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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

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. This cell line is a spontaneously immortalized HBEC that does not contain measurable level of ER α or ER β. Short-term treatment of these cells with physiological doses of 17-β estradiol induces anchorage independent growth, colony formation in agar methocel, and reduces ductulogenic capacity in collagen gel, all phenotypes whose expression is indicative of neoplastic transformation. Physiological doses of 4-OH-E_2 (0.007nM) induces transformation phenotypes that are not abrogated by the pure antiestrogen ICI. The fact that the MCF10F cells are both ER-α and ERβ negative, argue in favor of a metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. 2-OH-E_2 was unable to induce significant increase in colony formation, although small colonies less than 60 μm in diameter were observed, whereas none were found in the MCF10F cells treated with either DMSO or Cholesterol. The data support the conclusion that estrogen and its metabolites are transforming agent and that this effect is not estrogen receptor mediated.

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Estrogens and Breast Cancer
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INTRODUCTION

Epidemiological and clinical evidence indicate that breast cancer risk is associated with prolonged exposure to female ovarian hormones [1-4]. Breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors [5,6], from which estrogens have been demonstrated to be of essential importance in this phenomenon as it is observed in postmenopausal hyperestrogenism resulting from the use of estrogenic hormone replacement therapy and obesity [7,8].

Estrogens, that are necessary for the normal development of both reproductive and non-reproductive organs, exert their physiological effects by binding to their specific receptors, the estrogen receptors (ER) α or β. Estrogens might act as well through alternate non-receptor mediated pathways [17]. E_2, under the effect of 17 α-oxidoreductase is continuously interconverted to estrone (E_1), and both are hydroxylated at C-2, C-4, or C-16 α positions by cytochrome P450 isoenzymes, i.e., CYP1A1, CYP1A2, or CYP1B1, to form catechol estrogens [18-23]. The demonstration that the catecholestrogen 4-hydroxyestradiol (4-OH-E_2) induces an estrogenic response in the uterus of ER α null mice, and the fact that this response is not inhibited by the antiestrogen ICI-182, 780 [9], indicate that this catecholestrogen does not exert its effect on the ER. The metabolic activation of estrogens can be mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. An increase in CE 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 via a Michael addition and thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidatic activation of CE. Thus, estrogen and 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 [18-23]. Although this pathway has been demonstrated in other systems [18-20], it still needs to be demonstrated in human breast epithelial cells.

Furthermore, if estrogen is carcinogenic in the human breast through the above mentioned pathway it would induce in breast epithelial cells in vitro transformation phenotypes indicative of neoplasia 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 [24-39]. In order to test this hypothesis we have evaluated the transforming potential of E_2 on human breast epithelial cells (HBEC) in vitro, utilizing the spontaneously immortalized HBEC MCF-10F [40,41]. This cell line lacks both ER- α and ER-β although this latter receptor is induced in cells transformed by chemical carcinogens [42].

In the present work we report that the same phenotypes and characteristics that were expressed by MCF-10F cells transformed by the chemical carcinogen benz (a) pyrene (BP), oncogenes [43-46] and E_2 treated cells [47] are also induced by metabolites of E_2, such as 4-OH-E_2 and that the transformation phenotypes were not abrogated by an antiestrogen receptor.

6-BODY

Direct mutagenic and transforming activities of estrogens in cultured Syrian hamster embryonic cells were documented more than a decade ago, but whether these results are relevant to human situation has been subject to debate because rodent cells in general are easier to be transformed than human cells. Therefore, we have investigated the carcinogenic potential of estrogens in human breast epithelial cells in vitro. Using our established in vitro cell transformation model we have proposed two specific aims 1-To determine whether estrogens induce neoplastic transformation of normal HBEC MCF10Fcells through receptor mediated hormonal activities and 2-To determine whether estrogens promote neoplastic progression of estrogen or chemical carcinogen transformed derivatives of MCF10F cells. **This progress report covers the period of August 1 2001 to July 30, 2002, period in which we have been able to accomplish the Specific Aim 1 as indicated below.**

1-The experimental model.

MCF-10F cells were cultured in DMEM:F-12 medium containing 1.05 mM calcium (Ca^{2+}), antibiotics, antimycotics, hormones, growth factors, and equine serum as previously described [44]. Culture media were prepared by the Central Center Tissue Culture Facility at the Fox Chase Cancer (Philadelphia, PA). MCF-10F cells were treated with 0.00, 0.007 nM, 70 nM, or 1 μM of 17-β-estradiol (E_2) (Aldrich, St. Louis, MO), dimethylstilbestrol (DM-DES) (Sigma Chemical Co., St. Louis, MO); 2 hydroxy estradiol (2-OH-E_2), 4-hydroxy-estradiol (4-OH-E_2), 16-α-hydroxy-estradiol (16α-OH-E_2), benz (a) pyrene (BP), and cholesterol (Sigma Chemical Co., St. Louis, MO). The pure antiestrogen ICI-182,780 in combination with E2 or 4-OH-E_2 at the same doses described above were also used in this experiment. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic analysis (Figure 1).

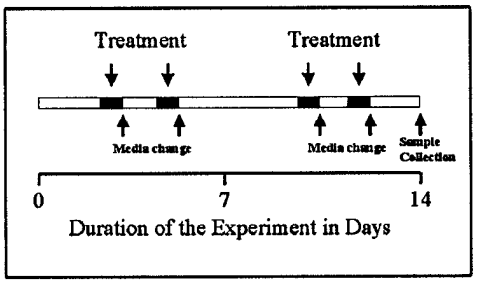


Figure 1. MCF-10F cells were treated with the different compounds: cholesterol, E_2, 2-OH-E_2, 4-OH-E_2, 16-α-E_2, DES or BP at 72 hrs and 120 hours post plating for 24 hours each followed by change of the culture media. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic 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 assayed for colony efficiency (CE), colony size (CS), and ductulogenic capacity in collagen gel and invasiveness in matrigel, as described in previous publications [44-45].

ii- Effect of estrogen and its metabolites on colony assay

E_2, DES, 2-OH-E_2, 4-OH-E_2, 16α-OH-E_2 and BP induce the formation of colonies in agar methocel, whereas cells treated with cholesterol were unable to produce colonies (Figure 2). The size of the colonies was over 60μm in diameter, although the size was significantly smaller ($P<0.01$) in those cells treated with 2-OH-E2 (Figure 3). 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 greater colony efficiency at a dose of 0.007nM (Figure 4). The dose response for E_2 and BP were very similar, whereas DES, 16-α-

OH E_2 and 2-OH-E_2 were weaker carcinogenic agent at 0.007nM and 70nM, than 4-OH-E_2, E_2 and BP (Figure 4).

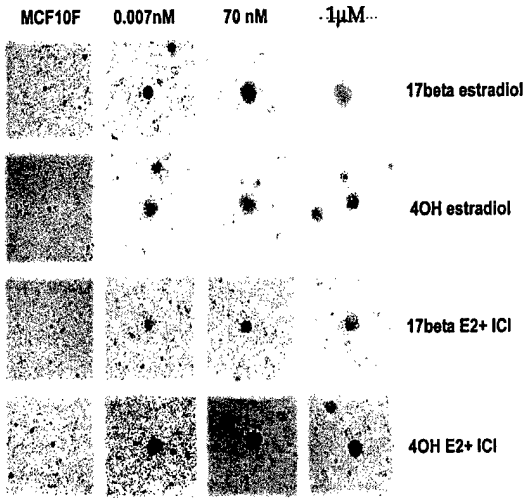


Figure 2: MCF-10F cells plated in agar-methocel for colony assay. Phase contrast microscopy X 4.

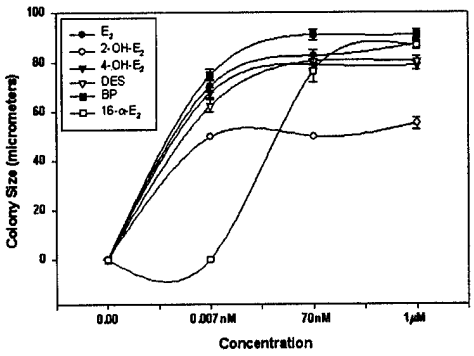


Figure 3 Plot showing the colony size of the cells treated at different concentrations of the compounds tested. 2-OH E_2 is the only compound in which the colony size was consistently below the 60-micrometer range

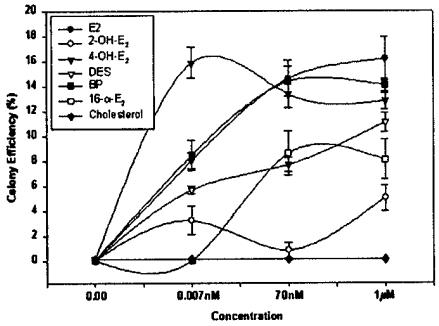


Figure 4: Plot showing the dose effect of 17-β-estradiol (E_2), 2-OH-estradiol (2-OH-E_2), 4-OH-estradiol (4-HO-E_2), Diethylstilbestrol (DES), Benz (a) pyrene (BP), 16-α-OH-estradiol (16-α-E_2) and cholesterol on colony efficiency

iii-Effect of estrogen and its metabolites on ductulogenesis

Ductulogenesis was quantitatively evaluated by estimating the ability of the cell plated in collagen to form tubules or spherical masses(SM) (Figure5).

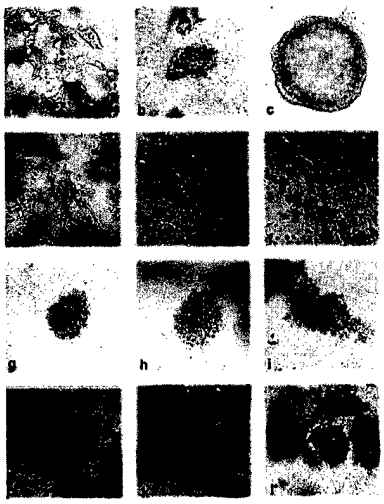


Figure 5a; MCF 10F cells treated with Solvent (DMSO) or cholesterol forming well defined ductular structures in collagen matrix; b, 0.007nM of E2 induces alteration in the ductular pattern; c, 70nM of E2 induces the loss of ductular formation in collagen matrix; d to f E2+ICI at 0.007nM, 70nM and 1μM respectively; h to I, 4-OH-E2 at 0.007nM, 70nM and 1μM respectively; J to l, 4-OH-E2 + ICI at 0.007nM, 70nM and 1μM respectively. Phase contrast microscope X10.

Non transformed cells produce ductules like structure and transformed cells produce solid masses of cells. Cells treated

with DMSO, or cholesterol at different concentrations were unable to alter the ductular pattern (Figure 5). E2, BP, DES, 2-OH-E_2, 4-OH-E_2 and 16-α-OH-E_2 treated cells induce the loss in MCF10F cells to produce ductules in a dose dependent fashion and the number of solid masses paralleled the formation of colonies in agar methocel (Figures 5-10).

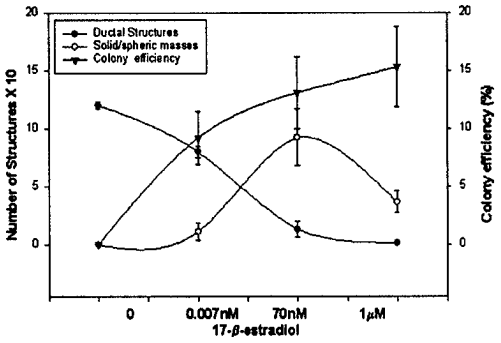


Figure 5: Plot showing the dose effect of 17- β -estradiol on MCF-10F cells forming either ductular structures or solid masses in collagen matrix per 10,000 cells plated in comparison with the colony efficiency.

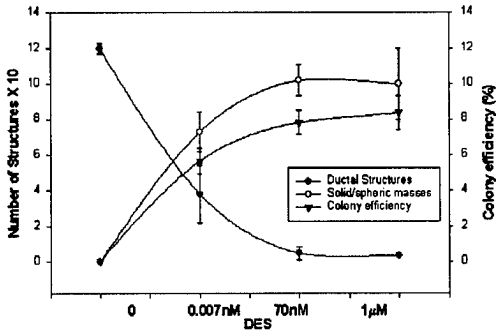


Figure 6: Plot showing the dose effect of DES on MCF-10F cells forming either ductular structures or solid masses in collagen matrix per 10,000 cells plated in comparison with the colony efficiency.

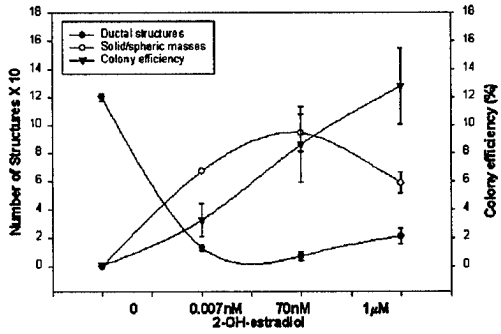
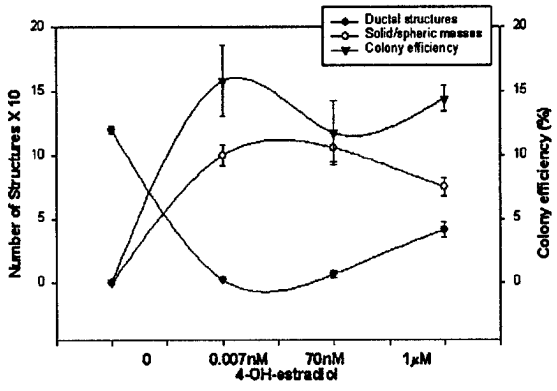


Figure 7: Plot showing the dose effect of 2-OH-estradiol on MCF-10F cells forming either ductular structures or solid masses in collagen matrix per 10,000 cells plated in comparison with the colony efficiency.

Figure 8: Plot showing the dose effect of 4-OH-estradiol on MCF-10F cells forming either ductular structures or solid masses in collagen matrix per 10,000 cells plated in comparison with the colony efficiency.



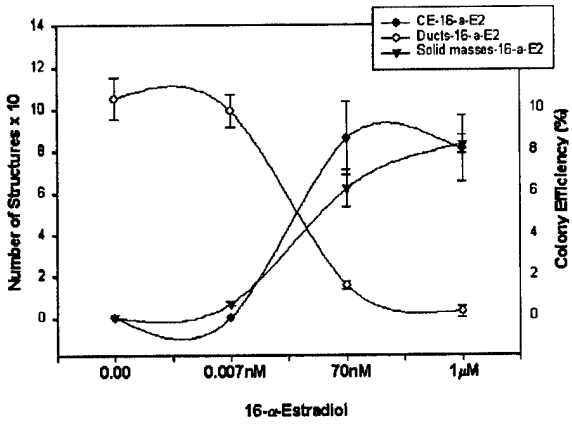


Figure 9: Plot showing the dose effect of 16-α-OH-estradiol on MCF-10F cells forming either ductular structures or solid masses in collagen matrix per 10,000 cells plated in comparison with the colony efficiency.

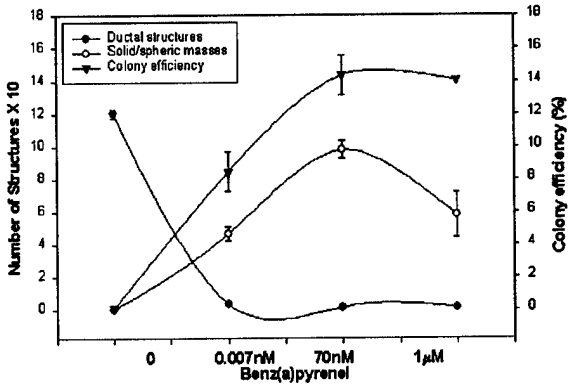


Figure 10: Plot showing the dose effect of benz(a)pyrene on MCF-10F cells forming either ductular structures or solid masses in collagen matrix per 10,000 cells plated in comparison with the colony efficiency.

4-OH-E2 at a dose of 0.007nM induces significant changes in the ductulogenic capacity and maximal number of solid masse was observed (Figure 8), when compared with the other compounds. Cells treated with 2-OH-E2 (Figure 6) or 16-α-OH-E2 (Figure 9) are less efficient in altering the ductulogenic capacity. The pattern of formation of solid masses in collagen matrix followed the same pattern of formation of colonies in agar-methocel (Figures 5-10).

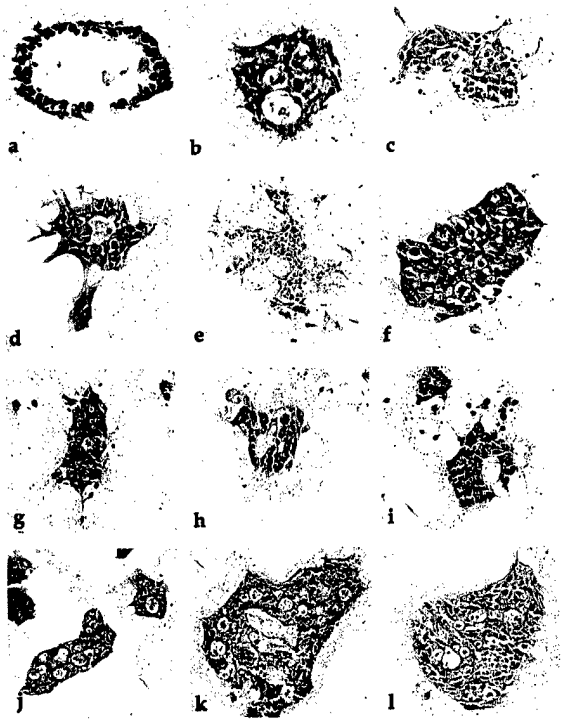


Figure 11. Histological sections of cells growing in collagen gel. The cells have been fixed in buffered formalin, embedded in paraffin and the sections stained with hematoxylin and eosin. a; MCF 10F cells treated with solvent (DMSO) or cholesterol forming well defined ductular structures lined by a single cuboidal layer of cells; b, 0.007nM of E2 induces alteration in the ductular pattern forming solid masses; c, 70nM of E2 induces the loss of ductular formation in collagen matrix and the solid masses are composed of large cuboidal cells. The doses of 1μM of E2 produce the same effect. BP a chemical carcinogen induce the same morphological changes; d, e and f solid masses of MCF10F cells transformed with 17-β-estradiol and incubated at the same time and doses with ICI. The doses used were 0.007nM, 70nM and 1μM respectively; g, h and I shows the histological appearance of MCF10F cells transformed with 4-OH-Estradiol at 0.007,70nM and 1μM respectively; j, k and l histological appearance of MCF10F cells transformed with 4-OH-Estradiol in presence of ICI at the concentration of 0.007,70nM and 1μM respectively. Bright field microscope X10.

Histological analysis of the paraffin embedded ductules and solid masses of cells growing in collagen matrix shows that MCF10-F cells form ductules in collagen matrix that are lined by a single layer of cuboidal epithelial cells (Figure 11a), this pattern was not disturbed by cholesterol or DMSO treatment. E_2, DES, BP and all the metabolites tested significantly impair the formation of ductules replacing them by structures filled by large cuboidal cells (Figures 11b,c, g, h, i). Most of the cells are actively proliferating as detected buy Immunostaining with Ki67(data not shown).

iv-Effect of estrogen and its metabolites on invasion

Based on the data that 70nM concentration was optimal for all the compounds to induce maximal colony formation, this was the doses utilized for measuring invasiveness in a matrigel membrane. Significant number of cells invading the matrigel was observed in MCF10F cells treated with E2, DES, 4-OH-E_2 and BP (Figures 12a-h and 13). The number of cells invading the matrigel membrane was significantly lower when treated with 2-OH-E2 and 16-α–OH E_2 (Figure 13).

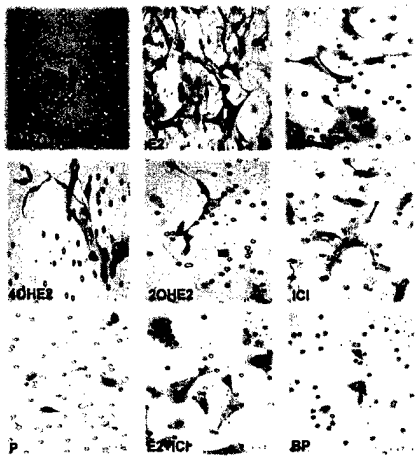


Figure 12: Invaded cells stained by Diff- Quick stain are seen in the membrane. a; MCF 10F cells treated with solvent (DMSO) or cholesterol; b, 0.007nM of E2; c, 70nM of E2; d, e and f MCF10F cells transformed with 17-β-estradiol and incubated at the same time and doses with ICI; g, h and I MCF10F cells transformed with 4-OH-Estradiol at 0.007,70nM and 1μM respectively; j, k and l MCF10F cells transformed with 4-OH-Estradiol in presence of ICI at the concentration of 0.007,70nM and 1μM respectively. Bright field microscope X10.

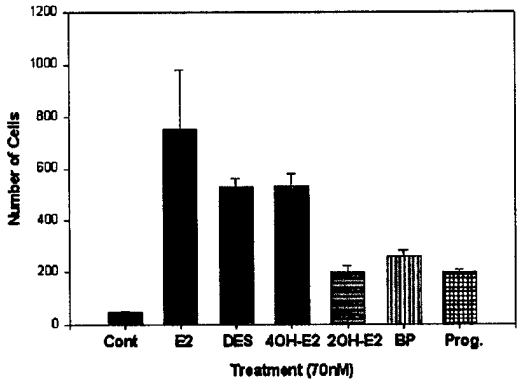


Figure 13: Histogram depicting the number of cells that has migrated the matrigel membrane.

v-Effect of the pure antiestrogen ICI-182,780 on the expression of transformation phenotypes

The transformation effect of 17-β-estradiol measured by colony formation in agar methocel was abrogated by ICI-182,780 (Figure 14). The ductulogenic capacity was restored in presence of ICI, however the histological appearance of the ductules was not. The cross section of the structures are filled with large cuboidal cells (Figures 11 d, e and f) similar to those cells treated with estradiol (Figures 11b and c). At difference of 17 β-estradiol, 4-OH E_2 transforming

phenotypes were not abrogated by ICI (Figure 15). Interestingly the cross sections of the ductules and solid masses in collagen have larger diameter and greater number of cuboidal epithelial cells (Figure 11 j, k and l). Of interest was the fact that neither ICI nor tamoxifen were able to abrogate the invasive phenotype induced by E2 or 4-OH-E_2 (Figure 16).

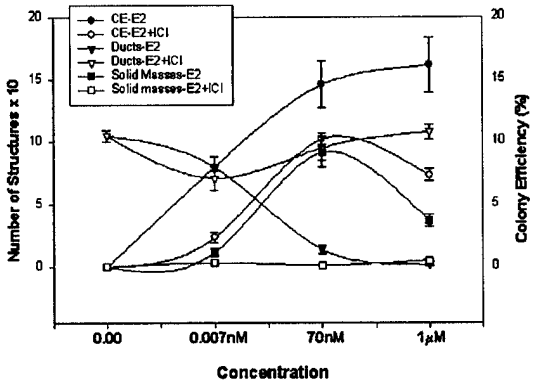


Figure 14: Plot showing the dose effect of 17-β estradiol alone or in combination with ICI on MCF-10F cells forming either ductular structures or solid masses in collagen matrix per 10,000 cells plated in comparison with the colony efficiency

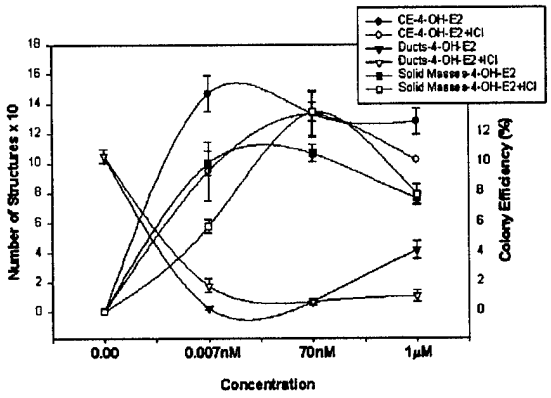


Figure 15: Plot showing the dose effect of 4-OH-estradiol alone or in combination with ICI on MCF-10F cells forming either ductular structures or solid masses in collagen matrix per 10,000 cells plated in comparison with the colony efficiency.

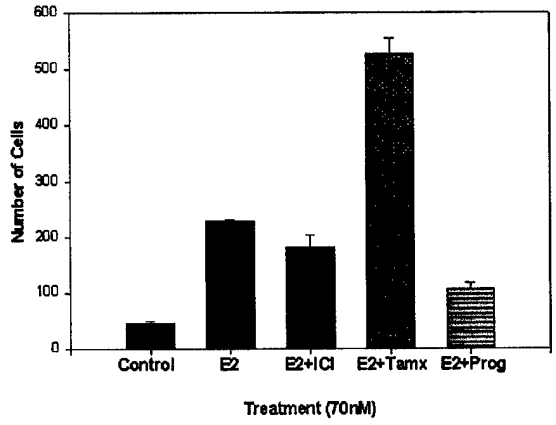


Figure 16: Histogram depicting the number of cells that has migrated the matrigel membrane.

iv Data interpretation

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 MCF-10F. This cell line is a spontaneously immortalized HBEC with most of the properties of a normal cell including anchorage dependent growth, growth factor dependency and a diploid karyotype [40,41]. MCF-10F cell line does not contain measurable level of ER α or ER β and has been successfully transformed with BP [42-45], radiation [48], and polyciclic aromatic hydrocarbons

[44]. Short term treatment of these cells with physiological doses of 17-β estradiol or DES 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 [47,49]. We have shown that 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. The ductulogenic pattern was not impaired by progesterone but the luminal size was smaller that those found in the MCF10F cells [47]. Since invasion of the extracellular matrix and the basement membrane by tumor cells is a crucial event in tumor metastasis the ability of the transformed cells to invade Matrigel in vitro was assessed. The reconstituted basement membrane **invasion assay** has been use successfully to measure transformation phenotypes with different cell lines [50-58] and in the present work we clearly shows that 17-β-estradiol at 70nM is able to increase significantly the number of cells invading the matrigel. The same effect was observed with DES, BP and all the metabolites tested.

Altogether these data clearly indicate that HBEC when treated with 17-β-estradiol produces significant morphogenetic changes. The fact that the MCF10F cells are both ERα and β negative, argue in favor of a metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. The transforming effect of E_2 is observed even at doses of 0.007nM that is in the physiological range, although maximal effect is observed at 70nM. The role of E_2 concentration is supported by data in which excessive synthesis of E_2 by overexpression of CYP19 in target tissues [59-63] and/or the presence of excess sulfatase that converts stored E_1 sulfate to E_1 [64] can be achieved at the organ site. 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 [63]. In animal models such as the Syrian golden hamster and ACI rat, implantation of E_2 produces tumors in the kidney [65,66] and mammary gland [67]. In fact, in the kidney of Syrian golden hamsters treated chronically with E_2 is due to less protective methylation of 2-CE and more pronounced oxidation of CE to CE-Q [68].

