER and an exon deleted ER. Such expression of ER variants might contribute to breast cancer progression toward hormone independence. Although more studies are needed, the findings that, in the normal breast, the

proliferating and steroid hormone receptor positive cells are different open new possibilities for clarifying the mechanisms through which estrogens might act on the proliferating cells to initiate the cascade of events leading to cancer.

The mechanism of oxidative metabolism of estrogen in cancer initiation

17β-estradiol (E_2) is biologically the most active estrogen in breast tissue. Circulating estrogens originate mainly from ovarian steroidogenesis in premenopausal women and peripheral aromatization of ovarian and adrenal androgens in postmenopausal women [18, 20]. The importance of ovarian steroidogenesis in the genesis of breast cancer is highlighted by the fact that occurring naturally or induced early menopause before age 40 significantly reduces the risk for developing breast cancer [18, 20]. However, the uptake of E_2 from the circulation does not contribute significantly to the total content of estrogen in breast tumors, because most estrogen in the tumor tissues is derived from *de novo* biosynthesis. In fact, the concentrations of E_2 in breast cancer tissues do not differ among premenopausal and postmenopausal women, even though plasma levels of E_2 decrease by 90% following menopause [20]. This phenomenon might be explained by *in situ* metabolism of estrogens. The three main enzyme complexes involved in the synthesis of biologically active estrogen in the breast are: (i) aromatase that converts androstenedione to estrone, (ii) estrone sulfatase that hydrolyses the estrogen sulfate to estrone, and (iii) 17β-estradiol-hydroxysteroid dehydrogenase that preferentially reduces estrone to 17β-estradiol in tumor tissues [18, 20].

There is evidence that oxidative catabolism of estrogens mediated by various cytochrome P450 (CYP) complexes constitutes a pathway of their metabolic activation and generates reactive free radicals that can cause oxidative stress and genomic damage. 17β-estradiol- and estrone, which are continuously interconverted by 17β-estradiol-hydroxysteroid dehydrogenase (or 17β-oxidoreductase), are the two major endogenous estrogens. They are generally metabolized via two major pathways: hydroxylation at C-16α position and at the C-2 or C-4 positions. The carbon position of the estrogen molecules to be hydroxylated differs among tissues and each reaction is probably catalyzed by various CYP isoforms. For example, in MCF-7 human breast cancer cells, which produce catechol estrogens in culture, CYP 1A1 catalyzes hydroxylation of estradiol-17β at C-2, C-15α and C-16α, CYP 1A2 predominantly at C-2, and a member of the CYP 1B subfamily at C-4. CYP3A4 and CYP3A5 also play a role in the 16α-hydroxylation of estrogens in humans [18, 20].

The hydroxylated estrogens are catechol estrogens that are easily autooxidated to semiquinones and, subsequently, quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA. Thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidatic activation of catechol estrogens. In addition, the reversible formation of the semiquinones and quinones of catechol estrogens catalyzed by microsomal P450 and cytochrome P450-reductase can locally generate superoxide and hydroxyl radicals to produce additional DNA damage. Furthermore, catechol estrogens have been shown to interact synergistically with nitric oxide present in human breast, generating a potent oxidant that induces DNA strand breakage [18, 20-25].

Steady-state concentrations of catechol estrogens are determined by the cytochrome P450-mediated hydroxylations of estrogens and monomethylation of catechols catalyzed by blood-borne catechol *o*-methyltransferase. Increased formation of catechol estrogens as a result of elevated hydroxylations of 17β-estradiol at C-4 and C-16α [18, 20] positions occurs in patients with breast cancer and in women at a higher risk for developing this disease. Lactoperoxidase, present in milk, saliva, tears and mammary glands, catalyzes the metabolism of 17β-estradiol to its phenoxyl radical intermediates, with subsequent formation of superoxide and hydrogen peroxide that might be involved in estrogen-mediated oxidative stress. A substantial increase in base lesions observed in the DNA of invasive ductal carcinoma of the breast [18, 20] has been postulated to result from the oxidative stress associated with metabolism of 17β-estradiol. A direct evidence of the carcinogenic and genotoxic effect of E_2 has been demonstrated *in vitro* [20]. Human breast epithelial cells treated with E_2, 2-OH-E_2, 4-OH-E_2, and 16α-OH-E_2 formed colonies in agar methocel with impairment in the formation of ductules in collagen matrix and increase in the invasive capacity [20].