From all the estrogen metabolites tested 2-OH-estradiol is the less transforming agent with very low colony efficiency, and the colonies thus formed were smaller. This is in agreement from what we know about this compound. [69-71]. The 2-OH-E2 due to their metabolic redox cycling could generate reactive estrogen quinones and free radicals, which are highly cytotoxic [72]. This could explain also the lower number of solid masses generated in collagen matrix at 1μM. Although 2-OH-E2 can undergo metabolic redox cycling to generate free radicals such as superoxide and the chemically reactive estrogen semiquinone-quinone intermediates [73-75], which may damage DNA and other cellular constituents [76-78], little or not tumorigenic activity towards the male Syrian hamster kidney [79-80] has been reported. Of interest is the fact that HBEC also behaves in the same fashion, but can not rule out its transforming effect in the ductulogenic pattern that is abrogated by this compound and by the invasive capacity of the transformed cells that scored at the same level than BP. At the present time we do no have an explanation for this discrepancy. The low carcinogenicity of 2-OH-E2 could be due to a faster rate of metabolization by catechol-O-methyltransferase catalyzed O methylation [81,82], a more rapid clearance in vivo [83] and possess weaker hormonal potency in estrogen target tissues

[69,70,84-89]. Moreover, 2-methoxiestradiol, (a product of subsequent enzymatic O-methylation of 2-OH-E2) is a very potent inhibitor of tumor cell proliferation [90-95] and angiogenesis [93,95] which may be an important reason for the lack transforming ability in HBEC.

The estrogen metabolite 4-OH estradiol is the most efficient in inducing colonies in agar methocel and the abrogation of ductulogenesis, forming solid masses of cells in collagen matrix even at the lowest doses tested 0.007nM. Also the compound scored high number of invading cells in matrigel. Interestingly 4-hydroxylation of estradiol is a dominant pathway for catechol estrogen formation in several extrahepatic targeting tissues [96,97] including human breast and uterus [98-100]. 4-OH E2 is similar to estradiol in its ability to bind and activate the classical estrogen receptor [66,70,71,84]. However, in our study we demonstrated that a pure antiestrogen ICI-182,780 was unable to abrogate at the different doses used the transforming effect of 4-OH-E2. Instead ICI-182,780 was able to abrogate the transforming effect of 17-β-estradiol. Although, the main difference between 4-OH-E2 and E2 in the interaction with the estrogen receptor is that 4-OH E2 interaction occurs with a reduced dissociation rate compared with estradiol [71,101], suggesting that the association of 4-OH-E2 with the ER may last longer than for the parent hormone, estradiol. This is supporting a direct effect of 4-OH-E_2 independently of the typical ER pathway. The uterotrophic potency of 4-OH-E_2 is slightly weaker than estradiol [102], possible to the faster metabolic clearance than estradiol. However, 4-OH-E_2 had stronger activity than estradiol in inducing progesterone receptor formation in the rat pituitary under experimental conditions [71] but in our experimental system we have observed that 4-OH-E_2 does not induce the formation of PgR (Unpublished observations). More importantly 4-OH-E_2 undergoes metabolic redox cycling to generate free radicals such as superoxide and the chemically-reactive estrogen semiquinone/quinone intermediates. These metabolite intermediates may damage DNA and other cellular constituents [103], induce cell transformations [104] and initiate tumorigenesis [72,74,80,105-109]. Although direct injection of estrone-3,4-quinone into the rat mammary gland did not induce the formation of mammary tumors [110] it does in liver [106]. The genotoxic effect of 4-OH-E_2 is demonstrated by the induction of LOH in chromosome 17 (Russo, J. et al., unpublished observations). The relevance of the in vitro study is also supported by the elevated levels of 4-hydroxylase activity observed in human breast cancer compared with to normal breast tissue [111] and that 4-OH-E_2 appears to be the most abundant estrogen metabolite in human breast cancer [112]. The local concentration of 4-OH-estradiol for increasing cell proliferation and tumor development that has been emphasized by other authors [102-109] is supported by the levels of P4501B1 [113,114] that catalyze the formation of 4-OH-E_2, explaining the transforming effect of DES and E2 in these cells. In the present work we show for the first time evidences that transformation of HBEC can be induced by this metabolite.

The transforming ability of 16-a-OH-E_2 is demonstrated at the 70 nM and 1μM range indicating that even though is a transforming agent is less efficient than 4-OH estradiol and even the parent compound. Several publications indicate the possible role of 16-α-hyrdroxylation in mammary tumor formation [115-117]. Supporting the carcinogen effect is the finding the estrogen 16 α hydroxylation was higher in terminal duct lobular units in cancerous or non cancerous tissues from women with breast cancer compared with breast tissue (reduction mammoplasties) from women without cancer [118]. Enhanced 16-α-hydroxylation was also detected in healthy

women at high risk for breast cancer (from cancer-prone families) [119,120]. Treatment of cultured human mammary tissue with 7,12-dimethylbenz[a]anthracene (DMBA) coordinately increased the ras protooncogene expression and estradiol 16-α-hydroxylation in terminal duct lobular units [121]. 16-α-Hydroxyestrone increased unscheduled DNA synthesis and anchorage-independent growth of mouse mammary epithelial cells in culture, which suggests possible genotoxicity for this estrogen metabolite [122]. In addition, l6-α-hydroxyestrone (but not 2-hydroxyestrone) enhanced the carcinogen-initiated growth stimulation of cultured mouse mammary epithelial cells [122,123]. Animal studies showed that in several different strains of mice with varying incidence of spontaneous mammary tumors, the extent of 16-α-hydroxylation of estradiol was positively correlated with their mammary tumor incidence [124]. All these reports are supporting our finding that at the doses tested it induces anchorage independent growth and alter the ductulogenic capacity of MC10 F cells. On the other hand other reports suggested that increased urinary excretion of catechol estrogens but not 16-α-hydroxylated estrogens is correlated with an increased risk for non-familial breast cancer [125-127]. More recent studies showed that a Finnish population (with high risk for developing breast cancer) had an increased urinary excretion of catechol estrogens relative to 16-α–hydroxylated estrogens when compared with an oriental population at a lower risk [126,127]. Finally, several additional questions concerning the possible etiological role of 16-α-hydroxylated estrogens in hormonal cancer still need to be addressed one of them is the very weak carcinogenic effect induced hamster kidney tumor [79,80,108]. Although pregnant women produce very large amounts of 16-α-hydroxyestradiol (estriol) and 16-α-hydroxyestrone during normal pregnancy [128,129], full term pregnancy does not increase their breast cancer risk, but actually decreases their risk of breast cancer [130-132].

Altogether our work shows an in vitro model of transformation of human breast epithelial cells in which several phenotypes are affected by E_2 and its metabolites and demonstrating that the catechol estrogen 4-OH- E_2 is a the metabolite with the highest transforming properties that are not abrogated by the pure antiestrogen, indicating first that this metabolite is a carcinogenic agent and second that estrogen receptors are not required in the initiation of neoplastic transformation of breast epithelial cells.

7-KEY RESEARCH ACCOMPLISHMENTS

a- In the present work we have capitalized on the availability in our laboratory of an *in vitro* model of transformation of immortalized HBEC by the chemical carcinogen BP for comparison with phenotypic and genomic changes induced by the natural estrogen 17β-estradiol (E_2) and its metabolites mainly 4-OH-E_2.

b- Short term treatment of these cells with physiological doses of 4-OH-E_2 induces anchorage independent growth, colony formation in agar methocel, reduced ductulogenic capacity in collagen gel, and increased invasiveness in a reconstituted basement membrane, all phenotypes whose expression is indicative of neoplastic transformation, and that are induce by BP under the same culture conditions. The fact that the MCF10F cells are both ER-α and ERβ negative, argue in favor of a metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates.

c- 2-OH-E_2 was unable to induce significant increase in colony formation, although small colonies less than 60 μm in diameter were observed, whereas none were found in the MCF10F cells treated with either DMSO or Cholesterol. The ductulogenic pattern was impaired by 2-OH-E_2 but the invasive phenotypes was lower than DES or 17β-estradiol.

d- We have found that antiestrogen like tamoxifen or ICI (a pure antiestrogen) are unable to abrogate the invasive phenotype of cell treated either with E2 or 4-OH-E_2.

8-REPORTABLE OUTCOMES

8a-Peer Review Publications :

1. Russo, J., Hu, Y.F., Tahin, Q., Mihaila, D., Slater, C., Lareef, M.H. and Russo, I.H. Carcinogenicity of Estrogens in Human breast epithelial cells. Acta Pathologica, Microbiologica Immunologica Scandinavica (APMIS) 109:39-52, 2001.

2. Russo, J., Santen, R., and Russo, I.H. Hormonal control of the breast development. In: Endocrinology (Fourth Edition) Edited by L. J. DeGroot and J.L. Jameson. W.B. Saunders Company. Philadelphia, Vol.3 pp.2181-2188, 2001.

3. Russo, J., Lareef, M.H., Tahin, Q., Hu, Y-F., Slater, C.M., Ao, X., and Russo, I.H. 17 beta estradiol is carcinogenic in human breast epithelial cells. Journal of Steroid Biochemistry and Molecular Biology 1656:1-14, 2002

4. Russo, J., Lareef, M.H., Tahin, Q., Hu, Y-F., Slater, C.M., and Russo, I.H. The role of estrogen in human breast cancer: a mechanistic view. In: Menopause Hormones and Cancer (Ed. Neves-e-Castro), Parthenon Publishing, England, 2002).

5. Russo, J. Tahin, Q., Lareef, H.M., Hu, YF. Russo, I.H. Neoplastic transformation of human breast epithelial cells by estrogens and chemical carcinogens. Environmental and Molecular Mutagenesis. 39:254-263,2002.

8-b.Abstracts Published:

1. Russo, J., Tahin., Q., Mihaila, D., Hu, Y-F., and Russo, I.H. Estrogens induced loss of heterozygosity in chromosomes 3 and 11 in human breast epithelial cells. Proc. Am. Assoc. Cancer Res.41: 4704a, 2000.

2. Lareef, M.H., Russo, I.H., Slater, C.M., Rogatko, A., and Russo, J. Estrogen induces transformation phenotypes in the estrogen receptor negative MCF10F cells. Proc. Am. Assoc. Cancer Res. 42:4743a, 2001.

3. Lareef, H.M. Russo I.H. Sheriff, F., Slater, C. and Russo, J. Estrogen and its metabolites are carcinogenic in the human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 43:5276a, 2002.

9-CONCLUSIONS

Our work shows an in vitro model of transformation of human breast epithelial cells in which several phenotypes are affected by E_2 and its metabolites and demonstrating that the Catechol estrogen 4-OH-E_2 is a the metabolite with the highest transforming properties that are not abrogated by the pure antiestrogen, indicating first that this metabolite is a carcinogenic agent and second that estrogen receptors are not required in the initiation of neoplastic transformation of breast epithelial cells.

10-REFERENCES.

1. Pike MC, Spicer DV, Dahmoush L, Press MF. Estrogens, progesterone, normal breast cell proliferation and breast cancer risk. Epidemiol Rev 1993; 15:17-35.
2. Kelsey JL, Gammon MD, John EM. Reproductive factors and breast cancer. Epidemiol Rev 1993; 15:36-47.
3. Bernstein L, Ross RK. Endogenous hormones and breast cancer risk. Epidemiol Rev 1993; 15:48-65.
4. Henderson BE, Ross R and Bernstein L. Estrogens as a cause of human cancer: the Richard & Hinda Rosenthal Foundation Award Lecture. Cancer Res 1988; 48: 246-53.
5. Topper YJ, Sankaran L, Chomczynski P, Prosser C, Qasba P. Three stages of responsiveness to hormones in the mammary cell. In: Angeli A, Bradlow HL, Dogliotti L (eds), Endocrinology of the Breast: Basic and Clinical Aspects. Annals of the New York Academy of Sciences 1986; 464: 1-10.
6. Lippman ME, Huff KK, Jakesz R, Hecht T, Kasid A, Bates S, Dickson RB. Estrogens regulate production of specific growth factors in hormone-dependent human breast cancer. In: Angeli A, Bradlow HL, Dogliotti L (eds), Endocrinology of the Breast: Basic and Clinical Aspects. Annals of the New York Academy of Sciences 1986; 464: 11-6.
7. Dupont WD, Page DL. Menopausal estrogen replacement therapy and breast cancer. Arch Int Med 1991; 151: 67-72.
8. Price MA, Tennant CC, Smith RC, Kennedy SJ, Butow PN, Kossoff MB, Dunn SM. Predictors of breast cancer in women recall following screening. Australian & New Zealand Journal of Surgery 1999; 69: 639-46.
9. Couse JF, Korach KS. Estrogen receptor null mice: What have we learned and where will they lead us? Endocrine Reviews 1999; 20: 358-417.

10. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998; 95: 927-37.
11. McDonnell DP. The molecular pharmacology of SERMs. TEM 1999; 10: 301-11.
12. Tsai MJ, O'Malley BW. Molecular mechanisms of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994; 63:451-86.
13. Katzenellenbogen BS. Dynamics of steroid hormone receptor action. Annu Rev Physiol 1980; 42:17-35.
14. Mosselman S, Polma J, Dijkema R. ER β: identification and characterization of a novel human estrogen receptor. FEBS Lett 1996; 392:49-53.
15. Kuiper GGJM, Carlsson B, Grandien K, Enmark E, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997; 138:863-70.
16. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 1997; 277: 1508-10.
17. Chen X; Danes C, Lowe M; Herliczek TW, Keyomarsi K. Activation of the estrogen-signaling pathway by p21 WAF1/CIP1 in estrogen receptor negative breast cancer cells. J. Natl. Cancer Inst. 92P1403-13, 2000.
18. Liehr JG, Ulubelen AA, Strobel HW. Cytochrome P-450-mediated redox cycling of estrogens. J Biol Chem 1986; 261:16865-70.
19. Roy D, Liehr JG. Temporary decrease in renal quinone and reductase activity induced by chronic administration of estradiol to male Syrian hamsters- increased superoxide formation by redox cycling of estrogen. J Biol Chem 1988; 263:3646-51.
20. Yan Z-J, Roy D. Mutations in DNA polymerase P mRNA of stilbene estrogen-induced kidney tumors in Syrian hamster. Biochem Mol Biol Int 1997; 37: 175-83.
21. Ball P, Knuppen R. Catecholestrogens (2- and 4-hydroxy-oestrogens). Chemistry, biosynthesis, metabolism, occurrence and physiological significance. Acta Endocrinol (Copenh) 1980; 232(suppl): 1:127.

22. Zhu BT, Bui QD, Weisz J, Liehr JG. Conversion of estrone to 2- and 4- hydroxyestrone by hamster kidney and liver microsomes: Implications for the mechanism of estrogen-induced carcinogenesis. Endocrinology 1994; 135:1772-79.

23. Ashburn SP, Han X, Liehr JG. Microsomal hydroxylation of 2- and 4-fluoroestradiol to catechol metabolites and their conversion to methyl ethers: Catechol estrogens as possible mediators of hormonal carcinogenesis. Mol Pharmacol 1993; 43:534-41.

24. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: Correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. Science 1987; 235:177-82.

25. Escot C, Theillet C, Lidereau R, Spyratos F, Champeme M-H, Gest J, Callahan R. Genetic alteration of the *c-myc* proto-oncogene (*MYC*) in human primary breast carcinomas. Proc Natl Acad Sci USA 1986; 83:4834-38.

26. Ali IU, Merio G, Callahan R, Lidereau R. The amplification unit on chromosome 11 q13 in aggressive primary human breast tumors entails the bcl-1, int-2 and hst loci. Oncogene 1989; 4:89-92.

27. Theillet C, Adnane J, Szepetowski P, Simon MP, Jeanteur P, Birnbaum D, Gaudray P. BCL-1 participates in the 11q13 amplification found in breast cancer. Oncogene 1990; 5: 147-9.

28. Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J, Callahan R. Loss of a *c-H-ras-1* and aggressive human primary breast carcinomas. Cancer Res 1986; 46:4776-81.

29. Lundberg C, Skoog L, Cavenee WK, Nordenskjoid M. Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. Proc Natl Acad Sci USA 1987; 84:2372-76.

30. Mackay J, Steel CM, Elder PA, Forrest APM, Evans HJ. Allele loss on short arm of chromosome 17 in breast cancers. Lancet 1988; 2:1384-5.

31. Ali IU, Lidereau R, Callahan R. Presence of two members of *c-erbAB* and *c-erbA2* in smallest region of somatic homiozygosity on chromosome 3p2l-p25 in human breast carcinoma. J Natl Cancer Inst 1989; 81:1815-20.

32. Chen L-C, Dolibaum C, Smith H. Loss of heterozygosity on chromosome lq in human breast cancer. Proc Natl Acad Sci USA 1989; 86:7204-7.

33. Callahan R, Campbell A. Mutations in human breast cancer: an overview. J Natl Cancer Inst 1989; 81:1780-6.

34. Sato T, Saito H, Swensen J, Olifant A, Wood C, Danner D, Sakamoto T, Takita K, Kasumi F, Miki Y, Skolnick M, Nakamura Y. The human prohibitin gene located on chromosome 17q2l is mutated in sporadic breast cancer. Cancer Res 1992; 52:1643-6.

35. Chen LC, Kurisu W, Ljung BM, Goldman ES, Moore D 2d, Smith HS. Heterogeneity for allelic loss in human breast cancer. J Natl Cancer Inst 1992; 84: 506-10.

36. Genuardi M, Tsihira N, Anderson DE, Saunders GF. Distal deletion of chromosome lq in ductal carcinoma of the breast. Am J Hum Genet 1989; 45:73-89.

37. Crop CS, Lidereau R, Campbell G, Champene M-H, Callahan R. Loss of heterozygosity on chromosomes 17 and 18 in breast carcinoma: two additional regions identified. Proc Natl Acad Sci USA 1990; 87:7737-41.

38. Sato T, Tanigami A, Yamakawa K, Akiyama F, Kasumi F, Sakamoto G, Nakamura Y. Allelotype of breast cancer: Cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res 1990; 50:7184-9.

39. Sato T, Akiyama F, Sakamoto G, Kasumi F, Nakamura Y. Accumulation of genetic alterations and progression of primary breast cancer. Cancer Res 1991; 51:5794-9.

40. Soule HD, Maloney TM, Wolman SR, Peterson Jr WD, Brenz R, McGrath CM, Russo J, Pauley R, Jones RF, Brooks SC. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 1990; 50: 6075-86.

41. Tait L, Soule H, and Russo J. Ultrastructural and immunocytochemical characterizations of an immortalized human breast epithelial cell line MCF-10. Cancer Res 1990; 50: 6087-99.

42. Hu YF, Lau KM, Ho SM, Russo J. Increased expression of estrogen receptor-β in chemically transformed human breast epithelial cells. Int J Oncol 1998; 12:1225-8.

43. Russo J, Calaf G, Sohi N, Tahin Q, Zhang PL, Alvarado ME, Estrada S, Russo IH. Critical steps in breast carcinogenesis. The New York Academy of Sciences 1993; 698:1-20.

44. Calaf G, Russo J. Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis 1993; 14:483-92.

45. Russo J, Calaf G, Russo IH. A critical approach to the malignant transformation of human breast epithelial cells. CRC Critical Reviews in Oncogenesis 1993; 4: 403-17.

46. Calaf G, Zhang PL, Alvarado MV, Estrada S, and Russo J. C-Ha ras enhances the neoplastic transformation of human breast epithelial cells treated with chemical carcinogens. Int J Oncol 1995; 6: 5-11.

47. Russo, J., Lareef, M.H., Tahin, Q., Hu, Y-F., Slater, C.M., Ao, X., and Russo, I.H. 17 beta estradiol is carcinogenic in human breast epithelial cells. Journal of Steroid Biochemistry and Molecular Biology 1656:1-14, 2002.

48. Roy D. Calaf G. Hei TK. Frequent allelic imbalance on chromosome 6 and 17 correlate with radiation-induced neoplastic transformation of human breast epithelial cells. Carcinogenesis. 22(10):1685-92, 2001 Oct.

49. Russo, J., Hu, Y.F., Tahin, Q., Mihaila, D., Slater, C., Lareef, M.H. and Russo, I.H. Carcinogenicity of Estrogens in Human breast epithelial cells. Acta Pathologica, Microbiologica Immunologica Scandinavica (APMIS) 109:39-52, 2001.

50. Yokoyama K. Kamata N. Hayashi E. Hoteiya T. Ueda N. Fujimoto R. Nagayama M. Reverse correlation of E-cadherin and snail expression in oral squamous cell carcinoma cells in vitro. Oral Oncology. 37(1):65-71, 2001.

51. Al-Mulla F. MacKenzie EM. Differences in in vitro invasive capacity induced by differences in Ki-Ras protein mutations. Journal of Pathology. 195(5):549-56, 2001 Dec.

52. Park BK. Zeng X. Glazer RI. Akt1 induces extracellular matrix invasion and matrix metalloproteinase-2 activity in mouse mammary epithelial cells.Cancer Research. 61(20):7647-53, 2001 Oct 15.

53. Oda T. Tanaka M. Sasaki TCytotoxicity of synthetic estrogen and related compounds in various tumor-derived cells. Biological & Pharmaceutical Bulletin. 24(10):1142-4, 2001 Oct.

54. Premzl A. Puizdar V. Zavasnik-Bergant V. Kopitar-Jerala N. Lah TT. Katunuma N. Sloane BF. Turk V. Kos J. Invasion of ras-transformed breast epithelial cells depends on the proteolytic activity of cysteine and aspartic proteinases. Biological Chemistry. 382(5):853-7, 2001 May.

55. Shen ZY. Xu LY. Li C. Cai WJ. Shen J. Chen JY. Zeng Y. A comparative study of telomerase activity and malignant phenotype in multistage carcinogenesis of esophageal epithelial cells induced by human papillomavirus. International Journal of Molecular Medicine. 8(6):633-9, 2001 Dec.

56. Callow MG. Clairvoyant F. Zhu S. Schryver B. Whyte DB. Bischoff JR. Jallal B. Smeal T. Requirement for PAK4 in the anchorage-independent growth of human cancer cell lines.Journal of Biological Chemistry. 277(1):550-8, 2002 Jan 4.

57. Kuppumbatti YS. Rexer B. Nakajo S. Nakaya K. Mira-y-Lopez R. CRBP suppresses breast cancer cell survival and anchorage-independent growth.Oncogene. 20(50):7413-9, 2001 Nov 1.

58. Moizhess TG. Vasiliev JM. Substrate-induced polarisation of cultured epitheliocytes and fibroblasts: non-reactivity of Ras-transformed cells. Cell Biology International. 5(9):931-4, 2001.

59. Brooks,S.C. and Horn,L. (1971) Hepatic sulfonation of estrogen metabolites. *Biochim. Biophys. Acta,* 231, 233-241.

60. Payne,A.H. and Singer,S.S. (1979) The role of steroid sulfatase and sulfotransferase enzymes in the metabolism of C2L and CL9 steroids. In Hobkirk,R. (ed.) *Steroid Biochenzistry,* vol 1. CRC Press, Boca Raton, FL, pp. 111-145.

61. Hobbirk,R. (1985) Steroid sulfotransferases and steroid sulfate sulfatases: Characteristics and biological roles. *Can.* J. *Biochem. Cell Biol.,* 63, 1127-1144.

62. Watanabe,K., Takanashi,K. and Yoshizawa,l. (1988) Determination of estradiol-17-sulfate in human urine by a direcl radioimmunoassay: unnary levels throughout the menstrual cycle. *Steroids,* 52, 123-136.

63. Hernandez,l.S., Watson,R.W.G., Wood,T.C. and Weinshilboum,R.M. (1992) Sulfation of estrone and 17p,-esiradiol in human liver. Catalysis by thermostable phenol sulfotransferase and by dehydroepiandrosterone sulfotransferase. *Drug Metab. Dispos.,* 20, 413-422.

64. Axelrod,J. and Tomchick,R. (1958) Enzymatic O-methylation of epinephnne and other catechols. J. *Biol. Chem.,* 233, 702-705.

65. Guldberg,H.C. and Marsden,C.A. (1975) Catechol-O-methyltransferase: pharmacological aspects and physiological role. *PhanmacoL Rev.,* 27, f 135-206.

66. Ball,R and Knuppen,R. (1980) Catecholoestrogens (2- and 4~'hydroxyoestrogens): chemistry, biogenesis, metabolism, occurrence and physiological significance. *Acta EndocrinoL,* 232, 1-127.

67. Fishman,J. and Nonon,B. (1975) Catechol estrogen formation in the central nervous system of the rat. *Endocrinology,* 96, 1054-1059.

68. Ball,R, Knuppen,R., Haupt,M. and Breuer,H. (1972) Interactions between estrogens and catechol amines. lil. Studies on the methylation of catechol estrogens, catechol amines and other catechols by the catechol Omethyltransferase of human liver. J. *Clin. Endocrinol. Metab.,* 34, 736 746.

69. Emons,G., Merriam,R., Pfeiffer,D., Loriaux,D.L., Ball,P and Knuppen,R.(1987) Metabolism of exogenous 4- and 2-hydroxyestradiol in human male. J. *Steroid Biochem.,* 28, 499-504.

70. Fishman,J. and Martucci,C. (1979) Absence of measurable catechol estrogens in the rat brain evidence for rapid turnover. *J. Clh'. Endocnnol.Metab., 49,* 940-942.

71. Zhu,B.T. and Lichr,J.G. (1996) Inhibition of catechol O-methyltransferase-catalyzed O-methylation of 2- and 4-hydroxyestradiol by quercetin. *J. Biol. Chem.,* 271, 1357-1363.

72. Yager,J.D. and Liehr,J.G. (1996) Molecular mechanisms of estrogen carcinogenesis. Anl. *Rev Pharmacol. Toxicol.,* 36, 203-232.

73. Liehr, J.G., Ulubelen,A.A. and Strobel,H.W. (1986) Cytochrome P-450~,mediated redox cycling of estrogens. J. *Biol. Chem.,* 261, 16865-16870.

74. Liehr,J.G. and Roy,D. (1990) Free radical generation by redox cycling of estrogens. *Free Radical BioL Med., 8,* 415~23.

75. Liehr,J.G. (1990) Genotoxic effects of estrogens. *Mutat. Res.,* 238,269-276.

76. Nutter,L.M., Ngo,E.O. and Abul-Hajj,Y.J. (1991) Characterization of :,/ DNA damage induced by 3,4-estrone-o-quinone in human cells. J. *BioL ~'~\Chem.,* 266, 16380-16386.

77. Han,X. and Liehr,J.G. (1994) DNA single-strand breaks in kidneys of I Syrian hamsters treated with steroidal estrogens: hormone-induced free radical damage preceding renal malignancy. *Carcinogenesis,* 15, 9971000.

78. Han,X. and Liehr,J.G. (1994) 8-Hydroxylation of guanine bases in kidney and liver DNA of hamsters treated with estradiol: Role of free radicals in estrogen-induced carcinogenesis. *Cancer Res.,* 54, 5515-5517.

79. Li, J.J. and Li, S.A. (1987) Estrogen carcinogenesis in Syrian hamster tissues: role of metabolism. *Fed. Proc.,* 46, 1858-1863.

80. Liehr,J.G., Fang,W.E, Sirbasku,D.A. and Ulubelen,A.A. (1986) Carcinogenicity of catechol estrogens in Syrian hamsters. J. *Steroid Biochem.,* 24, 353-356.

81. Li,S.A., Purdy,R.H. and Li,J.1. (1989) Variations in catechol-o-methyltransferase activity in-rodent tissues: possible role in estrogen carcinogenicity. *Carcinogenesis,* 10, 63-67.

82. Roy, D., Weisz, J. and Liehr,J.G. (1990) The O-methylation of 4-hydroxyestradiol is inhibited by 2-hydroxyestradiol: implications for estrogen-induced carcinogenesis. *Carcinogenesis,* II, 459-462.

83. Lipsett.M.B., Merriam,G.R., Kono,S., Brandon.D.D., Pfeiffer,D.G. and Loriaux,D.L, (1983) Metabolic clearance of catechol estrogens. In: Merriam,G.R. and Lipsett,M.B. (eds) *Catechol Estrogens.* Raven Press, New York, pp. 105-114.

84. Fishman,J. (1981) Biological action of catecholestrogens. J. *Endocr.,* 85,59P-65R

85. Schutze,N., Vollmer,G., Tiemann,l., Geiger,M. and Knuppen,R. (1993)Catecholestrogens are MCF-7 cell estrogen agonists. J *Steroid Biochem.Mol. Biol., 46,* 781-789.

86. Schutze,N., Vollmer,G. and Knuppen,R. (1994) Catecholestrogens are agonists of estrogen receptor-dependent gene expression in MCF-7 cells. *J. Steroid Biochem. Mol. Biol., 48,* 453-461.

87. Schneider, L., Huh,M.M., Bradlow,H.L. and Fishman,J. (1984) Antiestrogen action of 2-hydroxyestrone on MCF-7 human breast cancer cells. *J. Biol. Chem.,* 259, 4840 4845.

88. Vandewalle,B. and Lefebvre,J. (1989) Opposite effects of estrogen and catecholestrogen on hormone-sensitive breast cancer cell growth and differentiation. *Mol. Cell. Endocrti201.,* 61, 239-246.

89. Martucci,C. and Fishman,J. (1976) Uterine estrogen receptor binding of catecholestrogens and of estetrol (1.3,5(10)estratriene-3,15r~x,16ry.17p- tetrol). *Steroids,* 27, 325-333.

90. Seegets,J.C., Aveling,M.-L., van Aswegen,C.H., Cross,M., Koch,E and Joubert,W.S. (1989) The cytotoxic effects of estradiol-17p,catecholestradiols and methoxyestradiols on dividing MCF-7 and Hela cells. J. *Steroid Biochem.,* 32, 797-809.

91. Lottering,M.-L., Haag,M. and Seegers,J.C. (1992) Effects of 17,B-estradiol metabolites on cell cycle events in MCF-7 cells. Cancer *Res., 52,* 5926 5932.

92. D'Amato,R.J., Lin,C.M., Flynn,E., Folkman,J. and Hamel,E. (1994) 2Melhoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. *Proc. Natl Acad Sci. USA,* 91, 3964-3968.

93. Fotsis,T., Zhang,Y., Pepper,M.S., Adlercreutz,H., Montesano,R.,Nawroth,RR and Schwelgerer,L. (1994) The endogenous oestrogen~ metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumor growth. *Nature,* 368, 237-239.

94. Hamel,E., Lin,C.M., Flynn,E. and D'Amato,R.J. (1996) Interaction of 2-methoxyestradiol, an endogenous mammalian metabolite, with unpolymerized tubulin and tubulin polymers. *Biochemistry,* 35, 1304-1310.

95. Klauber,N., Parangi,S., Flynn,E., Hamel,E. and D'Amato,R.J. (1997) Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and taxol. *Cancer Res.,* 57, 81-86.

96. Bui,Q.D. and Weisz,J. (1988) Monooxygenase mediating catecholestrogen formation by rat anterior pituitary is an estrogen-4-hydroxylase. *Endocrinology, 124,* 1085-1087.

97. Liehr,J.G., Ricci,M.J., Jefcoate,C.R., Hannigan,E.V., Hokanson,J.A. and Zhu,B.T. (1995) 4-Hydroxylation of estradiol by human uterine myometrium and myoma microsomes: Implications for the mechanism of uterine tumorigenesis. *Proc. Natl Acad. Sci. USA,* 92, 9220-9224.

98. Hayes,C.L., Spink,D.C., Spink,B.C., Cao.J.Q., Walker,N.J. and Sutter,T.R.(1996) 17~-Estradiol hydroxylation catalyzed by human cytochrome P450 IBI. Proc. *NatlAcad Sci. USA,* 93, 9776-9781.

99. Weisz,J. (1991) Metabolism of esLrogens by target cells: Diversification and amplification of hormone action and the catechol estrogen hypothesis. In Hochberg R.B. and Naftolin, E (eds), *Neiv Biology of Steroid Honnones.* Raven Press, New York, pp. 101-112

100. Weisz,J. (1994) Biogenesis of catecholestrogens: metaboiic activation of estrogens by Phase I enzymes. *Polycyclic Aromatic Comp.,* 6, 241-251.

101. Barnea,E.R., MacLusky,N.J. and Naftolin,E (1983) Kinetics of catechol estrogen-estrogen receptor dissociation: a possible factor underlying Ndifferences in catechol estrogen biological activity. *Steroids, 41,* 643.

102. Tranks,S., MacLusky,N.J. and Naftolin,E (1982) Comparative pharmacology of oestrogens and catecholestrogens: actions on the immature uterus *irr vivo* and *in vitrtr. J. Errdocrinol., 94,* 91-98.

103. Cavalieri,E.L., Stack,D.E., Devanesan,RD., Todorovic,R., Dwiredy,l.,J Higginbotham,S., Johansson,S.L., Patil,K.D., Gross,M.L., Gooden,J.K., Ramanathan.R., Cerny,L. and Rogan,E.G. (1997) Molecular origin of cancer: catechol estrogen-3.4-quinones as endogenous tumor initiators.*Pr-oc. Natl Acad. Sci. USA, 94,* 10937-10942.

104. ayashi.N., Hasegawa,K., Komine,A.,Tanaka,Y., McLachlan,J.A.,Barrett,J.C. and Tsutsui,T. (1996) Estrogen-induced cell transformation and DNA-adduct formation in cultured Syrian hamster embryo cells. *Mol. Carcinogenesis,* 16, 149-156.

105. Liehr,J.G. (1994) Mechanism of metabolic activation and inactivation of catechol estrogens: a basis of genotoxicity. *Polycyclic Aromatic Corrrpourrds,* 6, 229-239.

106. Cavalieri,E.L. (1994) Minisymposium on endogenous carcinogens: the catechol estrogen pathway. Polycyclic *Arortratic Corrrporrrrds,* 6, 223-228.

107. Li,J.J., Gonzalez,S., Banerjee,S.K. and Li,S.A. (1993) Estrogen carcinogenesis in the hamster kidney: role of cytotoxicity and cell proliferation. *Environ. Hltir Perspect.,* 101 (Suppl. 5), 259-264.

108. Li,J.J., Li,S.A., Oberley,T.D. and Parsons,J.A. (1995) Carcinogenic activities of various steroidal and nonsteroidal estrogens in the hamster kidney: Relation to hormonal activity and cell proliferation. *Carrcer Res.,*55, 4347-4351.

109. Li.J.J. and Li,S.A. (1995) Estrogen carcinogenesis in the hamster kidney: a hormone-driven multi-step process. In Huff,J. Boyd,J. and Barrett,J.C. (eds) *Cellrrlar arrd Molecular Mechanisrrrs rrf Honrronal Carcinogenesis:Errvirorrrrrerrtal Irrfluences.* Wiley-Liss Inc., Philadelphia, pp. 247-259.

110. EI-Bayoumy,K., Ji,B.-Y., Upadhyaya,P, Chae,Y.-H., Kurtzke.,C.,Rivenson,A., Reddy,B.S., Amin,S. and Hecht,S.S. (1996) Lack of tumorigenicity of cholesterol epoxides and estrone-3,4-quinone in the rat mammary gland. *Canrcer Res.,* 56, 1970-1973.

111. Liehr,J.G. and Ricci,M.J. (1996) 4-Hydroxylation of estrogens as marker of human mammary tumors. *Proc. Natl Acad. Sci. USA,* 93, 3294-3296.

112. Castastagnetta,L.A., Granata,O.M., Arcuri,EP, Polito,L.M., Rosati,E and Cartoni,G.P (19921 Gas chromatography/mass spectrometry of catechol estrogens. *Steroids' 57,* 437-443.

113. Savas,U., Bhattaeharyya,K.K., Christou,M., Alexander,D.L. and Jefeoate,C.R. (1994) Mouse eytoehrome P-450EF, representative of a new IB subfamily of eytoehrome P-450s. J. *BioL Chem., 269,14905*-14911.

114. Hakkola,J,, Pasanen,M., Pelkonen,O., *et aL (1997)* ExpressionofCYPIBI in human adult and fetal tissues and differential inducibility of CYPIBI and CYPIAI by Ah receptor ligand in human placenta and cultured cells.*Carcinoger~esis,* 18, 391-397.

115. Bradlow,H.L., Herschcopf,R.J. and Fishman,J. (1986) Oestradiol 16ahydroxylase: a risk marker for breast cancer. Cancer *S`uv.,* 5, 573-583.

116. Bradlow,H.L., Sepkovic,D.W., Telang,N.T. and Osborne,M.R (1995) Indole-3-carbinol: A novel approach to breast cancer prevention. Ann. *NYAcad. Sci.,* 768, 180 200.

117. Fishman,J., Osborne,M.R and Telang,N.T. (1995) The role of estrogen in mammary carcinogenesis. Ann. *NYAcad. Sci.,* 768, 91-100.

118. Telang,N.T., Axelrod,D.M., Wong,G.Y., Bradlow,H.L. and Osborne,M.P. (1991) Biotransformation of estradiol by explant culture of human mammary tissue. *Steroids,* 56, 37 43.

119. Fishman,J., Schneider,J., Hershcopf,R.J. and Bradlow,H.L. (1984) Increased estrogen 16a-hydroxylase activity in women with breast and endometrial cancer. J. *Steroid. Bioche/?~. Mol. Biol.,* 20, 1077-1081.

120. Bradlow,H.L. and Michnovicz,J.J. (1989) A new approach to the .prevention of breast cancer. *Proc. Roy. Soc. Edinbu/gh,* 95B, 77-86.

121. Telang,N.T., Basu,A., Modak,M.J., Bradlow,H.L. and Osborne,M.P(1988) Parallel enhancement of ras protooncogene expression and of estradiol-16a-hydroxylation in human mammary terminal duct-lobular units (TDLU) by a carcinogen. *Breast Cuncer Res. Treat.,* 12, 138.

122. Telang,N.T., Sato,A., Wong,G.Y., Osborne,M.R and Bradlow,H.L. (1992) J Induction by estrogen metabolite 16a-hydroxyestrone of genotoxic damage and aberrant proliferation in mouse mammary epitheiial cells. J. *Natl Cuncer Inst.,* 84, 630638.

123. Suto,A,, Bradiow,H.L., Wong,G.Y., Osborne,M.P. and Telang,N.T. (1993) Experimental down-regulation of intermediate biomarkers of carcinogenesis in mouse mammary epithelial cells. *Breust Cuncer Res. Treat.,* 27, 193-202.

124. Bradlow,H.L., Hershcopf,R.J., Martucci,C.R and Fishman,J. (1985) Estradiol 16a-hydroxylation in the mouse correlates with mammary tumor incidence and presence of mammary tumor virus: A possible model for the hormonal etiology of breast cancer h1 humans. *Proc. Natl Acad. Sci.* USA, 82, 6295-6299.

125. Lemon,H.M., Heidel,J.W. and Rodriguez-Sierra,J.E (1992) Increased catechol estrogen metabolism as a risk factor for nonfamilial breast cancer, *Cancer 69,* 457-465.

126. Adlercreutz,H. Gorbach,S.L., Goldin,B.R., Woods,M.N., Dwyer,J.T. and Hamblainen,E. (1994) Estrogen metabolism and excretion in Oriental and Caucasian womcn. *J. Natl* Canter *h~st.,* 86. 1076-1()82.

127. Adlercreutz,H., Gorbach,S.L., Goldin.B.R., Woods,M.N., Dwyer,J.T., Hbckerstedt,K., Wahala,K., Hase,T., Hamalainen,E. and Fotsis,T. (1994) Diet and urinary estrogen profile in various populations. A preliminary report. *PO[yCN'CtiC A'omatic Compounds, 6,* 261-273.

128. Merrill,R.C. (1958) Estriol: a review. *Physiol. Rev.,* 38, 463-480.

129. Fotsis,T. (1987) The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC-MS-II. Fractionation and quantitation of the main groups of estrogen conjugates. *J. Steroid Biochem.,* 28, 215-226.

130. MacMahon,B., Cole,R, Lin,T.M., Lowe,C.R., Mirra,A.R, Ravnihar,B., Salber,E.J., Valaoras,V.G. and Yuasa,S. (1970) Age at first birth and breast cancer risk. *Bull. lVld Hlth* Org., 43, 209-221.

131. Yuan,J.-M., Yu,M.C., Ross,R.K., Gao,Y.T. and Henderson,B.E. (1988) Risk factors for breast cancer in Chinese women in Shanghai. *Cancer Res.,* 48, 1949-1953.

132. Henderson,B.E., Pike,M.C., Bemstein,L. and Ross,R.K. (1996) Breast cancer. In Schottenfeld.D. and Fraumeni,J.F.,Jr (eds) *Cancer Epidemiology and Prevention,* 2nd edn. Oxford University Press, New York, Oxford, pp 1022-1039.

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APPENDIX:

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2-Russo, J., Santen, R., and Russo, I.H. Hormonal control of the breast development. In: Endocrinology (Fourth Edition) Edited by L. J. DeGroot and J.L. Jameson. W.B. Saunders Company. Philadelphia, Vol.3 pp.2181-2188, 2001.

3-Russo, J., Lareef, M.H., Tahin, Q., Hu, Y-F., Slater, C.M., Ao, X., and Russo, I.H. 17 beta estradiol is carcinogenic in human breast epithelial cells. Journal of Steroid Biochemistry and Molecular Biology 1656:1-14, 2002.

4-Russo, J., Lareef, M.H., Tahin, Q., Hu, Y-F., Slater, C.M., and Russo, I.H. The role of estrogen in human breast cancer: a mechanistic view. In: Menopause Hormones and Cancer (Ed. Neves-e-Castro), Parthenon Publishing, England, 2002.

5-Russo, J. Tahin, Q., Lareef, H.M., Hu, YF. Russo, I.H. Neoplastic transformation of human breast epithelial cells by estrogens and chemical carcinogens. Environmental and Molecular Mutagenesis. 39:254-263,2002.

6-Russo, J., Tahin., Q., Mihaila, D., Hu, Y-F., and Russo, I.H. Estrogens induced loss of heterozygosity in chromosomes 3 and 11 in human breast epithelial cells. Proc. Am. Assoc. Cancer Res.41: 4704a, 2000.

7-Lareef, M.H., Russo, I.H., Slater, C.M., Rogatko, A., and Russo, J. Estrogen induces transformation phenotypes in the estrogen receptor negative MCF10F cells. Proc. Am. Assoc. Cancer Res. 42:4743a, 2001.

8-Lareef, H.M. Russo I.H. Sheriff, F., Slater, C. and Russo, J. Estrogen and its metabolites are carcinogenic in the human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 43:5276a, 2002.

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Carcinogenicity of estrogens in human breast epithelial cells[1]

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Epidemiological and clinical evidences indicate that breast cancer risk is associated with prolonged ovarian function that results in elevated circulating levels of steroid hormones. Principal among these is estrogen, which is associated with two important risk factors, early onset of menarche and late menopause. However, up to now there is no direct experimental evidence that estrogens are responsible of the initiation of human breast cancer. We postulate that if estrogens are causative agents of this disease, they should elicit in human breast epithelial cells (HBEC) genomic alterations similar to those exhibited by human breast cancers, such as DNA amplification and loss of genetic material representing tumor suppressor genes. These effects could result from binding of the hormone to its nuclear receptors (ER) or from its metabolic activation to reactive metabolites. This hypothesis was tested by treating with the natural estrogen 17β-estradiol (E_2) and the synthetic steroid diethylstilbestrol (DES) MCF-10F cells, a HBEC line that is negative for ER. Cells treated with the chemical carcinogen benzo (a) pyrene (BP) served as a positive control of cell transformation. BP-, E_2-, and DES-treated MCF-10F cells showed increases in survival efficiency and colony efficiency in agar methocel, and loss of ductulogenic capacity in collagen gel. The largest colonies were formed by BP-treated cells, becoming progressively smaller in DES- and E_2-treated cells. The loss of ductulogenic capacity was maximal in BP-, and less prominent in E2- and DES-treated cells. Genomic analysis revealed that E_2- and DES-treated cells exhibited loss of heterozygosity in chromosomes 3 and 11, at 3p21, 3p21–21.2, 3p21.1–14.2, and 3p14.2–14.1, and at 11q23.3 and 11q23.1–25 regions, respectively. It is noteworthy that these loci are also affected in breast lesions, such as ductal hyperplasia, carcinoma in situ, and invasive carcinoma. Our data are the first ones to demonstrate that estrogens induce in HBEC phenotypic changes indicative of cell transformation and that those changes are associated with significant genomic alterations that might unravel new pathways in the initiation of breast cancer.

Key words: breast cancer; epithelial cells; estrogens; carcinogenesis.

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Epidemiological and clinical evidence indicate that breast cancer risk is associated with prolonged exposure to female ovarian hormones, mainly since a greater incidence of this disease is associated with early onset of menarche and late menopause, two conditions directly regulated by ovarian function (1–4). Although breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors (5, 6), estrogens have been demonstrated to be of essential importance in this phenomenon. This postulate has been further supported by the greater cancer risk observed in postmenopausal hyperestrogenism resulting from the use of estrogenic hormone replacement therapy and obesity (7, 8).

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Estrogens, that are necessary for the normal development of both reproductive and non-reproductive organs, exert their physiological effects by binding to their specific receptors, the estrogen receptors (ER) α or β (9–15). ERα resides in the nucleus of target cells in an inactive form associated with a large inhibitory protein complex. Both endogenous and exogenous estrogens, such as 17β-estradiol (E_2) and the synthetic nonsteroidal estrogen diethylstilbestrol (DES), bind to the C-terminal ligand-binding domain (LBD) of the ERα activating the receptor, which undergoes a conformational change. The activated receptor undergoes dimerization, participating in the regulation of target gene transcription by one of two mechanisms, a) binding to transcription factors, such as AP-1, forming a complex that recruits transcriptional co-activators, i.e., the steroid receptor coactivator protein 1 (SRC-1) (16), or b) the ER can form a ternary complex with a co-activator protein after its direct interaction with specific regulatory sequences within target gene promoters (9–11). Estrogens might act as well through alternate non-receptor mediated pathways. It has been recently found that overexpression of p21 in a p21-negative, ER negative cell line induced both the ER and ERE promoters in an estrogen-responsive manner. Stable p21 clones that also lack the expression of wild type ERE were responsive to the growth inhibitory effect of ICI 182,780, a potent antiestrogen, and the growth stimulatory effects of 17 β estradiol (17).

E_2, under the effect of 17β-oxidoreductase is continuously interconverted to estrone (E_1), and both are hydroxylated at C-2, C-4, or C-16α positions by cytochrome P450 isoenzymes , i.e., CYP1A1, CYP1A2, or CYP1B1, to form catechol estrogens (18–23). The demonstration that the catecholestrogen 4-hydroxyestradiol (4-OH-E_2) induces an estrogenic response in the uterus of ERα null mice, and the fact that this response is not inhibited by the antiestrogen ICI-182,780 (9), indicate that this catecholestrogen does not exert its effect on the ER. There is evidence as well that estrogen may not need to activate its nuclear receptors to initiate or promote breast carcinogenesis. The metabolic activation of estrogens can be mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. An increase in CE 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 via a Michael addition and thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidatic activation of CE. Thus, estrogen and 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 (18–23). Although this pathway has been demonstrated in other systems, it still needs to be demonstrated in normal breast epithelial cells.

Breast cancers exhibit genomic alterations, such as DNA amplification and loss of genetic material that may represent tumor suppressor genes (24–39). Although their role in the causation of the disease has not been clearly established, it is generally accepted that the accumulation of genetic alterations promotes tumor progression (38, 39). Specific types of genetic alterations, then, might identify essential steps in the initiation and/or progression of cancer. We postulate that if estrogens initiate the neoplastic process or are responsible for its progression, they would induce in the normal breast epithelium the same type of genomic alterations observed in spontaneous malignancies. In order to test this hypothesis we evaluated the transforming potential of E_2 and DES on human breast epithelial cells (HBEC) in vitro, utilizing the spontaneously immortalized HBEC MCF-10F (40, 41). This cell line lacks both ER-α and ER-β, although this latter receptor is induced in cells transformed by chemical carcinogens (42). The same phenotypes and characteristics that were expressed by MCF-10F cells transformed by the chemical carcinogen benz(a)pyrene (BP) and oncogenes (43–46) were evaluated in E_2 and DES treated cells: anchorage independent growth, colony formation in agar methocel, ductulogenic capacity in collagen gel, and invasiveness index in Matrigel. In addition, DNA of treated cells was analyzed for specific genomic alterations such as loss of heterozygosity (LOH) at chromosomal loci known to be affected in spontaneously occurring breast lesions, such as

ductal hyperplasia, carcinoma in situ, and invasive carcinoma (47–60).

MATERIAL AND METHODS

Cells and dose selection

MCF-10F cells at passage 113 were cultured in DMEM:F-12 medium containing 1.05 mM calcium (Ca^{2+}), antibiotics, antimycotics, hormones, growth factors, and equine serum as previously described (44). In order to determine the optimal doses for the expression of the cell transformation phenotype we treated the immortalized human breast epithelial cells (HBEC) MCF-10 F with 17β-estradiol (E_2) for testing whether they express colony formation in agar methocel, or colony efficiency (CE), and loss of ductulogenesis in collagen matrix, phenotypes also induced by the carcinogen benz (a) pyrene (BP) (44, 45). MCF-10F cells were treated with 0.0, 0.07 nM, 70 nM, or 0.25 mM of E_2 twice a week for two weeks. CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses. Ductulogenesis was 75±4.9 in control cells; it decreased to 63.7±28.8, 41.3±12.4, and 17.8±5.0 in E_2 treated cells, which also formed solid masses, whose numbers increased from 0 in controls to 18.5±6.7, 107±11.8 and 130±10.0 for each E_2 dose. Based upon these results a dose of 3.7uM (1μg/ml) was selected for testing the effect of E2 or DES.

Evaluation of the effect of estrogens on the expression of cell transformation phenotypes

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). MCF-10F cells were treated with 1.0 μg/ml E_2 (Aldrich, St. Louis, MO) or DES (Sigma Chemical Co., St. Louis, MO); control cells were treated with DMSO. MCF-10F cells treated with 1.0 μg/ml benz (a) pyrene (BP) served as positive controls for cell transformation assays. In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were first treated with E_2, DES, or BP at 72 hrs and 120 hours post plating. At the end of the first week of treatment, the cells were divided for evaluation of specific phenotypic characteristics or they were passaged for administration of another two periods of hormonal treatment. 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 doubling time (DT), survival efficiency (SE), colony efficiency (CE), colony size (CS), and ductulogenic capacity, as described in previous publications (44, 45).

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 Co, 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 10x magnification in 5 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.

Ductulogenesis in collagen matrix

This in vitro technique evaluates the capacity of cells to differentiate by providing evidence of whether treated cells form tridimensional 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 expression of specific differentiation genes.

Genomic analysis of treated cells

DNA isolation. To obtain DNA, treated and control cells were lysed in 5ml of TNE (0.5M Tris pH 8.9, 10mM 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 2 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 hour at 37°C and 100 μg/

TABLE 1. *Microsatellite DNA Polymorphism Analysis of MCF-10F Cells Treated with 17β-Estradiol (E2), Diethylstilbestrol (DES), or Benz(a)pyrene (BP)*

Ch	Marker	Location	MCF10-F	E2–1	E2–2	DES-1	DES-3	DES-4	DES-5	BP
1	D1S104	1p21–1p23	○	○	○	○	○	○	○	○
1	BAT-40	1p13.1	○	○	○	○	○	○	○	○
2	D2S171	2p24–21	○	○	○	○	○	○	○	○
2	D2S123	2p16	○	○	○	○	○	○	○	○
3	D3S1297		○	○	○	○	○	○	○	○
3	D3S1560	3p26–3p25	○	○	○	○	○	○	○	○
3	D3S1304	3p26–3p25	○	○	○	○	○	○	○	○
3	D3S1307	3p26-p25	○	○	○	○	○	○	○	○
3	D3S1289	3p23–3p21	○	○	○	○	○	○	○	○
3	D3S1449	3p22.3–3p21.3	○	○	○	○	○	○	●	○
3	D3S1478	3p21.3–21.2	○	○	○	○	○	○	●	○
3	D3S2384	3p21.3–21.2	○	○	○	○	○	○	●	○
3	D3S1450	3p21.1–3p14.2	○	○	○	○	○	○	●	○
3	D3S1217	3p21	○	○	○	○	○	○	●	○
3	D3S1447	3p21	○	○	○	○	○	○	○	○
3	D3S1241	3p21	○	○	○	○	○	○	○	○
3	D3S1448	3p21	○	○	○	○	○	○	○	○
3	D3S1480	3p14	○	○	○	○	○	○	○	○
6	ESR	6q24–27	○	○	○	○	○	○	○	○
8	MYCL-1	8q24.1	○	○	○	○	○	○	○	○
9	D9S199	9p23	○	○	○	○	○	○	○	○
9	D9S157	9p23–22	○	○	○	○	○	○	○	○
9	D9S171	9p21	○	○	○	○	○	○	○	○
9	D9S165	9p21	○	○	○	○	○	○	○	○
11	D11S988	1pter-qter	○	○	○	○	○	○	○	○
11	D11S922	11p15.5	○	○	○	○	○	○	○	○
11	H-RAS1	11p15.5	○	○	○	○	○	○	○	○
11	CCKBR	11p15.4	○	○	○	○	○	○	○	○
11	D11S1392	11p13	○	○	○	○	○	○	○	○
11	Int-2	11q13	○	○	○	○	○	○	○	○
11	D11S907	11p13	○	○	○	○	○	○	○	○
11	D11S911	11q13–11p23	○	○	○	○	○	○	○	○
11	D11S436	11p12–11p11.1	○	○	○	○	○	○	○	○
11	D11S614	11q22–11q23	○	○	○	○	○	○	○	○
11	D11S940	11q22	○	○	○	○	○	○	○	○
11	DRD2	11q23.1	○	○	○	○	○	○	○	○
11	D11S968	11q23.1–11q25	○	○	○	○	○	○	○	○
11	D11S29	11q23.3	○	●	●	○	○	○	○	○
11	D11S925	11q23.3–11q24	○	○	○	○	○	○	○	○
11	D11S912	11q24.2–11q25	○	●	●	○	○	○	○	○
12	IGF-1	12q22–12q23	○	○	○	○	○	○	○	○
13	D13S289	13q12.2	○	○	○	○	○	○	○	○
13	D13S260	13q12.3	○	○	○	○	○	○	○	○
13	D13S267	13q12.3	○	○	○	○	○	○	○	○
13	D13S171	13q12.3–13	○	○	○	○	○	○	○	○
13	D13S218	13q13–14.1	○	○	○	○	○	○	○	○
13	GABRB-1	13q14.2	○	○	○	○	○	○	○	○
13	D13S155	13q14.3–21.2	○	○	○	○	○	○	○	○
16	D16S540		○	○	○	○	○	○	○	○
17	D17S849	17p13.3	○	○	○	○	○	○	○	○
17	D17S796	17p13.1	○	○	○	○	○	○	○	○
17	D17S513	17p13.1	○	○	○	○	○	○	○	○
17	Tp53	17p13.1	○	○	○	○	○	○	○	○
17	D17S786	17p13.1	○	○	○	○	○	○	○	○
17	D17S793	17p13.1–17p11.2	○	○	○	○	○	○	○	○

TABLE 1 *(contd)*

Ch	Marker	Location	MCF10-F	E2–1	E2–2	DES-1	DES-3	DES-4	DES-5	BP
17	D17S945	17p13–12	O	O	O	O	O	O	O	O
17	D17S520	17p12	O	O	O	O	O	O	O	O
17	D17S800	17q11.1–12	O	O	O	O	O	O	O	O
17	THRA-1	17q11.2–12	O	O	O	O	O	O	O	O
17	D17S787	17q21–22	O	O	O	O	O	O	O	O
17	D17S855	17q21.2	O	O	O	O	O	O	O	O
17	D17S1323	17q21.2	O	O	O	O	O	O	O	O
17	D17S808	17q23.2	O	O	O	O	O	O	O	O
17	D17S789	17q24	O	O	O	O	O	O	O	O
17	D17S515	17q24.2–25.2	O	O	O	O	O	O	O	O
17	D17S785	17q25.2	O	O	O	O	O	O	O	O
18	D18S58	18q22.3–23	O	O	O	O	O	O	O	O

E_2, 17β-estradiol; DES, diethylestilbestrol; BP, benzo(a)pyrene; Ch, chromosome.