The detection of various types of DNA damage induced by estrogen metabolites in cell-free systems or in cells in culture and by parent hormones *in vivo* [5, 26] has led to the hypothesis of an additional role of estrogen as a mutagen and tumor initiator [27]. The induction of mutations by estrogens or their metabolites has been demonstrated [28], supporting the hypothesis that estrogens are mutagenic and that metabolic conversion of E_2 to cathecol estrogen is required for the induction of such mutations. In addition to mutations, E_2 also induces microsatellite instability. Changes in DNA fragments containing microsatellite repeat sequences have been detected in E_2-induced hamster kidney tumors, in surrounding kidney tissue [29] and in MCF-

10F HBEC transformed by E_2 [30]. Microsatellite instability (MSI) and loss of heterozygosity (LOH) are relatively common genetic modifications [31, 32], induced by the natural hormone E_2 in cells in culture [30], in Syrian hamster kidney tumors, and in surrounding tissues [29], in human vaginal tumors in daughters of women treated with diethylstilbestrol (DES) and in human breast tumors [33-40]. LOH has also been detected in ch13q12.2-12.3 (D13S893) and in ch17q21.1 (D17S800) in E_2, 2-OH-E_2, 4-OH-E_2-, E_2+ICI, and E_2+Tamoxifen-treated cells [41]. LOH in ch17q21.1-21.2 (D17S806) was also observed in E_2, 4-OH-E_2, E_2+ICI, and E_2+Tamoxifen -treated cells [41]. LOH was strongly associated with the invasion phenotype and was not abrogated by antiestrogens. More importantly, the changes detected in these chromosome loci are also found in primary breast cancer, for example LOH in the locus marked by D17S800 has been associated with grade III, high S phase and positive for p53 primary tumors [42]. MSI and LOH were observed in chromosome 17p13 using the marker D17S513 [40]. MSI and LOH on this locus has also been reported in ductal carcinoma of the breast and in other endocrine dependent organs like ovary and uterus [43-47]

Additional factors contributing to the carcinogenic effect of estrogen

The breast is an endocrine organ and can synthesize E_2 *in situ* from precursor androgens via the enzyme aromatase [18, 20]. Breast tissue contains aromatase and produces amounts of E_2 that exert biological effects on breast cell proliferation. The effects of local production exceed those exerted in a classic endocrine fashion by uptake of E_2 from plasma. One crucial factor is excessive synthesis of E_2 by overexpression of CYP19 in target tissues [48-50] and/or the presence of excess sulfatase that converts stored E_1 sulfate to E_1. The observation that breast tissue can synthesize E_2 *in situ* suggests that much more E_2 is present in some locations of target

tissues than would be predicted from plasma concentration [50]. A second crucial factor might be high levels of 4-CE due to overexpression of CYP1B1, which converts E_2 predominantly to 4-OHE_2 [51]. This could result in relatively large amounts of 4-CE and, subsequently, more extensive oxidation to their CE-3, 4-Q. A third factor could be a lack or low level of COMT activity. If this enzyme is insufficient, either through a low level of expression or its low activity allele, 4-CE will not be effectively methylated, but will be oxidized to the ultimate carcinogenic metabolite, CE-3, 4-Q. Fourth, a low level of GSH and/or low levels of quinone reductase and/or CYP reductase can leave available a higher level of CE-Q that might react with DNA. The effects of some of these factors have already been observed in analyses of breast tissue samples from women with and without breast cancer [52].

Altogether, both set of data support the concept that estrogen and its metabolites are at high concentration in the breast tissue, indicating a direct carcinogenic effect in the breast epithelial cells [52], and also give relevance to the *in vitro* study that shows that E_2 and its metabolite, 4-OH-E_2 are powerful transforming agent in HBE cells that are lacking ERα [22, 41]. Furthermore, more studies using relevant breast tissue models (i.e. in vitro techniques) and clinical samples from patients with and without breast cancers, like nipple aspirate and ductal lavage, in combination with genomic and proteomic technology, will further our understanding of the interaction of E_2 with crucial genetic pathways and protein interactions with key molecules controlling cell proliferation and differentiation leading to cancer.

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