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.

Detection of allelic loss. We evaluated for allelic losses the regions of chromosomes 1, 2, 3, 6, 8, 9, 11, 12, 13, 16, 17, and 18 most frequently reported to exhibit loss of heterozygosity (LOH) in spontaneous breast tumors (Table 1). 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 (61–63). 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 (64).

DNA fingerprinting. Before performing DNA amplification of microsatellite DNA polymorphisms to detect allelic losses present in E_2-, DES-, and BP-treated cells, we verified by DNA fingerprinting whether all the clones derived from MCF-10F treated cells were from the same lineage. Genomic DNA was extracted from the cells listed in Table 1. The identity of these cells was confirmed by Southern blot hybridization of genomic DNA with a cocktail of the three minisatellite probes D2S44, D14S13 and D17S74. Genomic DNAs were digested with HifI, and hybridized with probes under standard condition (64).

PCR analysis of microsatellites. Primers used for the analysis of microsatellite polymorphisms are given elsewhere (64). 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/mmole)

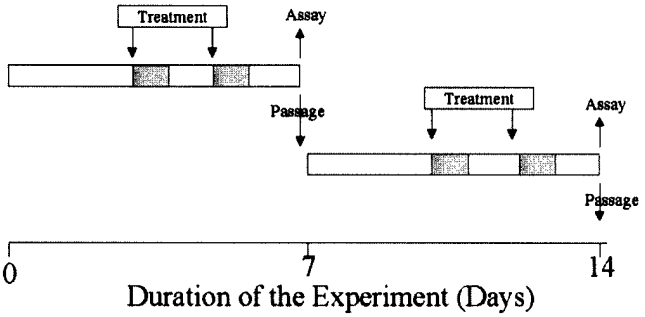

Fig. 1. MCF-10F cells were treated with E_2, DES, or BP at 72 hrs and 120 hours post plating. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis.

(Dupont, NEN, Boston, MA), and 0.5 units of Amplitaq DNA polymerase (Perkin Elmer Cetus) in 10 ml 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 (4ml) onto 5% to 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 to 24 h. Allele sizes were determined by comparison to M13mp18 sequencing ladders.

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.

RESULTS

Effect of estrogens on the expression of transformation phenotypes

The doubling time (DT) of MCF-10F cells was 93±5.6 hours. It was decreased, but no significantly, in E2- and DES-treated cells and their derived clones. A significant decrease, greater than 50%, was observed in BP-treated cells (Table 2). Evaluation of colony formation at the end of the second week of treatment revealed that MCF-10F cells treated with E_2, DES, or BP, formed colonies in agar-methocel, whereas MCF-10F control cells treated with DMSO did not (Table 2, Fig. 2). E_2 treated cells formed 24 colonies, from which six clones were expanded and maintained in culture. These clones were designated E_2-1 to E_2-6 (Table 2). DES treated MCF-10F cells formed 151 colonies, from which 24 colonies were isolated and seven clones survived. They were expanded and maintained in culture, being designated DES-1 to DES-7 (Table 2). BP treated cells formed 89 colonies, which had an average size of 670±46 μm in diameter. Those colonies formed by DES- and E_2-treated cells and their respective

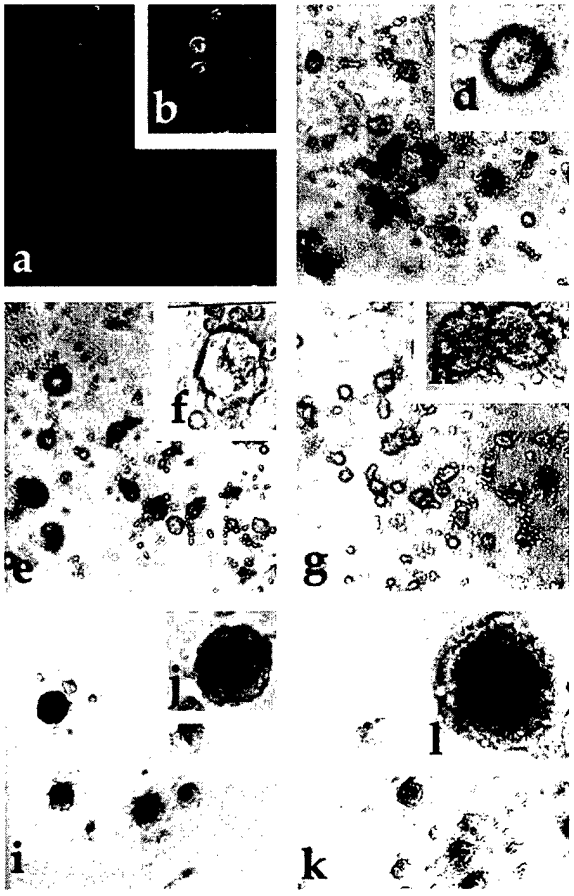

Fig. 2. MCF-10F cells plated in agar-methocel for colony assay. Control cells do not form colonies. Only isolated cells are present (a, 4×), (b, 10×). (c-k), colonies formed by E_2-, DES, and BP-treated MCF-10F cells. E2-induced colonies (c, 4×) (d, 10×). DES-induced colonies (e, 4×); (f, 10×). Colonies of E_2-2 clone (g, 4×) (h, 10×). Colonies of DES-3 clone (i, 4×) (j, 10×). Colonies of BP-treated cells (k, 4×) (l, ×10). Phase contrast.

derived clones were significantly smaller than those formed by BP transformed cells. However, DES-treated cell colonies were larger than those formed by E_2-treated cells and their derived clones (Table 2, Fig. 2).

Ductulogenesis was qualitatively evaluated by estimating the ability of the cells plated in collagen to form tubular and ductular structures. It was maximal (++) in MCF-10F cells, and completely negative (−) in BP-treated cells, which grew as a solid or cystic mass (Table 2). E_2-, DES-treated cells, and E_2-4 and DES-1 clones exhibited a moderately decreased ability to form ductules (+). It was interesting to observe that all the other clones derived from E_2- and DES-

TABLE 2. *Phenotypic markers of cell transformation induced in MCF-10F cells by 17β estradiol (E_2), Diethylstilbestrol (DES) and Benz (a) pyrene (BP)*

Cell Type	No. of Passages	Doubling time (DT)[a]	Colony Number (CN)[b]	Colony Efficiency (%) CE)[c]	Colony Size (CS) (μm)[d]	Ductulogenesis[g]
MCF-10F	113	93±5.6	0.0	0.0	0.0	++
BP	4	42±3.8	89	18±4.5	670±46	−
E_2	4	78±16.0	24[e]	4.8±0.9	170±34	+
DES	4	73±13	151[f]	30.20±8.9	190±23	+
E_2-1*	4	81±3.0	36	7.2±3.7	180±12	+/−
E_2-2*	4	68±10	45	9.0±2.0	150±6	+/−
E_2-3	5	66±8.0	39	7.9±5.6	190±9	−
E_2-4	3	82±6.0	20	3.5±1.1	134±5	+
E_2-5	6	61±5.6	63	12.6±3.0	193±12	+/−
E_2-6	4	73±3.0	54	10.8±4.9	189±5	−
DES-1*	4	73±9	167	33.17±6.3	278±40	+
DES-2	4	74±10	148	29.4±10.0	189±23	+/−
DES-3*	3	78±5	130	25.8±3.9	278±12	+/−
DES-4*	3	75±8	189	37.5±7.3	239±34	+/−
DES-5*	6	68±10	167	33.17±5.9	360±60	−
DES-6	5	67±3	150	29.8±10	290±32	+−/
DES-7	5	78±4	99	19.6±6.9	207±28	+−/

[a] Doubling time (DT) in hours, was determined as described in (43). DT was significantly different by Student's t-test between BP and all the other cells lines ($p<0.001$). [b] Colony number (CN); [c] Colony efficiency (CE), and [d] Colony size (CS). These three parameters were significantly different between MCF-10F and all other cell lines ($p=0.00001$). CS of DES clones was significantly different from E_2 and BP cells ($p=0.001$). [e] From 24 colonies derived from E2-treated cells, clones E_2-1, E_2-2, E_2-3, E_2-4, E_2-5 and E_2-6 were recovered and expanded. [f] From 151 colonies derived from DES treated cells, 24 colonies were isolated and clones DES-1, 2,3,4,5,6 and 7 were recovered and expanded. [g]Ductulogenesis, duct-like formation in collagen gel. * These cells have been used for detection of microsatellite DNA polymorphism.

treated cells exhibited an overall decrease of ductulogenic capacity. Clones E_2-3, E_2-6, and DES-5 had completely lost this property, being in this sense similar to BP-treated cells (Table 2).

Genomic changes induced in E_2 and DES transformed MCF-10 cells

DNA fingerprint analysis of parent, E_2-, DES-, and BP-treated cells and their derived clones revealed that their allelic pattern was identical in all the cell lines analyzed (Fig. 3). These results confirmed that all the cells tested had the same HBEC origin, and that they were free of contamination from other cell lines maintained in our laboratory.

Among 67 markers tested, which were selected based on chromosomal changes reported to be present in breast and other cancers, only clones DES-5, E_2-1 and E_2-2, exhibited LOH in chromosomes 3 and 11, respectively (Table 1). LOH in chromosome 3 was detected at three different loci, which were detected with five different markers, 3p21.3–21.2 (marker D3S1478 and D3S2384), 3p21.1–14.2 (marker D3S1450), and 3p21 (marker D3S1217 and D3S1447) (Fig. 4, Table 1). It was of interest that clone DES-5, in addition to exhibiting LOH in chromosome 3, was the one exhibiting the most marked expression of transformation phenotypes, i.e., larger colony size and absent ductulogenic ability in collagen gel (Table 2). Clones E_2-1 and E_2-2 identically expressed LOH in chromosome 11 at 11q23.3 (marker D11S29), and 11q24.2-q25 (marker D11S912). BP-treated cells did not exhibit LOH at any of the loci tested. Interestingly, we have found that all the clones of the cells transformed with either E2, DES or BP presented microsatellite instability (MSI), expressed as an allelic expansion at 3p21 locus (marker D3S1447) (data not shown). In order to determine whether these MSIs were related to alterations in mismatch repair genes, we performed microsatellite DNA analysis in loci 1p13.1, with marker BAT40, 2p16, with marker D2S123, and 18q22.3–23, with marker D18S58, which are related to mismatch repair genes.

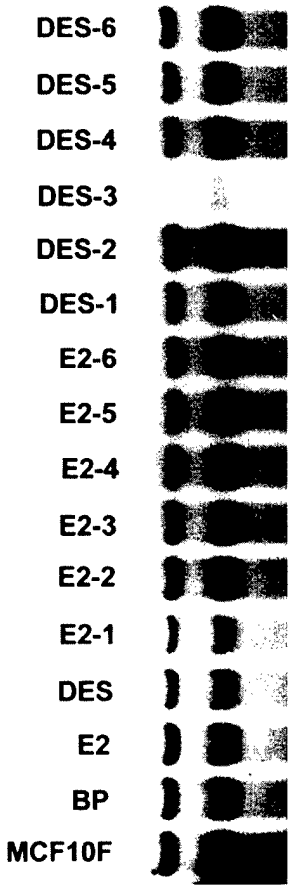

Fig. 3. Fingerprint analysis of genomic DNA obtained from the cells listed in table 2 using markers listed in Materials and Methods.

However, none of those markers showed alterations with this technique (Table 1).

DISCUSSION

In the present work we have capitalized on the availability in our laboratory of an in vitro model of transformation of immortalized HBEC by the chemical carcinogen BP for comparison with phenotypic and genomic changes induced by the natural estrogen 17β-estradiol (E_2) and the synthetic estrogen diethylstilbestrol (DES) in the same cells (44, 45). The immortalized human breast epithelial cells MCF-10F are negative for both ER-α and ER-β (42). Short term treatments of these cells with these two estrogenic compounds induce 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 induce by BP under the same culture conditions. It was of great interest that by the fourth passage after 4 treatments during a two-week period, clones derived from DES- and E_2-transformed cells exhibited loss of heterozygosity in chromosomes 3 and 11, respectively, whereas during the same period of time the chemical carcinogen BP did not induce genomic changes, even though we have previously re-

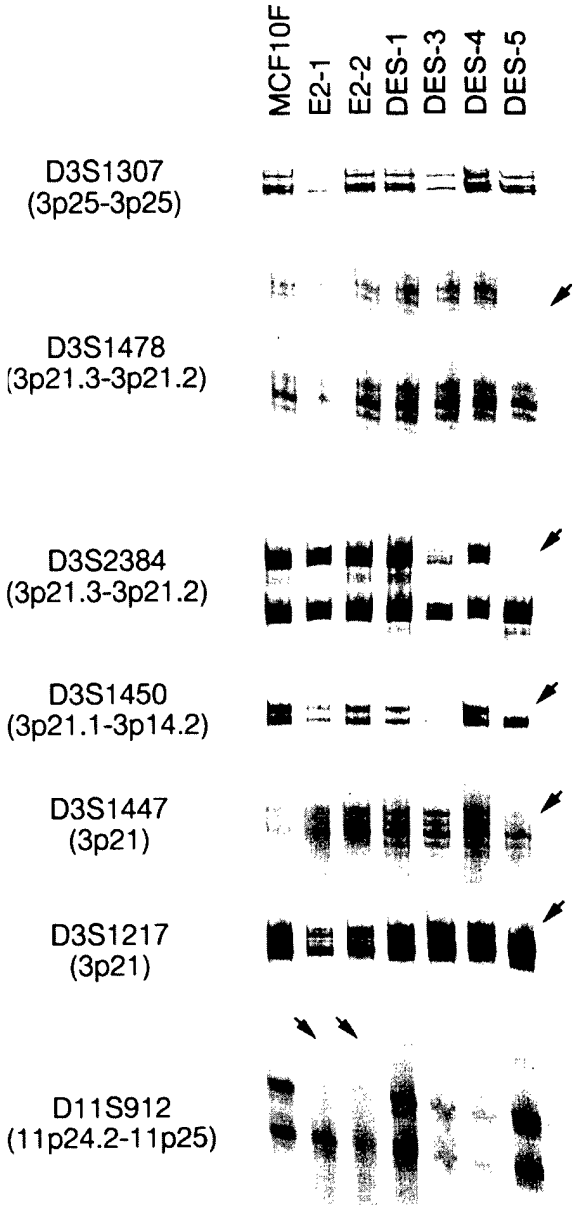

Fig. 4. LOH analysis of MCF-10F, and of clones E2–1, E2–2, and DES-1, DES-3, DES-4, and DES-5, derived from E_2 and DES-treated cells. Arrows indicate the loss of alleles in E_2-1, E_2-2, and DES-5 clones.

ported that this carcinogen induces LOH in chromosome 17 (43), in addition to tumorigenesis in a heterologous host after a larger number of passages and a more prolonged selection process in vitro (44, 45). The expression of LOH in chromosome 3 in DES-transformed breast epithelial cells acquired relevance in view of the light that frequent homozygous deletions, rearrangements, and hypermethylation at 3p21 loci have been reported to be present in spontaneously occurring breast lesions, such as ductal hyperplasia, carcinoma in situ, and invasive carcinoma (31, 47–50, 65–68). The existence of suppressor genes on 3p has also been suggested by transfection studies in which 3p DNA fragments inhibited tumorigenesis in nude mice (68, 69). We have observed LOH at 3p21 using markers D3S1217 and D3S1447, and in the region 3p21.1-p21.2 with markers D3S1478 and D3S2384. LOH in this region has been reported in nearly all-small cell lung carcinomas (70). Even though deletion in these regions is not considered to be specific for breast cancer, our observations might indicate that they represent a genetic event triggered by estrogens, which could play a key role in the development and progression of tumors originated from this type of epithelium. LOH in 3p21.3 has been found more frequently in breast cancer metastases than in primary tumors. Several putative "metastasis-related genes" are located in this region, such as 37LRP (71–74), CTNNB1, that encodes β catenin (75), and the αRLC gene, that encodes a new integrin subunit, identified by positional cloning, that shows homology with the α1 integrin involved in the metastatic process (70). We have also found LOH in the 3p21.1–14.2 (marker D3S1450). This region has been found to be associated with dysregulated cell proliferation rather than with tumor progression (50). It is also frequently deleted in in situ carcinoma, benign tumors, and familial breast cancers (51, 76, 77). The3p14.2 region contains a fragile site known as FRA3B, from which the FHIT gene has recently been cloned. It encodes a protein showing homology with a yeast hydrolase, and its transcripts show rearrangements in different cell lines and tumors (52, 77). Recently, telomerase-regulating genes have been located in 3p21.3-p22 and 3p12–21.1 using the microcell monochromosome transfer technique (78). We have also found that estrogen induces LOH in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2–25, respectively. It has been reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice (79). Several genes, such as HRAs, CTSD, ILK, TSG101 and KI1 have been reported to be located on the short arm of chromosome 11 (53–54, 79–85). A region of deletion on 11q22–23 has been described on the long arm of chromosome 11in 40 to 60% of breast tumors (51, 57, 59, 60, 86, 88). The ataxia telangiectasia susceptibility gene (ATM) is the most widely studied candidate gene in this region (89). ATM may act upstream of the TP53 gene in cell cycle regulation (90, 91) and its heterozygous mutation is associated with high incidence of early-onset breast cancer. This region has been reported to contain several tumor suppressor genes and genes involved in the metastatic process. In this latter group, the MMP genes encoding matrix metalloproteases involved in invasion, ETS1 encoding a transcription factor involved in angiogenesis, and VACM-1, encoding a protein probably involved in cell cycle regulation have been identified (92). Although some of these genes might be affected during the transformation of HBEC induced by estrogens, a more detailed allelotyping using multiple markers is required for better defining the significance of LOH in these cells.

Approximately 35% of breast cancers show LOH at the D11S29 and NCAM loci (93), and a higher frequency of LOH at this locus has also been found in melanomas (94). LOH has been found at frequencies of 25% and 29% at the distal D11S968 (11qter) and D11S29 (11q23.3 locus), slightly above the accepted baseline of 0–20 per cent in colorectal cancer. The fact that breast cancer, melanoma, and colorectal cancer have been found to be influenced by estrogens (95), give relevance to our data that treatment of MCF-10F cells with estrogens induces LOH in this specific locus. LOH at 11q23-qter occurs frequently in ovarian and other cancers (96, 97).

The most frequent allelic loss observed in breast cancer has been reported in chromosome 17p, suggesting that genes located in that chromosome arm, such as p53 oncogene, might be a likely target for this event. (33, 93–114). We have not been able up to now to demonstrate

any LOH in chromosome 17 in estrogen transformed MCF-10F cells. However, we have used a small number of markers, and the possibility that LOH might be located at sites not tested yet cannot be ruled out. Therefore, the study of allelic imbalances at 17q and 17p, as well as in chromosome 16 (99, 115, 116) in estrogen transformed HBEC must be carried out to provide further understanding of the functional involvement of these chromosomes in the process of cell transformation by E_2 and DES.

The observations that E_2, DES, and BP induce similar phenotypical, but different genomic alterations requires further investigation in order to elucidate the significance of timing of appearance of each type of changes with regards to cancer initiation and progression. There are several probable avenues for explaining these discrepancies. In this model, phenotypical changes are induced by both estrogens and the chemical carcinogen as an early event, whereas LOH is a rare event that is manifested in different chromosomes and only in few clones derived from E_2 and DES treated cells. The rarity of the phenomenon is in agreement with the low frequency of LOH observed in BP transformed cells, in which the phenomenon is manifested at a more advanced stage of neoplastic progression (43, 114). Altogether these observations suggest that these three compounds might act through different genetic events for inducing similar transformation phenotypes.

REFERENCES

1. Pike MC, Spicer DV, Dahmoush L, Press MF. Estrogens, progesterone, normal breast cell proliferation and breast cancer risk. Epidemiol Rev 1993;15:17–35.
2. Kelsey JL, Gammon MD, John EM. Reproductive factors and breast cancer. Epidemiol Rev 1993;15:36–47.
3. Bernstein L, Ross RK. Endogenous hormones and breast cancer risk. Epidemiol Rev 1993;15: 48–65.
4. Henderson BE, Ross R and Bernstein L. Estrogens as a cause of human cancer: the Richard & Hinda Rosenthal Foundation Award Lecture. Cancer Res 1988;48:246–53.
5. Topper YJ, Sankaran L, Chomczynski P, Prosser C, Qasba P. Three stages of responsiveness to hormones in the mammary cell. In: Angeli A, Bradlow HL, Dogliotti L (eds), Endocrinology of the Breast: Basic and Clinical Aspects. Ann N Y Acad Sci 1986;464:1–10.
6. Lippman ME, Huff KK, Jakesz R, Hecht T, Kasid A, Bates S, Dickson RB. Estrogens regulate production of specific growth factors in hormone-dependent human breast cancer. In: Angeli A, Bradlow HL, Dogliotti L (eds), Endocrinology of the Breast: Basic and Clinical Aspects. Ann N Y Acad Sci 1986;464:11–6.
7. Dupont WD, Page DL. Menopausal estrogen replacement therapy and breast cancer. Arch Int Med 1991;151:67–72.
8. Price MA, Tennant CC, Smith RC, Kennedy SJ, Butow PN, Kossoff MB, Dunn SM. Predictors of breast cancer in women recall following screening. Australian & New Zealand Journal of Surgery 1999;69:639–46.
9. Couse JF, Korach KS. Estrogen receptor null mice: What have we learned and where will they lead us? Endocrine Reviews 1999;20:358–417.
10. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–37.
11. McDonnell DP. The molecular pharmacology of SERMs. TEM 1999;10:301–11.
12. Tsai MJ, O'Malley BW. Molecular mechanisms of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994;63:451–86.
13. Katzenellenbogen BS. Dynamics of steroid hormone receptor action. Annu Rev Physiol 1980; 42:17–35.
14. Mosselman S, Polma J, Dijkema R. ER β: identification and characterization of a novel human estrogen receptor. FEBS Lett 1996; 392:49–53.
15. Kuiper GGJM, Carlsson B, Grandien K, Enmark E, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997;138:863–70.
16. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 1997;277: 1508–10.
17. Chen X; Danes C, Lowe M; Herliczek TW, Keyomarsi K.. Activation of the estrogen-signaling pathway by p21 WAF1/CIP1 in estrogen receptor negative breast cancer cells. J Natl Cancer Inst 92P1403–13, 2000.
18. Liehr JG, Ulubelen AA, Strobel HW. Cytochrome P-450-mediated redox cycling of estrogens. J Biol Chem 1986;261:16865–70.
19. Roy D, Liehr JG. Temporary decrease in renal quinone and reductase activity induced by chronic administration of estradiol to male Syrian hamsters – increased superoxide formation

by redox cycling of estrogen. J Biol Chem 1988; 263:3646–51.
20. Yan Z-J, Roy D. Mutations in DNA polymerase P mRNA of stilbene estrogen-induced kidney tumors in Syrian hamster. Biochem Mol Biol lnt 1997;37:175–83.
21. Ball P, Knuppen R. Catecholestrogens (2- and 4-hydroxy-oestrogens). Chemistry, biosynthesis, metabolism, occurrence and physiological significance. Acta Endocrinol (Copenh) 1980; 232(suppl):1:127.
22. Zhu BT, Bui QD, Weisz J, Liehr JG. Conversion of estrone to 2- and 4- hydroxyestrone by hamster kidney and liver microsomes: Implications for the mechanism of estrogen-induced carcinogenesis. Endocrinology 1994;135:1772–79.
23. Ashburn SP, Han X, Liehr JG. Microsomal hydroxylation of 2- and 4-fluoroestradiol to catechol metabolites and their conversion to methyl ethers: Catechol estrogens as possible mediators of hormonal carcinogenesis. Mol Pharmacol 1993;43:534–41.
24. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: Correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. Science 1987;235:177–82.
25. Escot C, Theillet C, Lidereau R, Spyratos F, Champeme M-H, Gest J, Callahan R. Genetic alteration of the *c-myc* proto-oncogene (*MYC*) in human primary breast carcinomas. Proc Natl Acad Sci USA 1986;83:4834–38.
26. Ali IU, Merio G, Callahan R, Lidereau R. The amplification unit on chromosome 11 q13 in aggressive primary human breast tumors entails the bcl-1, int-2 and hst loci. Oncogene 1989; 4:89–92.
27. Theillet C, Adnane J, Szepetowski P, Simon MP, Jeanteur P, Birnbaum D, Gaudray P. BCL-1 participates in the 11q13 amplification found in breast cancer. Oncogene 1990;5:147–9.
28. Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J, Callahan R. Loss of a *c-H-ras-1* and aggressive human primary breast carcinomas. Cancer Res 1986;46:4776–81.
29. Lundberg C, Skoog L, Cavenee WK, Nordenskjoid M. Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. Proc Natl Acad Sci USA 1987;84:2372–76.
30. Mackay J, Steel CM, Elder PA, Forrest APM, Evans HJ. Allele loss on short arm of chromosome 17 in breast cancers. Lancet 1988;2:1384–5.
31. Ali IU, Lidereau R, Callahan R. Presence of two members of *c-erbAB* and *c-erbA2* in smallest region of somatic homiozygosity on chromosome 3p2l-p25 in human breast carcinoma. J Natl Cancer Inst 1989;81:1815–20.
32. Chen L-C, Dolibaum C, Smith H. Loss of heterozygosity on chromosome lq in human breast cancer. Proc Natl Acad Sci USA 1989; 86:7204–7.
33. Callahan R, Campbell A. Mutations in human breast cancer: an overview. J Natl Cancer Inst 1989;81:1780–6.
34. Sato T, Saito H, Swensen J, Olifant A, Wood C, Danner D, Sakamoto T, Takita K, Kasumi F, Miki Y, Skolnick M, Nakamura Y. The human prohibitin gene located on chromosome 17q2l is mutated in sporadic breast cancer. Cancer Res 1992;52:1643–6.
35. Chen LC, Kurisu W, Ljung BM, Goldman ES, Moore D 2d, Smith HS. Heterogeneity for allelic loss in human breast cancer. J Natl Cancer Inst 1992;84:506–10.
36. Genuardi M, Tsihira N, Anderson DE, Saunders GF. Distal deletion of chromosome lq in ductal carcinoma of the breast. Am J Hum Genet 1989;45:73–89.
37. Crop CS, Lidereau R, Campbell G, Champene M-H, Callahan R. Loss of heterozygosity on chromosomes 17 and 18 in breast carcinoma: two additional regions identified. Proc Natl Acad Sci USA 1990;87:7737–41.
38. Sato T, Tanigami A, Yamakawa K, Akiyama F, Kasumi F, Sakamoto G, Nakamura Y. Allelotype of breast cancer: Cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res 1990;50:7184–9.
39. Sato T, Akiyama F, Sakamoto G, Kasumi F, Nakamura Y. Accumulation of genetic alterations and progression of primary breast cancer. Cancer Res 1991;51:5794–9.
40. Soule HD, Maloney TM, Wolman SR, Peterson Jr WD, Brenz R, McGrath CM, Russo J, Pauley R, Jones RF, Brooks SC. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 1990;50:6075–86.
41. Tait L, Soule H, and Russo J. Ultrastructural and immunocytochemical characterizations of an immortalized human breast epithelial cell line MCF-10. Cancer Res 1990;50:6087–99.
42. Hu YF, Lau KM, Ho SM, Russo J. Increased expression of estrogen receptor-β in chemically transformed human breast epithelial cells. Int J Oncol 1998;12:1225–8.
43. Russo J, Calaf G, Sohi N, Tahin Q, Zhang PL, Alvarado ME, Estrada S, Russo IH. Critical steps in breast carcinogenesis. N Y Acad Sci 1993;698:1–20.
44. Calaf G, Russo J. Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis 1993;14:483–92.
45. Russo J, Calaf G, Russo IH. A critical approach to the malignant transformation of hu-

man breast epithelial cells. CRC Critical Reviews in Oncogenesis 1993;4:403–17.
46. Calaf G, Zhang PL, Alvarado MV, Estrada S, and Russo J. C-Ha ras enhances the neoplastic transformation of human breast epithelial cells treated with chemical carcinogens. Int J Oncol 1995;6:5–11.
47. Chen L-C, Matsumura K, Deng G, Kurisu W, Ljung B-M, Lerman MI, Waldman FM, Smith HS. Deletion of two separate regions on chromosome 3p in breast cancers. Cancer Res 1994;54:3021–4.
48. Bergthorsson JT, Eiriksdottir G, Barkardottir RB, Egilsson V, Arason A, Ingvarsson S. Linkage analysis and allelic imbalance in human breast cancer kindreds using microsatellite markers from the short arm of chromosome 3. Human Genetics 1995;96:437–43.
49. Kerangueven F, Noguchi T, Wargniez V. Multiple sites of loss of heterozygosity on chromosome arms 3p and 3q in human breast carcinomas. Oncology Reports 1996;3:313–6.
50. Pandis N, Bardi G, Mitelman F, and Heim S. Deletion of the short arm of chromosome 3 in breast tumors. Genes Chrom Cancer 1997;18: 241–5.
51. Man S, Ellis I, Sibbering M, Blarney R, and Brook J. Highs level of allele loss at the FHIT and ATM genes in non-comedo ductal carcinoma in situ and grade I tubular invasive breast cancers. Cancer Res 1996;56:5484–9.
52. Negrini M, Monaco C, Vorechovsky I, Ohta M, Druck T, Baffa R, Huebner K, Croce CM. The FHIT gene at 3pl4.2 is abnormal in breast carcinomas. Cancer Res 1996;56:3173–9.
53. Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J, Callahan R. Loss of a c-H-*ras*-I allele and aggressive human primary breast carcinomas. Cancer Res 1986;46:4776–81.
54. Mackay J, Elder P, Porteous D I, et al. Partial deletion of chromosome 11p in breast cancer correlates with size of primary turnout and estrogen receptor level. Br J Cancer 1988;58:710–4.
55. Takita K-I, Sato T, Miyagi M, Watatani M, Akiyama F, Sakamoto G, Kasumi F, Abe R, Nakamura Y. Correlation of loss of alleles on the short arms of chromosomes 11 and 17 with metastasis of primary breast cancer to lymph nodes. Cancer Res 1992;52:3914–7.
56. Winqvist R, Mannermaa A, Alavaikko M, Blanco G, Taskinen PJ, Kiviniemi H, Newsham I, Cavenee W. Refinement of regional loss of heterozygosity for chromosome 11pl5.5 in human breast tumors. Cancer Res 1993;53:4486–8.
57. Gudmundsson J, Barkardottir RB, Eiriksdottir G, Baldursson T, Arason A, Egilsson V, Ingvarsson S. Loss of heterozygosity at chromosome 11 in breast cancer: association of prognostic factors with genetic alterations. Br J Cancer 1995;72:696–701.
58. Negrini M, Sabbioni S, Ohta M, Veronese ML, Rattan S, Junien C, Croce CM. Seven-megabase yeast artificial chromosome contig at region 11pl5: Identification of a yeast artificial chromosome spanning the breakpoint of a chromosomal translocation found in a case of Beckwith-Wiedmann syndrome. Cancer Res 1995;55:2904–9.
59. Carter S, Negrini M, Baffa R, Gillum DR, Rosenberg AL, Schwartz GF, Croce CM. Loss of heterozygosity at 11 q22-q23 in breast cancer. Cancer Res 1994;54:6270–4.
60. Koreth J, Bakkenist C, and McGee JOD. Allelic deletions at chromosome 11q22-q23.1 and 11q25-q term are frequent in sporadic breast but not colorectal. Cancers Oncogene 1997;14: 431–7.
61. Weber JL. Human DNA polymorphisms based on length variations in simple sequence tandem repeats. In: Genome Analysis Series (Tilghman, S, Davies K, eds) Genetic and Physical Mapping, New York, Cold Spring Harbor Laboratory Press 1990;1:159–181.
62. Litt M. PCR of TG microsatellites. In: McPherson MC, Quirke P, Taylor G (eds), PCR: A Practical Approach. Oxford Univ Press; 1991:85–99.
63. Weber JL. Informativeness of human (dC-dA)n (dG) n polymorphisms. Genomics 1990;7:524–30.
64. Huang Y, Bove B, Wu Y, Russo IH, Tahin Q, Yang X, Zekri A, Russo J. Microsatellite Instability during the Immortalization and Transformation of Human Breast Epithelial Cells In vitro. Mol Carcinog 1999;24:118–27.
65. Ben Cheickh, M, Rouanet P, Louason G, Jeanteur P, and Theillet C. An attempt to define sets of cooperating genetic alterations in human breast cancer. Int J Cancer 1992;51:542–7.
66. Sato T, Akivama F, Sakamoto G, Kasumi F, and Nakamura Y. Accumulation of genetic alterations and progression of primary breast cancer. Cancer Res 1991;51:5794–9.
67. Deng G, Chen LC, Schott DR, Thor A, Bhargava V, Ljung BM, Chew K, Smith HS. Loss of heterozygosity and p53 gene mutations in breast cancer. Cancer Res 1994;54:499–505.
68. Sanchez Y, el-Naggar A, Pathak S, and Killary A M. A tumor suppressor locus within 3pl4-pl2 mediates rapid cell death of renal cell carcinoma in vivo. Proc Natl Acad Sci USA 1994; 91:3383–7.
69. Killary A, Wolf M, Giambernardi T, and Naylor S. Definition of a tumor suppressor locus within human chromosome 3p21 –p22. Proc Natl Acad Sci USA 1992;89:10877–81.

70. Hibi Y, Yamakawa IC, Ueda R, Horio Y. Aberrant upregulation of a novel integrin α subunit gene at 3p21.3 in small cell lung cancer. Oncogene 1994;9:611–9.
71. Jackers P, Minoletti F, Belotti D, Clausse N, Sozzi G, Sobel ME, Castronovo V. Isolation from a multigene family of the active human gene of the metastasis-associated multifunctional protein 37LRP/p4O at chromosome 3p21.3. Oncogene 1996;13:495–503.
72. Wewer UM, Taraboletti G, Sobel ME, Albrechtsen R, Liotta LA. Role of laminin receptor in tumor cell migration. Cancer Res 1987;47:5691–8.
73. Martignone S, Menard S, Bufalino R, et al. Prognostic significance of the 67-kilodalton laminin receptor expression in human breast carcinomas. J Natl Cancer Inst 1993;85:398–402.
74. Maemura M, and Dickson RB. Are cellular adhesion molecules involved in metastasis of breast cancer. Breast Cancer Res Treat 1994; 32:239–60.
75. Trent JM, Wiltshire R, Su L, Nicolaides NC, Vogelstein B, Kinzler KW. The gene for the APC-binding protein beta-catenin (CTNNB1) maps to chromosome 3p22, a region frequently altered in human malignancies. Cytogenet Cell Genet 1995;71:343–4.
76. Dietrich CU, Pandis N, Teixeira MR, Bardi G, Gerdes AM, Andersen JA, Heim S. Chromosome abnormalities in benign hyper-proliferative disorders of epithelial and stromal breast tissue. Int J Cancer 1995;60:49–53.
77. Pennisi E. New gene forges link between fragile site and many cancers. Science 1996;272: 649.
78. Cuthbert AP, Bond J, Trott DA, Gill S, Broni J, Marriott A, Khoudoli G, Parkinson EK, Cooper CS, Newbold RF. Telomerase repressor sequences on chromosome 3 and induction of permanent growth arrest in human breast cancer cells. J Natl Cancer Inst 1999;91:37–45.
79. Negrini M, Sabbioni S, Haldar S, Possati L, Castagnoli A, Corallini A, Barbanti-Brodano G, Croce CM. Tumor and growth suppression of breast cancer cells by chromosome 17-associated functions. Cancer Res 1994;54:1818–24.
80. Borresen AL, Andersen TI, Garber J, Barbier-Piraux N, Thorlacius S, Eyfjord J, Ottestad L, Smith-Sorensen B, Hovig E, Malkin D. Screening for germ line TP53 mutations in breast cancer patients. Cancer Res 1992;52:3234–6.
81. Puech A, Henry I, Jeanpierre C, Junien C. A highly polymorphic probe on 11p15.5: L22.5.2 (D11S774). Nucleic Acids Research 1991;19: 5095–9.
82. Hannigan GE, Bayani J, Weksberg R, Beatty B, Pandita A, Dedhar S, Squire J. Mapping of the gene encoding the integrin-linked kinase, ILK, to human chromosome 11pl5.5-pl5.4. Genomics 1997;42:177–9.
83. Wang H, Shao N, Ding QM, Cui J, Reddy ES, Rao VN. BRCA1 proteins are transported to the nucleus in the absence of serum and splice variants BRCA1a, BRCA1b are tyrosine phosphoproteins that associate with E2F, cyclins and cyclin dependent kinases. Oncogene 1997;15:143–57.
84. Dong J-T, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT, Barrett JC. KA/1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science 1995 268:884–6.
85. Wei Y, Lukashev M, Simon D, et al. Regulation of integrin function by the urokinase receptor. Science 1996;273:1551–5.
86. Hampton GM, Mannermaa A, Winquist R, Alavaikko M, Blanco G, Taskinen PG, Kiviniemi H, Newsham I, Cavenee WK, Evans GA. Losses of heterozygosity in sporadic human breast carcinoma: A common region between 11q22 and 11q23.3. Cancer Res 1994;54:4586–9.
87. Negrini M, Rasio D, Hampton GM, Sabbioni S, Rattan S, Carter SM, Rosenberg AL, Schwartz GF, Shiloh Y, Cavenee WK, Croce CM. Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: Identification of a new region at 11 q23.3. Cancer Res 1995;55:3003–7.
88. Winqvist R, Hampton GM, Mannermaa A, Blanco G, Alavaiko M, Kiviniemi H, Taskinen PJ, Evans GA, Wright FA, Newsham I, Cavenee WK. Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. Cancer Res 1995;55:2660–4.
89. Elson A, Wang Y, Daugherty CJ, Morton CC, Zhou F, Campos-Torres J, Leder P. Pleiotropic defects in ataxia-telangiectasia protein-deficient mice. Proc Natl Acad Sci USA 1996;93:13084–9.
90. Westphal CH, Schmaltz C, Rowan S, Elson A, Fisher DE, Leder P. Genetic interactions between *atm* and *p53* influence cellular proliferation and irradiation-induced cell cycle checkpoints. Cancer Res 1997;57:1664–7.
91. Swift M, Morrel D, Massey R, Chase C. Incidence of cancer in 161 families affected by ataxia-telangiectasia. New Eng J Med 1991; 325:1831–6.
92. Byrd PJ, Stankovic T, McConville C M, Smith AD, Cooper PR, Taylor AM. Identification and analysis of expression of human VACM-1, a cullin gene family member located on chromosome 11 q22–23. Genome Res 1997;7:71–5.
93. Tomlinson IP, Nicolai H, Solomon E, Bodmer WF. The frequency and mechanism of loss of

heterozygosity on chromosome 11q in breast cancer. J Pathol 1996;180:38–43.
94. Tomlinson IP, Beck NE, Bodmer WF. All ele loss on chromosome 11q and microsatellite instability in malignant melanoma. European Journal of Cancer 1996;32A:1797–802.
95. Connolly KC, Gabra H, Millwater CJ, Taylor KJ, Rabiasz GJ, Watson JE, Smyth JF, Wvllie AH, Jodrell DI. Identification of a region of frequent loss of heterozygosity at 11 q24 in colorectal cancer. Cancer Res 1999;59:2806–9.
96. Launonen V, Stenback F, Puistola U, Bloi~u R, Huusko P, Kytola S, Kauppila A, Winqvist R. Chromosome 11q22.3-q25 LOH in ovarian cancer: association with a more aggressive disease course and involved subregions. Gynecol Oncol 1998;71:299–304.
97. Dahiva R, McCarville J, Lee C, Hu W, Kaur G, Carroll P, Deng G. Deletion of chromosome 1 lpl5, pl2, q22, q23–24 loci in human prostate cancer. Int J Cancer 1997;72:283–8.
98. Vogelstein B, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y, White R. Allelotype of colorectal carcinomas. Science 1989;244:207–11.
99. Sato T, Tanigami A, Yamakawa K, Akiyama F, Kasumi F, Sakamoto G, Nakamura Y. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res 1990;50:7184–9.
100. Futreal PA, Söderkvist P, Marks JR, Iglehart JD, Cochran C, Barrett JC, Wiseman RW. Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. Cancer Res 1992;52:2624–7.
101. Devilee P, Cornelisse CJ. Genetics of human breast cancer. Cancer Survey 1990;9:605–30.
102. Holistein M, Sidransky D, Vogelstein B, Harris CC. P53 mutations in human cancers. Science 1991;253:49–53.
103. Prosser J, Thompson AM, Cranston G, Evans HJ. Evidence that p53 behaves as a tumor suppressor gene in sporadic breast tumors. Oncogene 1990;1573–9.
104. Hovig E, Smith-Sorensen B, Brogger A, Borresen AL. Constant denaturant gel electrophoresis, a modification of denaturating gradient gel electrophoresis, in mutation detection. Mutation Research 1991;262:63–71.
105. Osborne RJ, Merlo GR, Mitsudomi T, Venesio T, Liscia DS, Cappa APM, Chiba I, Takahashi T, Nau MM, Callahan R, Minna JD. Mutations in the p53 gene in primary human breast cancers. Cancer Res 1991;51: 6194–8.
106. Thompson AM, Anderson TJ, Condie A, Prosser J, Chetty U, Carter DC, Evans HJ, Steel CM. P53 allele losses, mutations and expression in breast cancer and their relationship to clinico-pathological parameters. Int J Cancer 1992;50:528–32.
107. Varley JM, Brammar WJ, Lane DP, Swallow JE, Dolan C, Walker RA. Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinomas. Oncogene 1991;6:413–21.
108. Biggs PJ, Warren N, Venitt S, Stratton M.R. Does a genotoxic carcinogen contribute to human breast cancer-7. Mutagenesis 1993;8:275–83.
109. Kirchweger R, Zeillinger R, Schneeberger C, Speiser P, Louason G, Theillet C. Patterns of allele losses suggest the existence of five distinct regions of LOH on chromosome 17 in breast cancer. Int J Cancer 1994;56:193–9.
110. Jantke I, Jonat W, Maass H, Goedde HW. Human breast cancer: frequent p53 allele loss and protein overexpression. Human Genetics 1993; 90:635–40.
111. Anderson T, Gaustad A, Ottestad L, Farrants GW, Nesland JM, Tveit KM, Borresen AL. Genetic alterations of the tumor suppressor gene regions 3p, 11p, 13q, 17p, and 17q in human breast carcinomas. Genes, Chromosomes & Cancer 1992;4:113–1.
112. Goldman ES, More D 2nd, Balazs M, Li VE. Loss of heterozygosity on the shortarm of chromosome 17 is associated with high proliferative capacity and DNA aneuploidy in primary human breast cancer. Proc Natl Acad Sci 1991;88:3847–51.
113. Coles C, Thompson AM, Elder PA, Cohen BB, Mackenzie IM, Cranston G, Chetty U, Mackay J, Macdonald M, Nakamura Y. Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis. Lancet 1990;336:761–3.
114. Russo J, Hu YF, Yang X, Huang Y, Silva I, Bove B, Higgy N, Russo IH. Breast cancer multistage progression. Frontiers in Bioscience 1998;3:944–60.
115. Lindblom A, Rotstein S, Skoog L, Nordenskjöld M, Larsson C. Deletions on chromosome 16 in primary familial breast carcinomas are associated with development of distant metastases. Cancer Res 1993;53:3707–11.
116. Tsuda H, Callen DF, Fukutomi T, Nakamura Y, Hirohashi S. Allele loss on chromosome 16q24.2-qter occurs frequently in breast cancers irrespectively of differences in phenotype and extent of spread. Cancer Res 1994;54:513–7.

PART XIII

Endocrinology of the Breast

Editor: John C. Marshall

Chapter 161

Hormonal Control of Breast Development

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HYPOTHALAMIC-PITUITARY INFLUENCES, OVARIAN FUNCTION, AND BREAST DEVELOPMENT

In the nonpregnant female, the development of the mammary gland is rigorously controlled by the ovary. Although puberty is often considered to be the point of initiation of ovarian function, the development of the ovary, in fact, is a gradual process.[1] The complex regulatory mechanisms controlling ovarian function involve gonadotropin-releasing hormone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), the ovarian peptides inhibin, activin, and follistatin, as well as growth hormone (GH) and prolactin. The ovary is active during late fetal development and for a short time after birth but becomes relatively quiescent until the onset of puberty. At that time, ductal elongation and branching begin under the influence of estradiol as well as GH.[2,3] Although its exact mechanism of action is unclear, GH directly stimulates ductal growth in hypophysectomized-ovariectomized rats and might also act through its local mediator, insulin-like growth factor 1 (IGF-1). Normal duct development, however, requires the presence of estrogen and progesterone acting through the estrogen receptor (ER) and progesterone receptor (PR), which are present in the mammary gland. Estradiol acts locally to stimulate DNA synthesis and promote bud formation, probably through an ER-mediated effect. Prolactin plays an additional role, but its specific actions are not fully understood. 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 biologic characteristics of the gland.[2,3] The mammary gland, in turn, responds selectively to particular hormonal stimuli, depending on specific topographic differences in gland development, which modulate the expression of either cell proliferation or differentiation.

ADOLESCENCE

Although the main changes occurring in the mammary gland are initiated at puberty, development continues during adulthood and the final histomorphometry of the gland varies greatly from one woman to another.[2] Mammary gland development can be defined from the external appearance of the breast or by determination of the mammary gland area, volume, degree of branching, or level of differentiation of the gland (e.g., lobule type formation).[4]

The adolescent period begins with the first signs of sexual change at puberty and terminates with sexual maturity. Thelarche, defined as the initial clinical appearance of a breast bud, occurs at an average age of 11.2 (0.7 standard deviation [SD]) in white females and 1 year earlier in African-American females.[5,6] With the approach of puberty, the rudimentary mammae begin to show growth activity both in the glandular tissue and in the surrounding stroma.[2–4] Glandular increase is caused by the growth and division of small bundles of primary and secondary ducts (Fig. 161–1). These grow and divide in two ways. One involves repeated bifurcation of existing ducts, a process called "dichotomous" from the Greek word dichotomos or "to divide into two parts." The other involves localized buds on an expanding ductu-

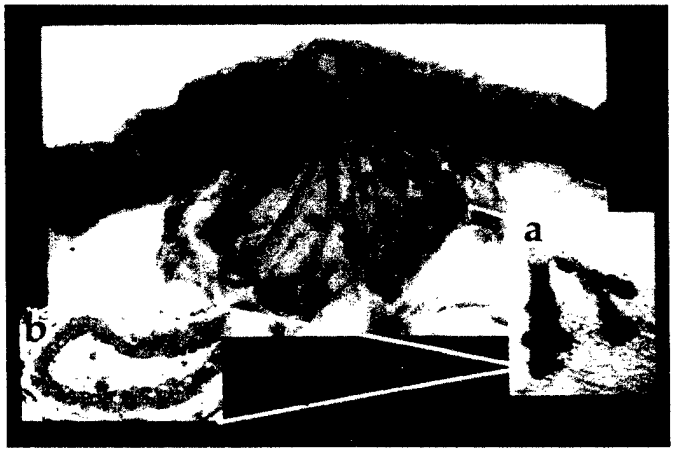

FIGURE 161–1. Mammary gland of the human female at birth formed by several excretory ducts, ending in terminal ducts: *a*, Detail of the *inset* showing the club-shaped terminal end bud from which lengthening and further divisions of the virginal ducts originate; *b*, cross-section at the level shown in "a"; the duct is lined by the two layers of cells. Proliferation takes place mainly in the basal cells. The inner cells have secretory properties from which the "witch milk" is formed. Toluidine blue ×25.

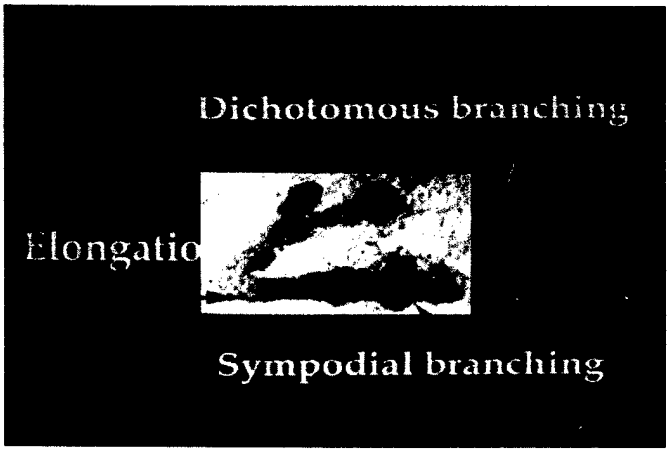

FIGURE 161–2. Before the onset of puberty in human females, the ducts grow and divide in a dichotomous and sympodial basis; a ball-shaped end bud sprouts from the duct, and new branches and twigs develop from the terminal and lateral end buds. Toluidine blue ×25.

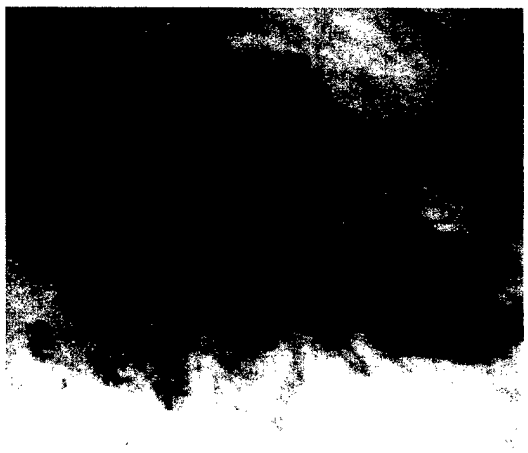

FIGURE 161–4. Whole mount preparation of human breast tissue of a 24-year-old nulliparous woman showing type 2 lobules. Toluidine blue ×25.

lar base, a process called *sympodial* from Greek *syn* plus *podion* base, (Fig. 161–2).

The ducts grow, divide, and form club-shaped terminal end buds. The structures give origin to new branches, twigs, and small ductules or alveolar buds (see Fig. 161–2). We have coined the term alveolar bud to identify those structures that are morphologically more developed than the terminal end bud but are yet more primitive than the terminal structure of the mature structure, which is called an acinus. Alveolar buds cluster around a terminal duct, forming the type 1 lobule or virginal lobule (Fig. 161–3). Each cluster consists of approximately 11 alveolar buds. Lobule formation in the female breast occurs within 1 to 2 years after onset of the first menstrual period. Full differentiation of the mammary gland is a gradual process that takes many years and, in some cases, if pregnancy does not supervene, is never attained.

THE MATURE BREAST

Study of normal breast tissue in adult women identifies two additional and more mature types of lobule, designated types 2 and 3 (Figs. 161–4 and 161–5). The transition from type 1 to the more mature types 2 and 3 represents a gradual process with sprouting of an increased number of new alveolar buds. In type 2 and 3 lobules, these are now called ductules; they increase in number from approximately 11 in the type 1 lobule to 47 in the type 2 lobule and 80 in the type 3 lobule, respectively (Fig. 161–6 and Table 161–1). The increase in number results in a concomitant increase in size of the lobules and a reduction in size of each individual structure.[2, 7]

NULLIPAROUS WOMEN. In these subjects, breast tissue contains more undifferentiated structures (e.g., terminal ducts and type 1 lobules), although occasionally type 2 and 3 lobules are seen. This pattern remains constant throughout the reproductive years unless pregnancy ensues. Type 2 lobules are present in moderate numbers during the early reproductive years but sharply decrease after the age of 23, whereas the number of type 1 lobules remains significantly higher. This observation suggests that a certain percentage of type 1 lobules might have progressed to type 2 lobules, but the number of type 2 lobules progressing to type 3 is significantly lower than in parous women.

PAROUS WOMEN. In this group, the predominant structure is the most differentiated lobule (i.e., type 3). The number of type 3 structures peaks during the early reproductive years and decreases after the fourth decade of life. A history of parity between the ages of 14 and 20 years correlates with a significant increase in the number of type 3 lobules that remain present as the predominant structure

FIGURE 161–3. Whole mount preparation of breast tissue of an 18-year-old nulliparous woman showing type 1 lobules. Toluidine blue ×25.

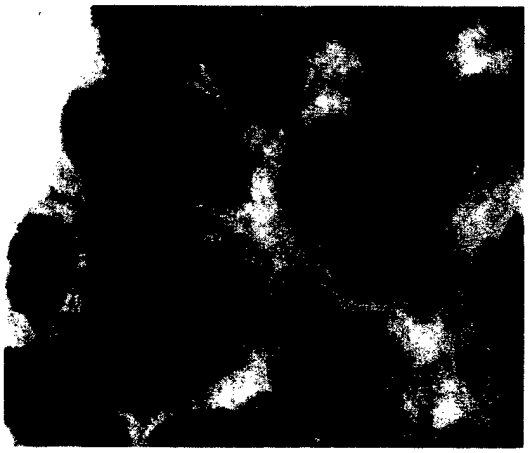

FIGURE 161–5. Whole mount preparation of human breast tissue of a 35-year-old parous woman containing type 3 lobules. Toluidine blue ×25.

FIGURE 161–6. Lobule types 1, 2, 3, and 4, which were taken at the same magnification (×2.5) and stained with hematoxylin and eosin (H&E).

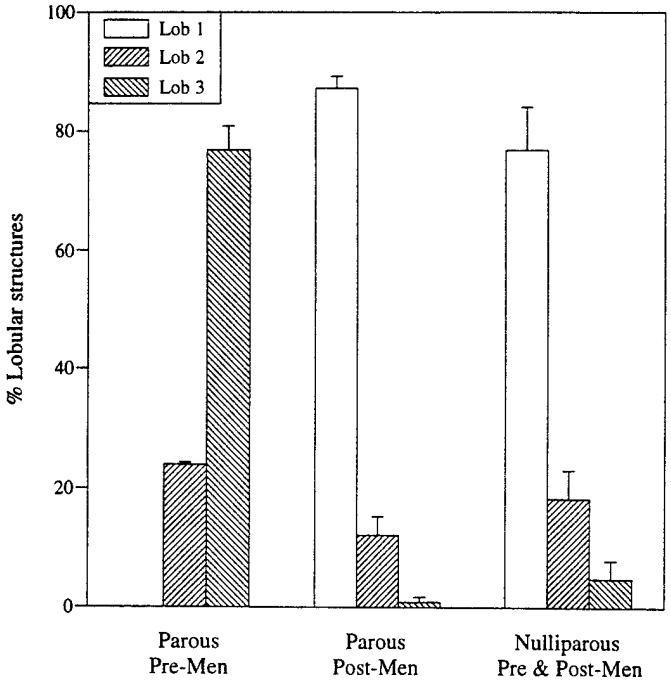

FIGURE 161–7. Percentage of lobule type structures in the breast of premenopausal (Pre-Men) and postmenopausal (Post-Men) parous women and also premenopausal and postmenopausal (Pre & Post-Men) nulliparous women. Type 1 (Lob 1), type 2 (Lob 2), and type 3 (Lob 3) lobules.

until the age of 40. At that time, the number of type 3 lobules decreases, probably owing to their involution to predominantly lobules type 1 (Fig. 161–7).[8]

PATTERN OF DISTRIBUTION OF CELLS POSITIVE FOR ESTROGEN RECEPTOR AND PROGESTERONE RECEPTOR IN RELATION TO PROLIFERATING CELLS IN THE MAMMARY GLAND[1]

Even though the breast is influenced by a myriad of hormones and growth factors,[9–16] estrogens are considered to play a major role in promoting the proliferation of both the normal and neoplastic breast epithelium.[17–23] Estrogens could influence the proliferative activity of mammary epithelial cells by at least three different mechanisms: (1) direct receptor-mediated[18, 24–31] stimulation, (2) indirect autocrine/paracrine loops,[21, 22] or (3) interruption of negative feedback factors (i.e., the effect of estrogen to remove one or more inhibitory factors present in the serum).[20, 32] Unfortunately, none of these mechanisms has been precisely defined with regard to their role in the normal development and differentiation of the breast or the initiation and progression of the neoplastic process.

A greater degree of complexity emerged with the cloning of a gene encoding a second type of ER, the ERβ. This receptor is present in the mouse, rat, and human and has an affinity for estradiol similar to that of the classical ER (now identified as ERα). In addition, it has now become possible to localize modulators of estrogen and progesterone receptor function in tissue. ER- and PR-mediated transcription often involves binding of coactivators to the transcription complex, a mechanism that enhances receptor function. One of these coactivators, SRC-1, has been cloned and can be localized with specific antibodies. Knockout of SRC-1 causes decreased growth and development of the uterus, prostate, testis, and mammary gland.[33] Examination of its localization in mammary tissue can provide insight into the local regulation of ER functionality.[34] These new findings have prompted a re-evaluation of the estrogen signaling system in mammary tissue.[35–37]

Cell proliferation is indispensable for the normal growth and development of the breast. 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 essential role of ERα and PR in mammary gland development has been confirmed by knockout mice lacking functional receptors. ERα knockout mice display grossly impaired ductal epithelial proliferation and branching. PR knockout mice display significant ductal development but decreased arborization and absence of alveolar differentiation.[38, 39]

Clinical studies have defined the relationship of cell proliferation to estrogen and progesterone production by correlation with phases of the menstrual cycle in women. The breast epithelium of sexually mature, normally cycling women does not exhibit maximal proliferation during the follicular phase of the menstrual cycle[13–17, 40–45] when estrogens reach peak levels of 200 to 300 pg/mL and progesterone is less than 1 ng/mL.[46] Maximal proliferative activity occurs during the luteal phase, when progesterone reaches levels of 10 to 20 ng/mL and estrogen levels are two- to three-fold lower than during the follicular phase.[46] In breast cells grown in vitro, or when breast tissues are implanted in athymic nude mice, however, estrogens alone stimulate cell proliferation and progesterone has no effect or even inhibits cell growth.[21, 22, 44, 45]

Our studies of the proliferative activity of the mammary epithelium in both rodents and humans have demonstrated that cell division varies with the degree of differentiation of the mammary parenchyma.[9–12, 17, 47–49] In women, the highest level of cell proliferation is observed in the undifferentiated type 1 lobules (Lob1), which are present in the breasts of young nulliparous subjects.[9–12, 17] The progressive differentiation of type 1 lobules into types 2 and 3 results in a concomitant

TABLE 161–1. Characteristics of the Lobular Structures of the Human Breast

Structure	Lobular Area* (μm²)	No. of Ductules/ Lobule†	No. of Cells/ Cross-Section‡
Lob 1	48 ± 44	11.2 ± 6.3	32.4 ± 14.1
Lob 2	60 ± 26	47 ± 11.7	13.1 ± 4.8
Lob 3	129 ± 49	81 ± 16.6	11 ± 2

*Student's t-tests were done for all possible comparisons. Lobular areas showed significant differences between lob 1 versus lob 3 and lob 2 versus lob 3 ($P < .005$).

†The number of ductules per lobule was different ($P < .01$) in all the comparisons.

‡The number of cells per cross-section was significantly different in ductules of lob 1 versus lob 2 and lob 3 ($P < .01$).

Reprinted from Russo J, Rivera R, Russo IH: Influence of age and parity on the development of the human breast. Breast Cancer Res Treat 23:211–218, 1992.

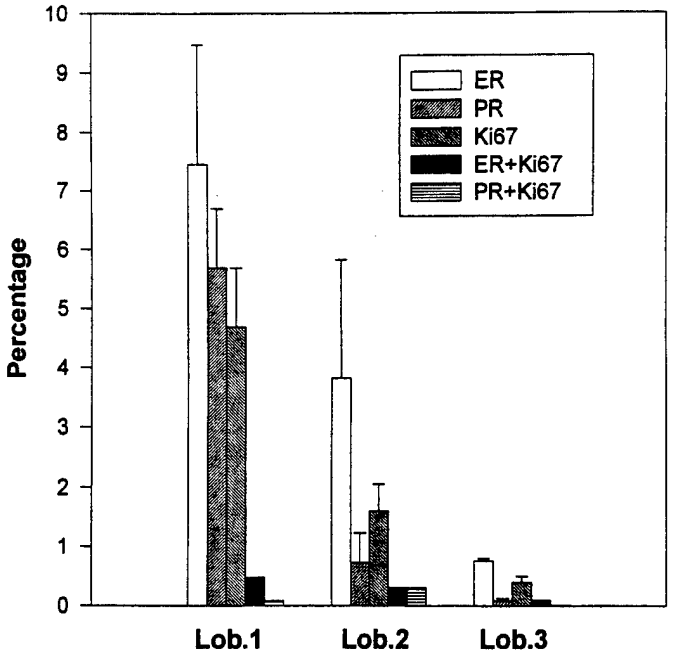

FIGURE 161–8. Percentage of cells positive for estrogen receptor (ER), progesterone receptor (PR), Ki67, and of cells positive for both ER and Ki67 (ER + Ki67), or PR and Ki67 (PR + Ki67) (ordinate). Cells were quantitated in type 1 (Lob 1), type 2 (Lob 2), and type 3 (Lob 3) lobules of the breast (abscissa).

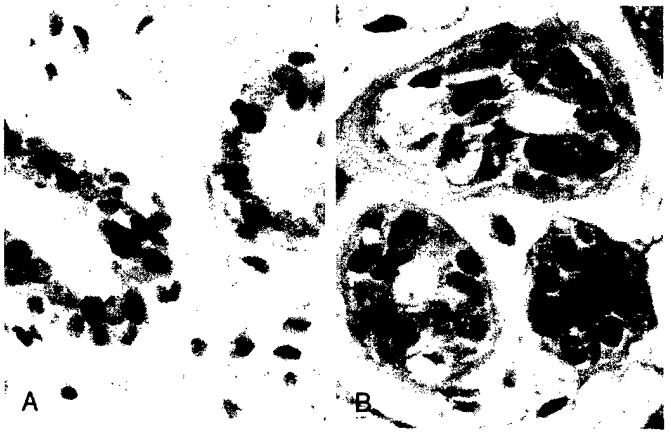

FIGURE 161–9. *A* and *B*, Lob 1 ductules of the human breast. *A*, The single-layered epithelium lining the ductule contains Ki67-positive cells (brown nuclei) and ER-positive cells (red-purple nuclei) (×40); *B*, The single-layered epithelium lining the ductule contains brown Ki67-positive cells and red-purple PR-positive cells. Sections were stained with DAB/alkaline phosphatase-vector red, with light hematoxylin counterstain and photographed at ×40.

reduction of the proliferative activity of the mammary epithelium.[8–12] Additional differentiation into type 4 lobules, characteristic of breast tissue during pregnancy, further lowers the rate of proliferation.

Understanding of the relationship between lobular differentiation, cell proliferation, and hormone responsiveness of the mammary epithelium is just beginning to be unraveled. An important finding is that ERα and progesterone receptor (PR) content in the lobular structures of the breast are directly proportional to the rate of cell proliferation. Proliferation and receptor content are maximal in the undifferentiated type 1 lobule and decrease progressively in types 2, 3, and 4.

DISTRIBUTION OF PROLIFERATING CELLS IN RELATION TO THE PRESENCE OF STEROID RECEPTORS AND THEIR COACTIVATORS

Use of Ki67 immunohistochemical techniques allows detection of proliferating cells in tissue. The highest percentage of positive cells occurs in type 1 lobules (Fig. 161–8 and Table 161–2); a three-fold lower percentage is found in type 2 lobules and a 10-fold decrement is found in type 3 (Fig. 161–9A, B; see also Fig. 161–8 and Table 161–2). The proliferating cells are found almost exclusively in the epithelium lining ducts and lobules, whereas only occasional positive cells are found in the myoepithelium or in the intralobular and interlobular stroma. The same pattern of reactivity is observed in tissue sections incubated with the ER and PR antibodies. Positive cells are found exclusively in the epithelium with type 1 lobules that contain the highest number of positive cells. Their number decreases progressively in type 2 and 3 lobules (see Fig. 161–8 and Table 161–2).

Use of double-staining procedures for Ki67 and ER or PR has allowed quantitation in the same tissue sections of the spatial relationship between proliferating and receptor positive cells. This can be done with techniques using two colors or by immunofluorescent methodology. The percentage of ER and PR positive cells in type 1 lobules does not differ significantly, 7.5% and 5.7%, respectively (see Table 161–2). In type 2 lobules, the percentage of ER- and PR-positive cells is reduced to 3.8% and 0.7%, respectively; and in type 3, their numbers become negligible (see Table 161–2). There are similarities in the relative percentages of Ki67, ER, and PR-positive cells and in the progressive reduction in the percentage of positive cells as the lobular differentiation progressed. However, cells that are positive for Ki67 are not the same that reacted positively for ER or PR (Fig. 161–9A, B; see also Table 161–2). Very few cells (<0.5% in type 1 lobules) and even fewer in type 2 and 3 cells appear positive for both Ki67 and ER (Ki67 plus ER) (see Table 161–2). Double reactivity with this technique is identified by the darker staining of the nuclei, which appear dark purple-brown. The percentage of cells exhibiting double labeling with Ki67 and PR antibodies (Ki67 plus PR) in type 1 lobules is lower than the percentage of double-labeled ER-positive cells (see Table 161–2).

The content of ER and PR in the normal breast tissue, as detected immunocytochemically, varies with the degree of lobular development

TABLE 161–2. Distribution of Ki67, ER, and PR Positive Cells in the Lobular Structures of the Human Breast

Lobule Type	No. of Cells	Ki67	ER	PR	Ki67 + ER	Ki67 + PgR
Lob1	19,339[a]	4.72 ± 1[d,e]	7.46 ± 2.88[h]	5.7 ± 1.36[k]	0.48 ± 0.28[n]	0.09 ± 0.01[o]
Lob2	8490[b]	1.58 ± 0.45[f]	3.83 ± 2.44[i]	0.73 ± 0.57[l]	0.31 ± 0.21	0.28 ± 0.27
Lob3	17,750[c]	0.4 ± 0.18[g]	0.76 ± 0.04[j]	0.09 ± 0.04[m]	0.01 ± 0.01	0.01 ± 0.01

[a]Total number of cells counted in type 1 (Lob1) lobules in the breast tissue samples of 12 donors.
[b]Total number of cells counted in type 2 (Lob2) lobules in the breast tissue samples of five donors.
[c]Total number of cells counted in type 3 (Lob3) lobules in the breast tissue samples of three donors.
[d]Proliferative activity determined by the percentage of Ki67-positive cells, expressed as the mean ± standard deviation. Differences were significant in Lob 1 versus [f]lob 2 ($t = 1.98$, $P < .05$), [f]Lob 2 versus [g]Lob3 ($t = 2.27$, $P < .04$), and [e]Lob 1 versus [g]Lob ($t = 2.56$, $P < .01$).
ER-positive cells were significantly different in [h]Lob 1 versus [j]Lob 3 ($t = 2.04$, $P < .05$)
PR-positive cells were significantly different in [k]Lob 1 versus [l]Lob 2 ($t = 2.27$, $P < .05$), and [k]Lob 1 versus [m]Lob 3 [$t = 2.60$, $P < .03$].
[n]Percentage of cells positive for both Ki67 and ER, expressed as the mean ± standard deviation.
[o]Percentage of cells positive for both Ki67 and PR, expressed as the mean ± standard deviation.

in a linear relationship with the rate of cell proliferation of the same structures. The utilization of a double-labeling immunocytochemical technique has allowed the determination whether the receptor positive cell population is the same population that is proliferating (i.e., Ki67-positive cells). Clearly, this was not the case as was also reported by other authors.[43] The findings that proliferating cells differ from those that are ER- and PR-positive support data, which indicate that estrogen controls cell proliferation by an indirect mechanism. A possible explanation is that ER-positive cells respond to estrogen with an increase in growth factor production, which acts in a paracrine fashion on neighboring ER-negative cells. This paracrine phenomenon has also been demonstrated using supernatants of estrogen-treated ER-positive cells that stimulate the growth of ER-negative cell lines in culture and in vivo in nude mice bearing ER-negative breast tumor xenografts.[50, 51] A paracrine mechanism can also explain inhibition of cellular proliferation. ER-positive cells treated with antiestrogens secrete transforming growth factor-α (TGF-α), which inhibits the proliferation of ER-negative cells.[28]

The proliferative activity and the percentage of ER- and PR-positive cells are highest in type 1 lobules in comparison with types 2 to 4, which consist of the normal breast. These findings provide a mechanistic explanation for the higher susceptibility of these structures to be transformed by chemical carcinogens in vitro[52, 53] and also support the observations that type 1 lobules are the site of origin of ductal carcinomas.[54] However, the relationship between ER-positive and ER-negative breast cancers is unclear.[55–57] It has been suggested that either ER-negative breast cancers result from the loss of the ability of the cells to synthesize ER during clinical evolution of ER-positive cancers or that ER-positive and ER-negative cancers are different entities.[55, 57] It is postulated that type 1 lobules contain at least three cell types—ER-positive cells that do not proliferate, ER-negative cells that are capable of proliferating, and a small proportion of ER-positive cells that can also proliferate (Fig. 161–10). Therefore, estrogen might stimulate ER-positive cells to produce a growth factor, which in turn stimulates neighboring ER-negative cells capable of proliferating (see Fig. 161–10). In the same fashion, the small number of cells that are ER-positive and can proliferate could be the source of ER-positive tumors. The possibility also exists that the ER-negative cells convert to ER positive cells. The conversion of ER-negative cells to ER-positive cells has been reported.[58, 59] The newly discovered ERβ opens the possibility that those cells traditionally considered to be ERα negative might be ERβ positive.[35, 36] It has been found that ER is expressed during the immortalization and transformation of ER-negative human breast epithelial cells, supporting the hypothesis of conversion from a negative to a positive receptor cell.[60]

The findings that proliferating cells in the human breast differ from those that contain steroid hormone receptors explain a great extent of the in vitro data.[61–64] Of interest are a series of observations regarding ER and cell proliferation. ER-positive MCF-7 cells respond to estrogen treatment with increased cell proliferation. Enhanced expression of the receptor by transfection also increases the proliferative response to estrogen.[61, 65] However, when ER-negative cells such as MDA-MB468 and others are transfected with the ER, estrogen now inhibits cell growth.[62–66] Although the negative effect of estrogen on those ER-negative cells transfected with the receptor has been interpreted as an interference of the transcription factor used to maintain estrogen independent growth,[65] there is no definitive explanation for their lack of survival. However, it can be explained by the finding that proliferating and ER-positive cells are two separate populations. Further support is the finding that when type 1 lobules of normal breast tissue are placed in culture, they lose the ER-positive cells, indicating that only proliferating cells that are also ER negative can survive and that these constitute the stem cells.[66, 67]

Until recently, it was believed that estrogens acted through a single nuclear estrogen receptor that transcriptionally activated specific target genes. However, there is increasing evidence that a membrane receptor combined with alternative second-messenger signaling mechanisms[68, 69] are also 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. Although more studies need to be done in this direction, it is clear that paracrine mechanisms are important in mediating cellular proliferation. In the normal breast, the proliferating and steroid hormone receptor–positive cells are different. This finding opens new possibilities for clarifying the mechanisms through which estrogens might act on the proliferating cells to initiate the cascade of events leading to cancer.

An additional complexity arises upon examination of the cell specific localization of steroid receptor coactivators in mammary tissue. Steroid receptor coactivators (e.g., SRC-1) enhance the transcriptional capabilities of both the ER and PR. Our studies demonstrate that SRC-1 is segregated in distinct subsets of cells within the epithelium of the estrogen-responsive rat mammary gland. This finding is in contrast to findings in the stroma, where significant numbers of cells coexpressed both ERα and SRC-1 (Fig. 161–11). These findings highlight the need to further explore whether stromal-epithelial interactions might be important in the human breast.[70–74] Prior studies have shown that in the uterus and prostate, receptors in stroma tissue are necessary for epithelial proliferation. In those organs, growth factors made by stroma in response to estrogens and androgens regulate the proliferation of

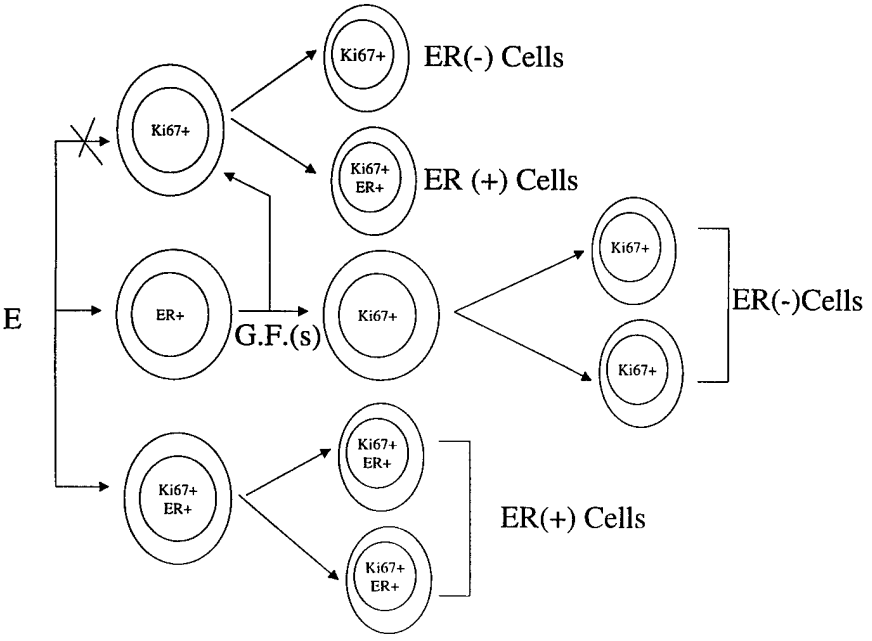

FIGURE 161–10. Schematic representation of the postulated pathways of estrogen actions on breast epithelial cells. Cells expressing three different phenotypes might be present in the epithelium: Estrogen receptor (ER)—negative Ki67-positive cells that are capable of proliferating, ER-positive cells that do not proliferate, and a small proportion of Er- and Ki67-positive cells. Estrogen might stimulate ER-positive cells to produce a growth factor that in turn stimulates neighboring ER-negative cells capable of proliferating. ER- and Ki67-positive cells can proliferate and could be stimulated by estrogen to originate ER-positive daughter cells or probably tumors. Er-negative cells may convert to ER-positive cells during neoplastic transformation.

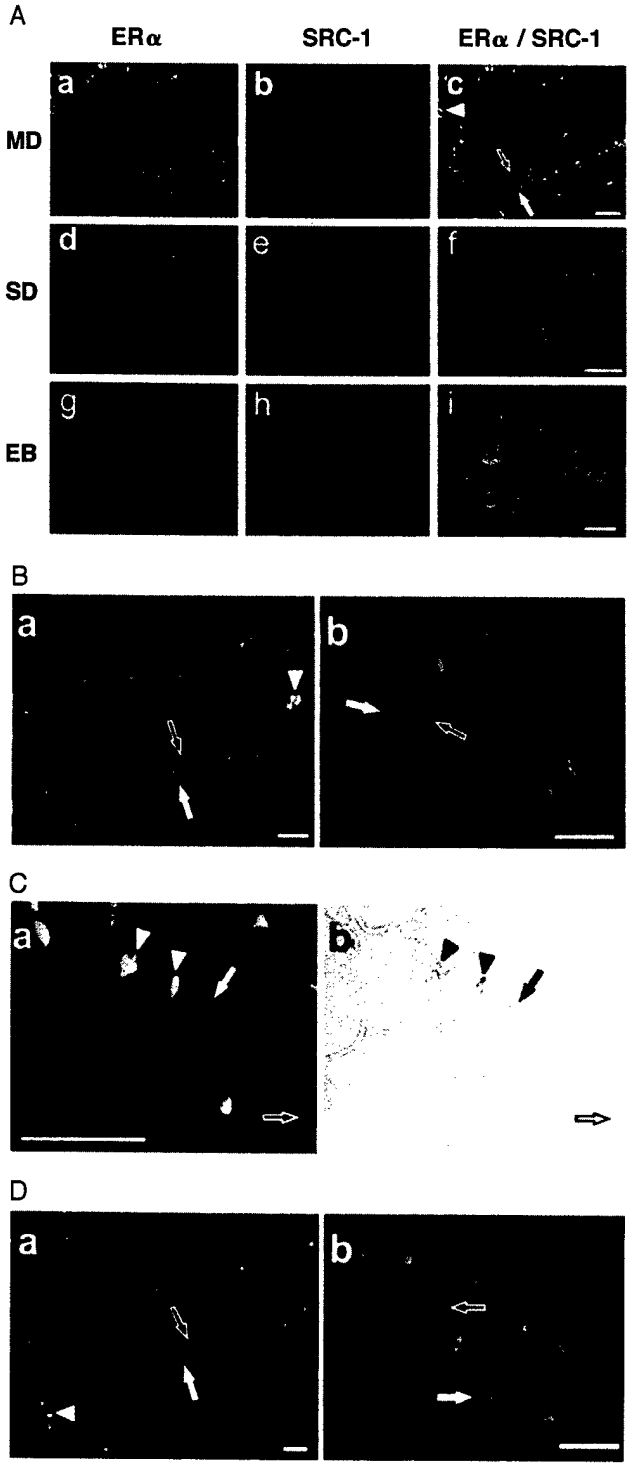

FIGURE 161–11. Segregation of SRC-1 expression from ER α-positive cells as illustrated by dual fluorescence labeling of SRC-1 and ERα. Mammary glands (A) from a 3-week-old virgin female rat were stained simultaneously for SRC-1 (b, e, and h; red) and ERα (a, d, and g; green). Green and red images were superimposed (c, f, and i). The main duct (MD), small duct (SD), and end bud (EB) are shown. The discrete distribution pattern (B) of ERα (green) and SRC-1 (red) was confirmed with the combination of two different antibodies (a and b). Stroma (C) expressing ERα alone (green), SRC-1 alone (red) or both (yellow). The phase contrast image from *a* is shown in *b*. Staining (D) from a 10-week-old virgin female rat mammary gland also demonstrated the segregation of SRC-1 from ERα in epithelial cells. *Solid arrow,* Cells expressing only ERα; *open arrow,* cells expressing only SRC-1; *solid arrowhead,* cells expressing both ERα and SRC-1. (*Bar* = 100 μm.)

epithelial cells. Although the presence of estrogen receptors in the stroma of human mammary tissue has not been established, further studies are required to fully understand potential stromal-epithelial interactions in the breast. This is particularly pertinent because of the demonstration that stromal tissue can synthesize estrogen through the aromatase enzyme in the human breast. The estrogen produced locally in stroma may then act in a paracrine fashion on surrounding epithelial cells.[70–74]

THE MENOPAUSAL BREAST

The menopause occurs at an average age of 51, when the ovaries stop synthesizing estradiol and amenorrhea ensues. The years that lead up to the final menstrual period constitute the perimenopause.[9] Many women ovulate irregularly during the perimenopausal transition. After menopause, the breast regresses both in nulliparous and parous women. This involutionary process is manifested by an increase in the number of type 1 lobules and a concomitant decline in type 2 and type 3 lobules. At the end of the fifth decade of life, the breasts of both nulliparous and parous women contain predominantly type 1 lobules (see Fig. 161–7). These observations led us to conclude that the understanding of breast development requires a horizontal study in which all the different phases of growth are taken into consideration. For example, the analysis of breast structures at a single given point (i.e., 50 years of age) might lead one to conclude that the breasts of both nulliparous and parous women are identical (see Fig. 161–7). However, the phenomena occurring in previous years might have

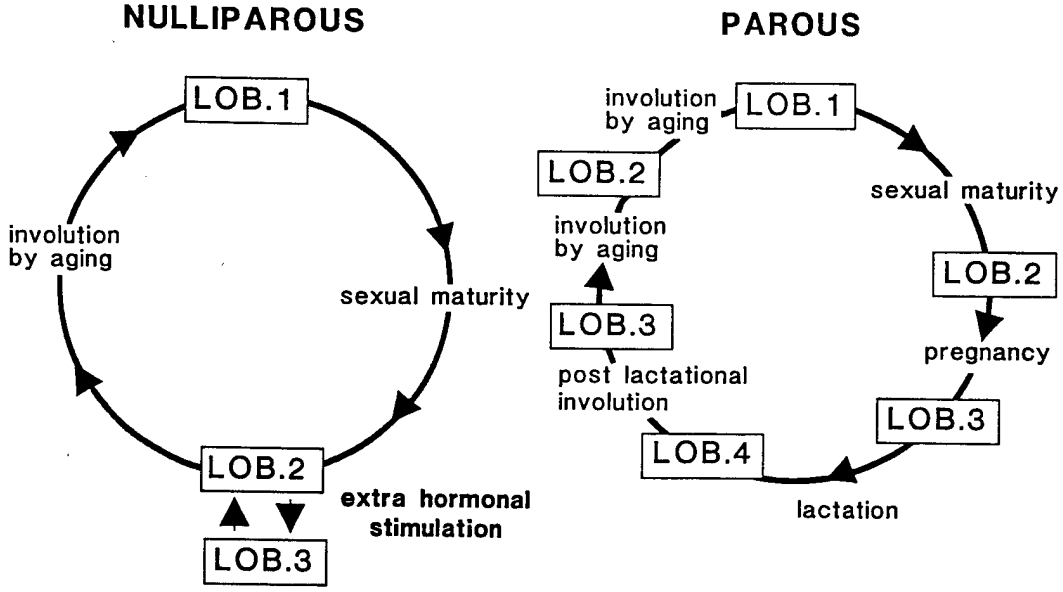

FIGURE 161–12. Schematic representation of breast development based on the relative percentage of lobules present. Nulliparous women's breasts contain primarily type 1 (Lob 1) lobules with some progression to type 2 (Lob 2) and only minimal formation of type 3 (Lob 3) lobules. Parous women undergo a complete cycle of development through the formation of type 4 (Lob 4) lobules, which later regress. (From Russo J, Rivera R, Russo IH: Influence of age and parity on the development of the human breast. Breast Cancer Res Treat 23:211–218, 1992.)

imprinted permanent changes on breast biology. This would affect the potential of the breast for neoplasm but would be no longer morphologically observable. Thus, from a quantitative point of view, the regressive phenomenon occurring in the breast at menopause differs in nulliparous and parous women. It should be recalled that in nulliparous breasts, the predominant structure is the type 1 lobule, which comprises 65% to 80% of the total lobule type components, a percentage independent of age. Type 2 lobules represent 10% to 35% and type 3, only 0% to 5% of the total lobular population. In breasts of premenopausal parous women, on the other hand, the predominant lobular structure is the type 3 lobule, which comprises 70% to 90% of the total lobule component. Only after menopause do the type 3 lobules decline in number, and the relative proportion of the three lobule types present approaches that observed in nulliparous women. These observations led us to conclude that early parous women truly underwent lobule differentiation, which was evident at a younger age, whereas nulliparous women seldom reached the lobule type 3 stage and never reached the lobule type 4 stage (Fig. 161–12; see also Fig. 161–6).

REFERENCES

1. Edwards RG, Howles CM, Macnamee C: Clinical endocrinology of reproduction. *In* Baulieu E-E, Kelly PA (eds): Hormones: From Molecules to Disease. New York and London, Chapman & Hall, 1990, pp 457–476.
2. Russo J, Russo IH: Development of human mammary gland. *In* Neville MC, Daniel CW (eds): The Mammary Gland: Development, Regulation, and Function. New York, Plenum Pub Corp, 1987, pp 67–93.
3. Dabelow A: Die Milchdruse. *In* Bargmann W (ed): Handbuch der Mikroskopischen Anatomic des Menchen, vol 3. Part 3: Haut and Sinnes Organs. Berlin, Springer-Verlag, 1957, pp 277–485.
4. Tanner JM (ed): The development of the reproductive system. *In* Growth at Adolescence. Oxford, Blackwell Scientific, 1962, pp 28–39.
5. Roche AF, Wellens R, Attie KM, Siervogel RM: The timing of sexual maturation in a group of US white youths. J Pediatr Endocrinol Metab 8:11–18, 1995.
6. Herman-Giddens ME, Slora EJ, Wasserman RC, et al: Secondary sexual characteristics and menses in young girls seen in office practice: A study from the Pediatric Research in Office Settings Network. Pediatrics 99:505–512, 1997.
7. Russo J, Tay LK, Russo IH: Differentiation of the mammary gland and susceptibility to carcinogenesis. Breast Cancer Res Treat 2:5–73, 1982.
8. Russo J, Rivera R, Russo IH: Influence of age and parity on the development of the human breast. Breast Cancer Res Treat 23:211–218, 1992.
9. Russo J, Russo IH: Role of hormones in human breast development: The menopausal breast. *In* Birkhäusen MH (ed): Progress in the Management of Menopause. London, Parthenon Publishing, 1997, pp 184–193.
10. Russo IH, Russo J: Role of hormones in cancer initiation and progression. J Mammary Gland Biol Neoplasia 3:49–61, 1998.
11. Russo J, Russo IH: Role of differentiation in the pathogenesis and prevention of breast cancer. Endocr Rel Cancer 4:7–21, 1997.
12. Calaf G, Alvarado ME, Bonney GE, et al: Influence of lobular development on breast epithelial cell proliferation and steroid hormone receptor content. Int J Oncol 7:1285–1288, 1995.
13. Lippman ME, Dickson RB, Gelmann EP, et al: Growth regulation of human breast carcinoma occurs through regulated growth factor secretion. J Cell Biochem 35:1–16, 1987.
14. Meyer JS: Cell proliferation in normal human breast ducts, fibroadenomas, and other duct hyperplasias, measured by nuclear labeling with tritiated thymidine. Hum Pathol 8:67–81, 1977.
15. Masters JRW, Drife JO, Scarisbrick JJ: Cyclic variations of DNA synthesis in human breast epithelium. J Natl Cancer Inst 58:1263–1265, 1977.
16. Ferguson DJP, Anderson TJ: Morphologic evaluation of cell turnover in relation to the menstrual cycle in the "resting" human breast. Br J Cancer 44:177–181, 1981.
17. Russo J, Russo IH: Estrogens and cell proliferation in the human breast. J Cardiovasc Pharmacol 28:19–23, 1996.
18. Kumar V, Stack GS, Berry M, et al: Functional domains of the human estrogen receptor. Cell 51:941–951, 1987.
19. King RJB: Effects of steroid hormones and related compounds on gene transcription. Clin Endocrinol 36:1–14, 1992.
20. Soto AM, Sonnenschein C: Cell proliferation of estrogen-sensitive cells: The case for negative control. Endocr Rev 48:52–58, 1987.
21. Huseby RA, Maloney TM, McGrath CM: Evidence for a direct growth-stimulating effect of estradiol on human-MCF-7 cells in vitro. Cancer Res 144:2654–2659, 1987.
22. Huff KK, Knabbe C, Lindsey R, et al: Multihormonal regulation of insulin-like growth factor-1-related protein in MCF-7 human breast cancer cells. Mol Endocrinol 2:200–208, 1988.
23. Dickson RB, Huff KK, Spencer EM, Lippman ME: Introduction of epidermal growth factor related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. Endocrinology 118:138–142, 1986.
24. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y: Proliferation, hormonal responsiveness and estrogen receptor content of MCF-7 human breast cancer cells growth in the short-term and long-term absence of estrogens. Cancer Res 47:4355–4360, 1987.
25. Petersen OW, Hoyer PE, van Deurs B: Frequency and distribution of estrogen receptor positive cells in normal, nonlactating human breast tissue. Cancer Res 47:5748–5751, 1987.
26. Jacquemier JD, Hassouin J, Torente M, Martin PM: Distribution of estrogen and progesterone receptors in healthy tissue adjacent to breast lesions at various stages: immunohistochemical study of 107 cases. Breast Cancer Res Treat 15:109–117, 1990.
27. McGuire W, Carbone P, Vollmer R (eds): Estrogen Receptors in Human Breast Cancer. New York, Raven Press, 1975.
28. Dickson R, Lippman M: Control of human breast cancer by estrogen, growth factors and oncogenes. *In* Lipman ME, Dickson RB (eds): Estrogen Receptors in Human Breast Cancer. New York, Raven Press, 1975.
29. Wittliff IL: Steroid-hormone receptors in breast cancer. Cancer 53:630–643, 1984.
30. Watts CKW, Handel ML, King RJB, Sutherland RL: Oestrogen receptor gene structure and function in breast cancer. J Steroid Biochem Mol Biol 41:5293–5536, 1992.
31. Aakvaag A, Utaacker E, Thorsen T, et al: Growth control of human mammary cancer cells (MCF-7 cells) in culture: Effect of estradiol and growth factors in serum containing medium. Cancer Res 50:7806–7810, 1990.
32. Dell'aquilla ML, Pigott DA, Bonaquist DL, Gaffney EV: A factor from plasma derived human serum that inhibits the growth of the mammary cell line MCF-7: Characterization and purification. J Natl Cancer Inst 72:291–298, 1984.
33. Xu J, Qiu Y, DeMayo FJ, et al: Partial hormone resistance in mice with disruption of the steroid receptor co-activator-1 (SRC-1) gene. Science 279:1922–1925, 1998.
34. Shim W-S, DiRenzo J, DeCaprio JA, et al: Segregation of steroid receptor coactivator-1 from steroid receptors in mammary epithelium. Proc Natl Acad Sci U S A 96:208–213, 1999.
35. Kuiper GGJM, Enmark E, Pelto-Huikko M, et al: Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93:5925–5930, 1996.
36. Byers M, Kuiper GGJM, Gustaffson J-ÅA, Park-Sarge OK: Estrogen receptor beta

mRNA expression in rat ovary: Down-regulation by gonadotropins. Mol Endocrinol 11:172–182, 1997.
37. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lupu R: Expression of estrogen receptor beta messenger RNA variant in human breast cancer. Cancer Res 58:210–214, 1998.
38. Lubahn DB, Moyer JS, Golding TS, et al: Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Soc Natl Acad Sci U S A 90:11162–11166, 1993.
39. Bocchinfuso WP, Korach KS: Mammary gland development and tumorigenesis in estrogen receptor knockout mice. J Mammary Gland Biol Neoplasia 2:323–334, 1997.
40. Longacre TA, Bartow SA: A correlative morphologic study of human breast and endometrium in the menstrual cycle. Am J Surg Pathol 10:382–393, 1986.
41. Going JJ, Anderson TJ, Battersby S: Proliferative and secretory activity in human breast during natural and artificial menstrual cycles. Am J Pathol 130:193–204, 1988.
42. Potten CS, Watson RJ, Williams GT: The effect of age and menstrual cycle upon proliferative activity of the normal human breast. Br J Cancer 58:163–170, 1988.
43. Clark RB, Howell A, Potter CS, Anderson E: Dissociation between steroid receptors expression and cell proliferation in the human breast. Cancer Res 57:4987–4991, 1997.
44. Laidlaw IJ, Clark RB, Howell A, et al: Estrogen and progesterone stimulate proliferation of normal human breast tissue implanted in athymic nude mice. Endocrinology 136:164–171, 1995.
45. Clarke RB, Howell A, Anderson E: Estrogen sensitivity of normal human breast tissue in vivo and implanted into athymic nude mice: Analysis of the relationship between estrogen-induced proliferation and progesterone receptor expression. Breast Cancer Res Treat 45:121–183, 1997.
46. Goodman HM: Basic Medical Endocrinology. New York, Raven Press, 1994, pp 288–290.
47. Russo J, Russo IH: Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. Cancer Res 40:2677–2687, 1980.
48. Russo J, Romero AL, Russo IH: Architectural pattern of the normal and cancerous breast under the influence of parity. J Cancer Epidemiol Biomarkers Prevent 3:219–224, 1994.
49. Russo J, Russo IH: Biology of disease: Biological and molecular bases of mammary carcinogenesis. Lab Invest 57:112–137, 1987.
50. Clarke R, Dickson RB, Lippman ME: Hormonal aspects of breast cancer: Growth factors, drugs and stromal interactions. Crit Rev Oncol Hematol 12:1–23, 1992.
51. Knabbe C, Lippman ME, Wakefield LM, et al: Evidence that transforming growth factor β is a hormonally regulated negative growth factor in human breast cancer cells. Cells 48:417–428, 1987.
52. Russo J, Reina D, Frederick J, Russo IH: Expression of phenotypical changes by human breast epithelial cells treated with carcinogens in vitro. Cancer Res 48:2837–2857, 1988.
53. Russo J, Calaf G, Russo IH: A critical approach to the malignant transformation of human breast epithelial cells. Crit Rev Oncog 4:403–417, 1993.
54. Russo J, Gusterson BA, Rogers A, et al: Biology of the disease: Comparative study of human and rat mammary tumorigenesis. Lab Invest 62:244–278, 1990.
55. Habel LA, Stamford JL: Hormone receptors and breast cancer. Epidemiol Rev 15:209–219, 1993.
56. Harlan LC, Coates RJ, Block G: Estrogen receptor status and dieting intakes in breast cancer patients. Epidemiology 4:25–31, 1993.
57. Moolgavkar SH, Day NE, Stevens RG: Two-stage model for carcinogenesis: Epidemiology of breast cancer in females. J Natl Cancer Inst 65:559–569, 1980.
58. Kodama F, Green GL, Salmon SE: Relation of estrogen receptor expression to clonal growth and antiestrogen effects on human breast cancer cells. Cancer Res 45:2720–2724, 1985.
59. Podhajcer OL, Bravo AL, Sorin I, et al: Determination of DNA synthesis, estrogen receptors, and carcinoembryonic antigen in isolated cellular subpopulations of human breast cancer. Cancer 58:720–729, 1986.
60. Hu YF, Lau KM, Ho SM, Russo J: Increased expression of estrogen receptor beta in chemically transformed human breast epithelial cells. Int J Oncol 12:1225–1228, 1998.
61. Foster JS, Wimalasena J: Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells. Mol Endocrinol 10:488–498, 1996.
62. Wang W, Smith R, Burghardt R, Safe SH: 17β Estradiol-mediated growth inhibition of MDA-MB 468 cells stably transfected with the estrogen receptor: Cell cycle effects. Mol Cell Endocrinol 133:49–62, 1997.
63. Levenson AS, Jordan VC: Transfection of human estrogen receptor (ER) cDNA into ER negative mammalian cell lines. J Steroid Biochem Mol Biol 51:229–239, 1994.
64. Weisz A, Bresciani F: Estrogen regulation of proto-oncogenes coding for nuclear proteins. Crit Rev Oncog 4:361–388, 1993.
65. Zajchowski DA, Sager R, Webster L: Estrogen inhibits the growth of estrogen receptor-negative, but not estrogen receptor-positive, human mammary epithelial cells expressing a recombinant estrogen receptor. Cancer Res 53:5004–5011, 1993.
66. Pilat MJ, Christman JK, Brooks SC: Characterization of the estrogen receptor transfected MCF-10A breast cell line 139B6. Breast Cancer Res Treat 37:253–266, 1996.
67. Calaf G, Tahin Q, Alvarado ME, et al: Hormone receptors and cathepsin D levels in human breast epithelial cells transformed by chemical carcinogens. Breast Cancer Res Treat 29:169–177, 1993.
68. Aronica SM, Kraus WL, Katzenellenbogen BS: Estrogen action via the cAMP signaling pathway. Stimulation of adenylate cyclase and cAMP regulated gene transcription. Proc Natl Acad Sci U S A 91:8517–8521, 1994.
69. Pappos TC, Gametahu B, Watson CS: Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. FASEB J 9:404–410, 1994.
70. Santner SJ, Pauley RJ, Tait L, et al: Aromatase activity and expression in breast cancer and benign breast tissue stromal cells. J Clin Endocrinol Metab 82:200–208, 1997.
71. Cunha GR, Hom YK: Role of mesenchymal-epithelial interactions in mammary gland development. J Mammary Gland Biol Neoplasia 1:21–35, 1996.
72. Cunha GR, Young P, Hom YK, et al: Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. J Mammary Gland Biol Neoplasia 2:393–402, 1997.
73. Hayward SW, Rosen MA, Cunha GR: Stromal-epithelial interactions in the normal and neoplastic prostate. Br J Urol 79 (suppl 2):18–26, 1997.
74. Cooke PS, Buchanan DL, Young P, et al: Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. Proc Natl Acad Sci U S A 94:6535–6540, 1997.

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17β-Estradiol is carcinogenic in human breast epithelial cells

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Abstract

The association found between breast cancer development and prolonged exposure to estrogen suggests that this hormone is of etiologic importance in the causation of this disease. In order to prove this postulate, we treated the immortalized human breast epithelial cells (HBEC) MCF-10F with 17β-estradiol (E_2) for testing whether they express colony formation in agar methocel, or colony efficiency (CE), and loss of ductulogenesis in collagen matrix, phenotypes also induced by the carcinogen benz[*a*]pyrene (BP). MCF-10F cells were treated with 0.0, 0.007, 70 nM, or 0.25 mM of E_2 twice a week for 2 weeks. CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses. Ductulogenesis was 75 ± 4.9 in control cells; it decreased to 63.7 ± 28.8, 41.3 ± 12.4, and 17.8 ± 5.0 in E_2-treated cells, which also formed solid masses or spherical formations lined by a multilayer epithelium, whose numbers increased from 0 in controls to 18.5 ± 6.7, 107 ± 11.8 and 130 ± 10.0 for each E_2 dose. MCF-10F cells were also treated with 3.7 μM of progesterone (P) and the CE was 3.39 ± 4.05. At difference of E_2, P does not impaired the ductulogenic capacity. Genomic analysis revealed that E_2-treated cells exhibited loss of heterozigosity in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2–25, respectively These results also indicate that E_2, like the chemical carcinogen BP, induces in HBEC phenotypes indicative of neoplastic transformation.

Keywords: 17β-Estradiol; HBEC; Estrogen; Breast

1. Introduction

Epidemiological and clinical evidence indicate that breast cancer risk is associated with prolonged exposure to female ovarian hormones [1–4]. Breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors [5,6], from which estrogens have been demonstrated to be of essential importance in this phenomenon as it is observed in postmenopausal hyperestrogenism resulting from the use of estrogenic hormone replacement therapy and obesity [7,8].

Estrogens, that are necessary for the normal development of both reproductive and non-reproductive organs, exert their physiological effects by binding to their specific receptors, the estrogen receptors (ER)-α or -β [9–16]. Estrogens might act as well through alternate non-receptor mediated pathways [17]. E_2, under the effect of 17β-oxidoreductase is continuously interconverted to estrone (E_1), and both are hydroxylated at C-2, C-4, or C-16α positions by cytochrome P450 isoenzymes, i.e. CYP1A1, CYP1A2, or CYP1B1, to form catechol estrogens [18–23]. The demonstration that the catecholestrogen 4-hydroxyestradiol (4-OH-E_2) induces an estrogenic response in the uterus of ER-α null mice, and the fact that this response is not inhibited by the antiestrogen ICI-182, 780 [9], indicate that this catecholestrogen does not exert its effect on the ER. The metabolic activation of estrogens can be mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. An increase in CE 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 via a Michael addition and thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidatic activation of CE. Thus, estrogen and 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 [18–23]. Although this pathway has been demonstrated in other systems [18–20], it still needs to be demonstrated in human breast epithelial cells (HBECs).

Furthermore, if estrogen is carcinogenic in the human breast through the above-mentioned pathway, it would induce

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in breast epithelial cells in vitro transformation phenotypes indicative of neoplasia 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 [24–39]. In order to test this hypothesis, we have evaluated the transforming potential of E_2 on HBEC in vitro, utilizing the spontaneously immortalized HBEC MCF-10F [40,41]. This cell line lacks both ER-α and ER-β, although this latter receptor is induced in cells transformed by chemical carcinogens [42]. In the present work, we report that the same phenotypes and characteristics that were expressed by MCF-10F cells transformed by the chemical carcinogen benz[*a*]pyrene (BP) and oncogenes [43–46] were expressed in E_2-treated cells. E_2 transformed cells exhibited loss of heterozygosity (LOH) in loci of chromosome 11, known to be affected in spontaneously occurring breast lesions, such as ductal hyperplasia, carcinoma in situ, and invasive carcinoma [47–60].

2. Material and methods

2.1. Cells and dose selection

MCF-10F cells at passage 125 were cultured in DMEM:F-12 medium containing 1.05 mM calcium (Ca^{2+}), antibiotics, antimycotics, hormones, growth factors, and equine serum as previously described [44]. In order to determine the optimal doses for the expression of the cell transformation phenotype, we treated the immortalized HBEC MCF-10F with 0.0, 0.07 nM, 70 nM, or 0.25 mM of 17β-estradiol (E_2) twice a week for 2 weeks (Fig. 1). Based upon these results a dose of 3.7 μM (1 μg/ml) was selected for testing the effect of E_2, progesterone (P), and BP.

2.2. Evaluation of the effect of estrogens and other compounds on the expression of cell transformation phenotypes

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 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). MCF-10F cells were treated with 1.0 μg/ml E_2 (Aldrich, St. Louis, MO), progesterone (Sigma Chemical Co., St. Louis, MO), control cells were treated with DMSO. MCF-10F cells treated with 1.0 μg/ml BP served as positive controls for cell transformation assays. In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were first treated with E_2, P, or BP at 72 and 120 h post-plating. At the end of the first week of treatment, the cells were passaged for administration of another two periods of hormonal treatment. 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 assayed for determination of survival efficiency (SE), colony efficiency (CE), colony size (CS), and ductulogenic capacity, as described in previous publications [44,45].

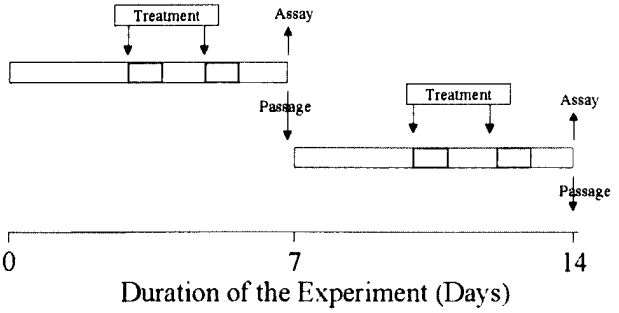

Fig. 1. MCF-10F cells were treated with E_2, DES, or BP at 72 and 120 h post plating. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis.

2.3. 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 Chemical Co., 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 50 μm in diameter, and expressed as a percentage of the original number of cells plated per well.

2.4. 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 determin-

ing 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.

2.5. Genomic analysis of treated cells

2.5.1. DNA isolation

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 2% v/v 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, pH 8.0), 1 mM EDTA.

2.5.2. Detection of allelic loss

We evaluated for allelic losses the regions of chromosomes 1, 2, 3, 6, 8, 9, 11, 12, 13, 16, 17, and 18 most frequently reported to exhibit LOH in spontaneous breast

Table 1
Microsatellite DNA polymorphism analysis of MCF-10F cells treated with E_2 or BP

Chromosome	Marker	Location	MCF10-F	E_2-1	E_2-2	BP
1	D1S104	1p21–1p23	○	○	○	○
1	BAT-40	1p13.1	○	○	○	○
2	D2S171	2p24–21	○	○	○	○
2	D2S123	2p16	○	○	○	○
3	D3S1297		○	○	○	
3	D3S1560	3p26–3p25	○	○	○	○
3	D3S1304	3p26–3p25	○	○	○	○
3	D3S1307	3p26–p25	○	○	○	○
3	D3S1289	3p23–3p21	○	○	○	○
3	D3S1449	3p22.3–3p21.3	○	○	○	○
3	D3S1478	3p2103–21.2	○	○	○	○
3	D3S2384	3p21.3–21.2	○	○	○	○
3	D3S1450	3p21.1–3p14.2	○	○	○	○
3	D3S1217	3p21	○	○	○	○
3	D3S1447	3p21	○	○	○	○
3	D3S1241	3p21	○	○	○	○
3	D3S1448	3p21	○	○	○	○
3	D3S1480	3p14	○	○	○	○
6	ESR	6q24–27	○	○	○	○
8	MYCL-1	8q24.1	○	○	○	○
9	D9S199	9p23	○	○	○	○
9	D9S157	9p23–22	○	○	○	○
9	D9S171	9p21	○	○	○	○
9	D9S165	9p21	○	○	○	○
11	D11S988	1pter–qter	○	○	○	○
11	D11S922	11p15.5	○	○	○	○
11	H-RAS1	11p15.5	○	○	○	○
11	CCKBR	11p15.4	○	○	○	○
11	D11S1392	11p13	○	○	○	○
11	Int-2	11p133	○	○	○	○
11	D11S907	11p13	○	○	○	○
11	D11S911	11q13–11p23	○	○	○	○
11	D11S436	11p12–11p11.1	○	○	○	○
11	D11S614	11q22–11q23	○	○	○	○
11	D11S940	11q22	○	○	○	○
11	DRD2	11q23.1	○	○	○	○
11	D11S968	11q2301–11q25	○	○	○	○
11	D11S29	11q23.3	○	●	●	○
11	D11S925	11q23.3–11q24	○	○	○	○
11	D11S912	11q24.2–11q25	○	●	●	○
12	IGF-1	12q22–12q23	○	○	○	○
13	D13S289	13q12.2	○	○	○	○
13	D13S260	13q12.3	○	○	○	○

Table 1 (*Continued*)

Chromosome	Marker	Location	MCF10-F	E_2-1	E_2-2	BP
13	D13S267	13q2.3	○	○	○	○
13	D13S171	13q12.3–13	○	○	○	○
13	D13S218	13q13–14.1	○	○	○	○
13	GABRB-1	13q14.2	○	○	○	○
13	D13S155	13q14.3–21.2	○	○	○	○
16	D16S540		○	○	○	
17	D17S849	17p13.3	○	○	○	○
17	D17S796	17p13.1	○	○	○	○
17	D17S513	17p13.1	○	○	○	○
17	Tp53	17p13.1	○	○	○	○
17	D17S786	17p13.1	○	○	○	○
17	D17S793	17p13.1–7p11.2	○	○	○	○
17	D17S945	17p13–12	○	○	○	○
17	D17S520	17p12	○	○	○	○
17	D17S800	17q11.1–12	○	○	○	○
17	THRA-1	17q11.2–12	○	○	○	○
17	D17S787	17q21–22	○	○	○	
17	D17S855	17q21.2	○	○	○	○
17	D17S1323	17q21.2	○	○	○	○
17	D17S808	17q23.2	○	○	○	○
17	D17S789	17q24	○	○	○	○
17	D17S515	17q24.2–25.2	○	○	○	○
17	D17S785	17q25.2	○	○	○	○
18	D18S58	18q22.3–23	○	○	○	○

tumors (Table 1). 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 [61–63]. 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 [64].

2.5.3. DNA fingerprinting

Before performing DNA amplification of microsatellite DNA polymorphisms to detect allelic losses present in E_2-, DES-, and BP-treated cells, we verified by DNA fingerprinting whether all the clones derived from MCF-10F-treated cells were from the same lineage. Genomic DNA was extracted from the cells listed in Table 1. The identity of these cells was confirmed by Southern blot hybridization of genomic DNA with a cocktail of the three minisatellite probes D2S44, D14S13 and D17S74. Genomic DNAs were digested with HifI, and hybridized with probes under standard condition [64].

2.5.4. PCR analysis of microsatellites

Primers used for the analysis of microsatellite polymorphisms are given elsewhere [64]. Conditions for PCR amplification were as follows: 30 ng of genomic DNA, 100 pmol of each oligonucleotide primer, 1 × PCR buffer (Perkin-Elmer Cetus), 5 μM each of TTP, dCTP, dGTP, and dATP, 1 mCi [^{32}P] dATP (300 mCi/mmol) (DuPont, NEN, Boston, MA), and 0.5 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus) in 10 ml 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 ml) 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.

2.5.5. 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.

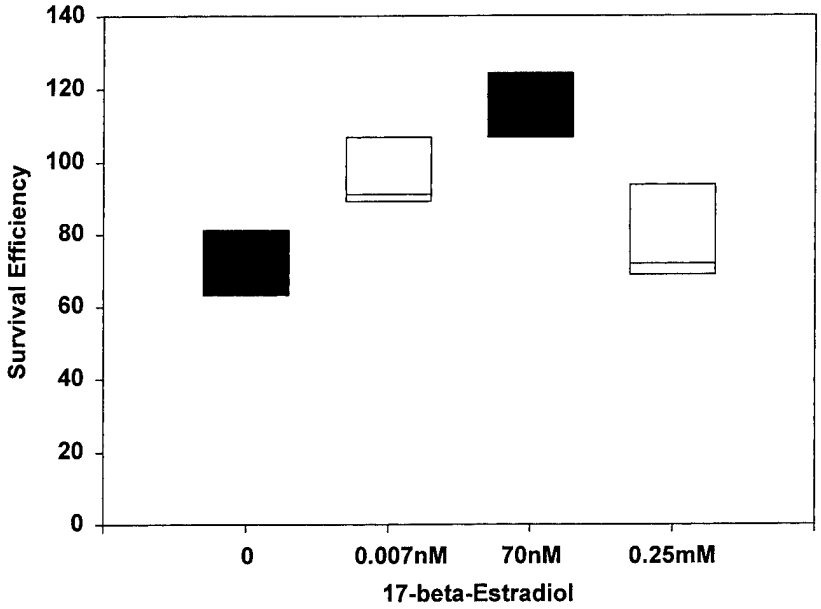

Fig. 2. Box plot showing the dose effect of 17β-estradiol on the survival efficiency in agar methocel of MCF-10F cells.

3. Results

3.1. Determination of the dose response curve to 17β-estradiol

In order to determine the optimal doses for the expression of the cell transformation phenotype we treated the immortalized HBEC MCF-10F with E_2 for testing the SE whether they express colony formation in agar methocel, or CE, and loss of ductulogenesis in collagen matrix. MCF-10F cells were treated with 0.0, 0.007, 70 nM, or 0.25 mM of E_2 twice a week for 2 weeks. The SE was increased with 0.007 and 70 nM of 17β-estradiol and decrease with 0.25 mM (Fig. 2). The cells treated with either doses of E_2 formed colonies in agar methocel and the size was not different among them (Fig. 3), however, the CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses (Fig. 4). Ductulogenesis or the number of ductules per 10,000 cells plated, was 75 ± 4.9 in control cells; it decreased to 63.7 ± 28.8, 41.3 ± 12.4, and 17.8 ± 5.0 in E_2-treated cells (Fig. 5), which also formed spherical like structures or solid masses (Fig. 6a–d), whose numbers increased from 0 in controls to

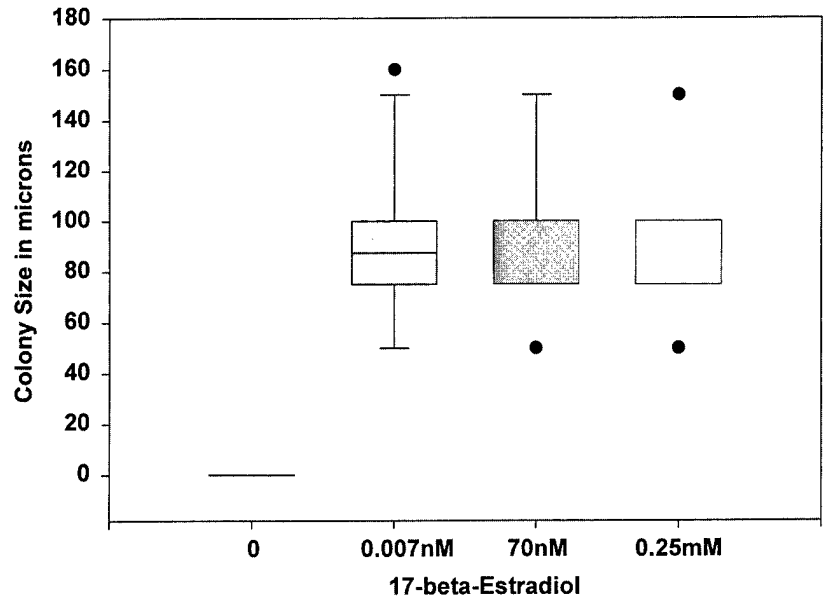

Fig. 3. Box plot showing the dose effect of 17β-estradiol on colony size.

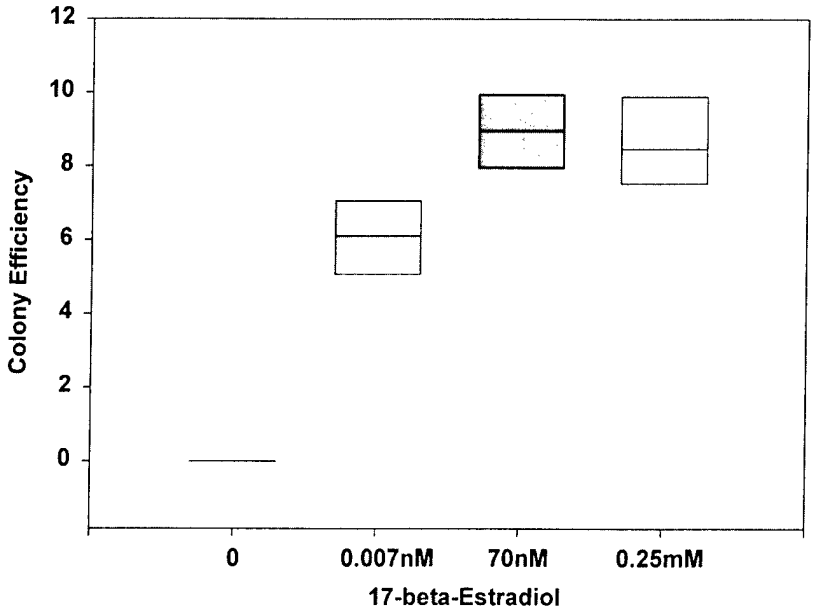

Fig. 4. Box plot showing the dose effect of 17β-estradiol on colony efficiency.

18.5 ± 6.7, 107 ± 11.8 and 130 ± 10.0 for each E_2 dose (Fig. 7).

3.2. Effect of estrogen, progesterone and benz[a]pyrene on the expression of transformation phenotypes

The SE of MCF-10F cells was increased with all the treatments (Fig. 8). Evaluation of colony formation at the end of the second week of E_2 and BP treatment revealed that MCF-10F cells formed colonies in agar-methocel over 60 μm in diameter, whereas those cells treated with progesterone the colonies are smaller (Fig. 9). MCF-10F control cells treated with DMSO did not form colonies (Fig. 9). The total CE was significantly increased by E_2 and BP, and significantly less by P (Figs. 10 and 11a–f).

3.3. Ductulogenic capacity

Ductulogenesis was qualitatively evaluated by estimating the ability of the cells plated in collagen to form tubular and ductular structures. It was maximal in MCF-10F

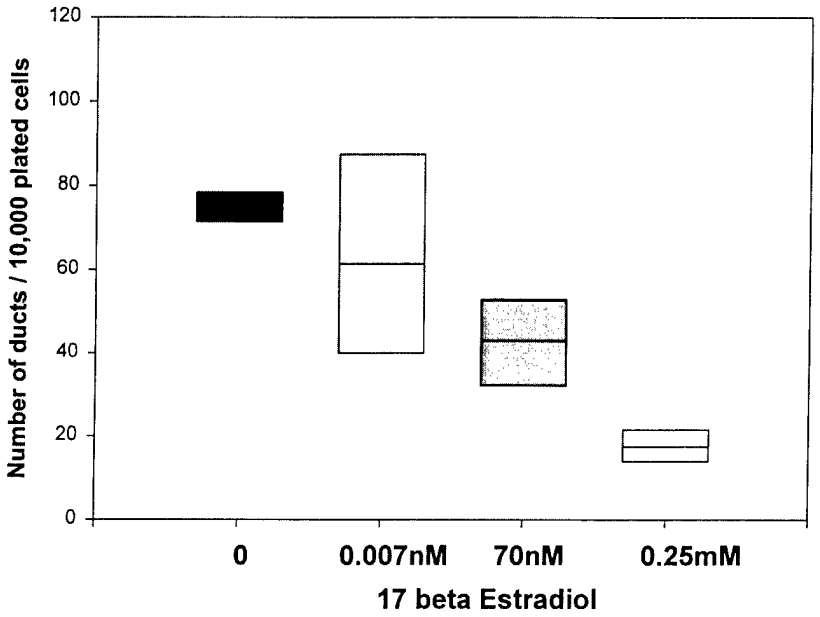

Fig. 5. Box plot showing the dose effect of 17β-estradiol on MCF-10F cells forming ductules in collagen matrix over 10,000 cells plated.

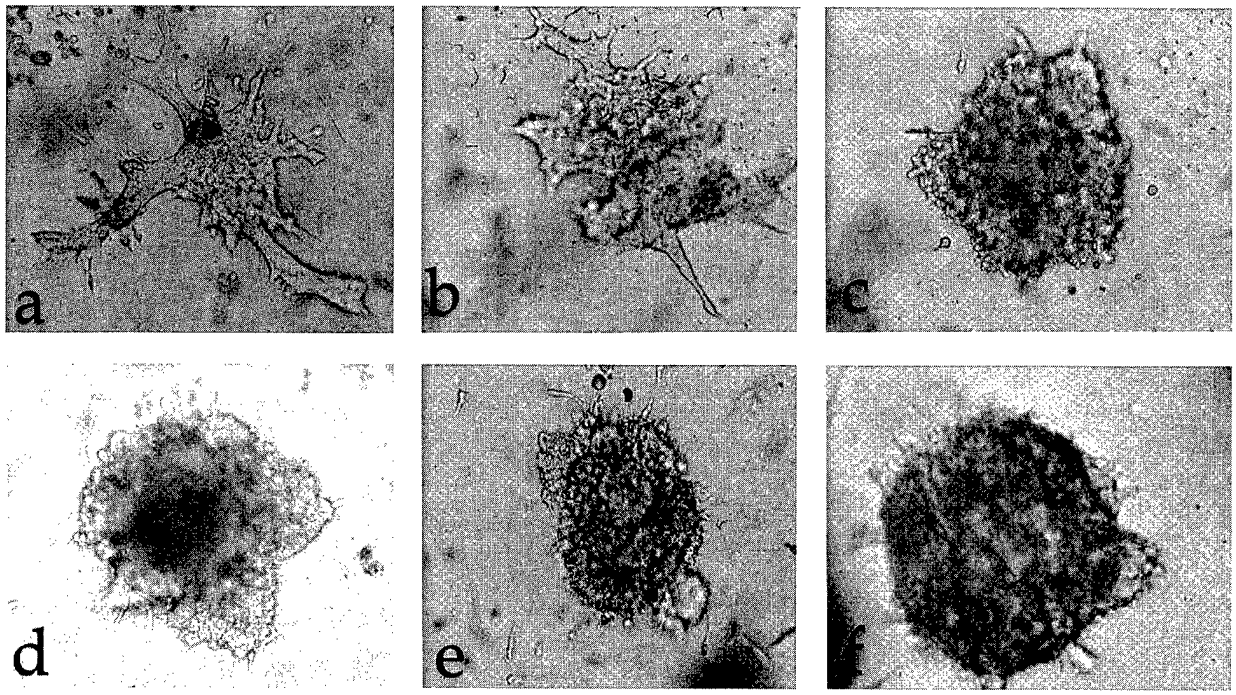

Fig. 6. (a) MCF-10F cells treated with solvent (DMSO) forming well defined ductular structures in collagen matrix; (b) 0.007 nM of E_2 induces alteration in the ductular pattern; (c) and (d) 70 nM of E_2 induces the loss of ductular formation in collagen matrix; (e) and (f) 1 μg of E_2 or BP, respectively, induces the formation of spherical masses in collagen matrix. Phase contrast microscope ×10.

cells (Fig. 6a), and completely negative (−) in BP-treated cells, which grew as a solid or cystic mass. All the cells treated with E_2 exhibited decreased ability to form ductules (Fig. 6b–e). Progesterone does not affect significantly the ductulogenic capacity. The collagen matrix embedded in paraffin and cross sectioned for determination of cell morphology showed that MCF-10F form a well-defined ductule lined by a monolayer of cuboidal epithelial cells (Fig. 12a), whereas those treated with E_2 the number of layers increase and in some cases the whole lumen is obliterated (Fig. 12b–d). BP also forms similar structures to those induced by estrogen, whereas the ductules formed by

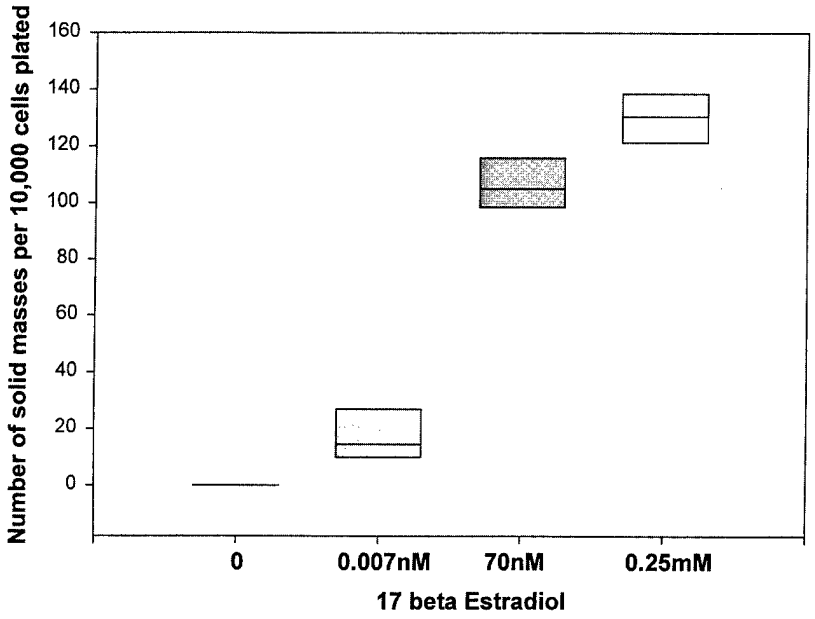

Fig. 7. Box plot showing the dose effect of 17β-estradiol on MCF-10F cells forming solid or spherical masses in collagen matrix per 10,000 cells plated.

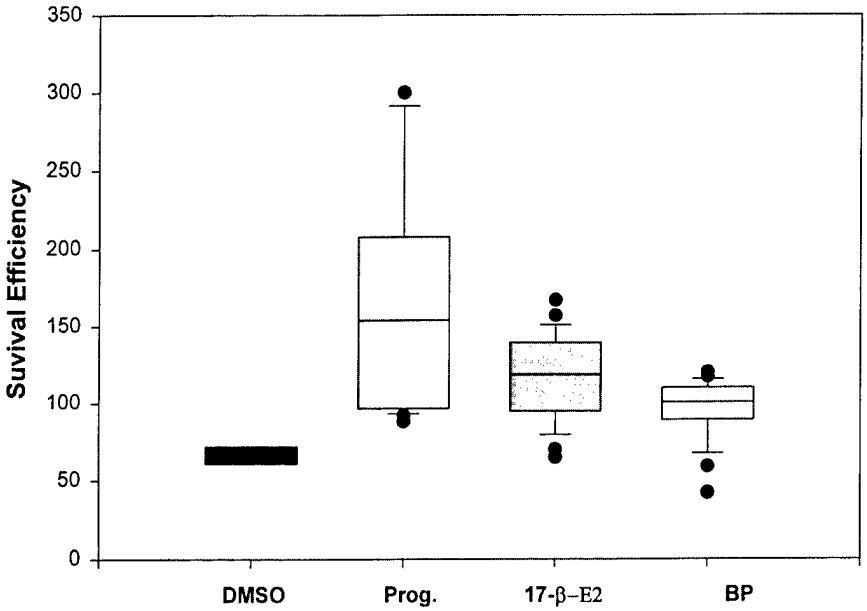

Fig. 8. Box plot showing the effect of different compounds on MCF-10F cells survival efficiency in agar methocel.

progesterone treatment are smaller with a reduced luminal size lined by a monolayer of cuboidal epithelial cells.

3.4. Genomic changes induced in E_2 and DES transformed MCF-10F cells

From the E_2-treated cells six clones out 24 colonies were expanded and maintained in culture. These clones were designated E_2-1–E_2-6 (Table 2). These clones were selected for genomic analysis. DNA fingerprint analysis of parent, E_2-, P-, and BP-treated cells and their derived clones revealed that their allelic pattern was identical in all the cell lines analyzed. These results confirmed that all the cells tested had the same HBEC origin, and that they were free of contamination from other cell lines maintained in our laboratory.

Among 67 markers tested, which were selected based on chromosomal changes reported to be present in breast and other cancers, only clones E_2-1 and E_2-2, exhibited LOH in chromosome 11 (Table 1). Clones E_2-1 and E_2-2 identically expressed LOH in chromosome 11 at 11q23.3 (marker D11S29), and 11q24.2–25 (marker D11S912). BP-treated cells did not exhibit LOH at any of the loci tested. Interestingly, we have found that all the clones of the cells transformed with either E_2-, BP-presented microsatellite

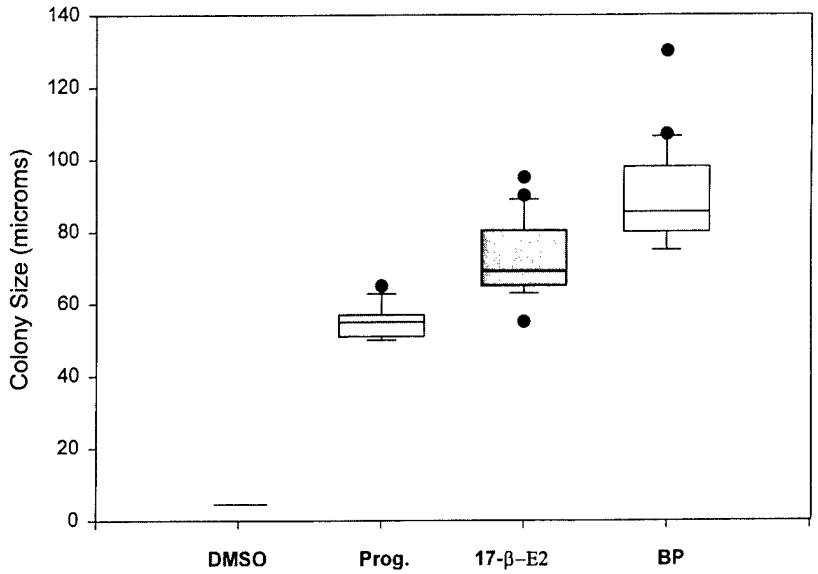

Fig. 9. Box plot showing the effect of different compounds on MCF-10F cells colony size growing in agar methocel.

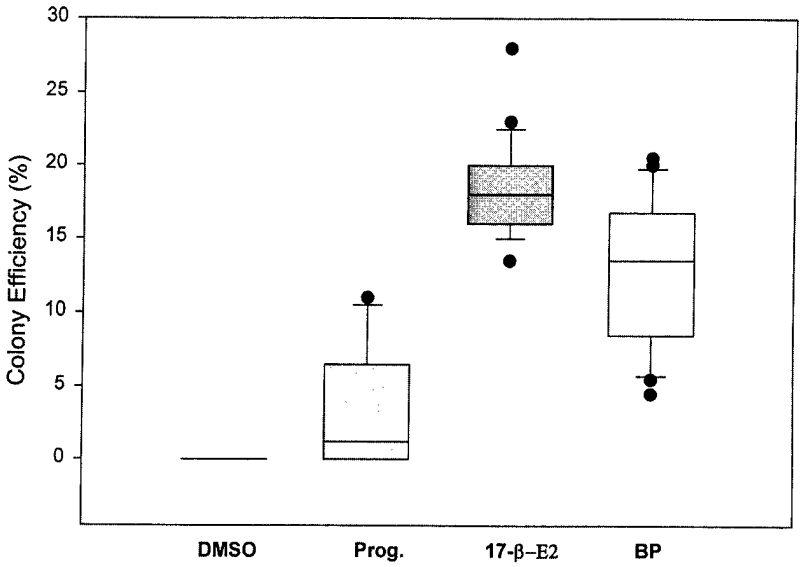

Fig. 10. Box plot showing the effect of different compounds on MCF-10F cells colony efficiency in agar methocel.

instability (MSI), expressed as an allelic expansion at 3p21 locus (marker D3S1447) (data not shown). In order to determine whether these MSIs were related to alterations in mismatch repair genes, we performed microsatellite DNA analysis in loci 1p13.1, with marker BAT40, 2p16, with marker D2S123, and 18q22.3–q23, with marker D18S58, which are related to mismatch repair genes. However, none of those markers showed alterations with this technique (Table 1).

4. Discussion

In the present work we have capitalized on the availability in our laboratory of an in vitro model of transformation of immortalized HBEC by the chemical carcinogen BP for comparison with phenotypic and genomic changes induced by the natural estrogen E_2. The immortalized HBEC MCF-10F are negative for both ER-α and ER-β [42]. Short term treatment of these cells with physiological doses of E_2 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 induce by BP under the same culture conditions. Progesterone was unable to induce significant increase in colony formation, although small colonies <60 mm in diameter were observed, whereas none were found in the MCF-10F cells treated with DMSO. The ductulogenic pattern was not impaired by progesterone but the luminal size was smaller that those found in the MCF-10F cells.

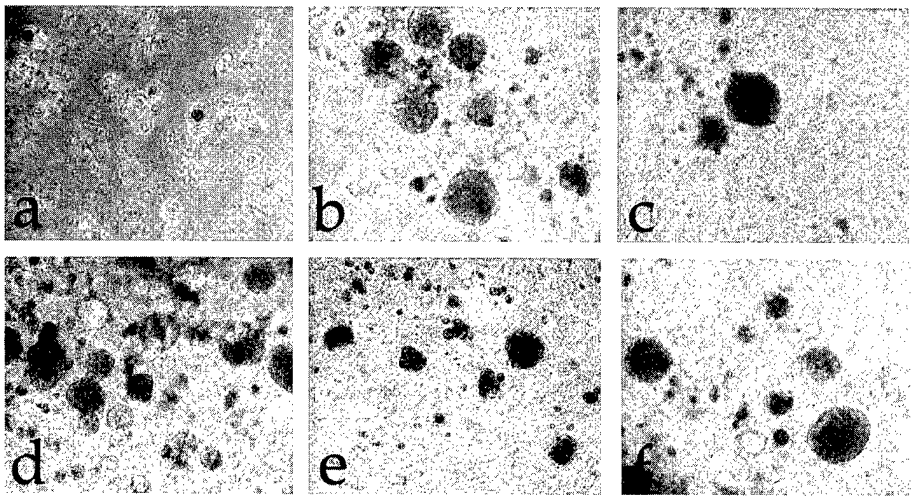

Fig. 11. MCF-10F cells plated in agar-methocel for colony assay: (a) control cells do not form colonies, only isolated cells are present; (b)–(d) colonies formed by E_2-treated MCF-10F cells at the doses of 0.007, 70 nM and 1 μM, respectively; (e) progesterone-treated cells; (f) BP-treated cells induces slightly larger colonies. Phase contrast microscopy ×4.

Fig. 12. Histological sections of cells growing in collagen gel. The cells have been fixed in buffered formalin, embedded in paraffin and the sections stained with hematoxylin and eosin. (a) MCF-10F cells treated with solvent (DMSO) forming well defined ductular structures lined by a single cuboidal layer of cells; (b) 0.007 nM of E_2 induces alteration in the ductular pattern forming spherical masses lined by two to three layers of cells; (c) 70 nM of E_2 induces the loss of ductular formation in collagen matrix and the solid spherical masses are composed of large cuboidal cells; (d) 1 μg of E_2 or BP induces the formation of spherical masses lined by multiple layers of cells. Phase contrast microscope ×10 Hematoxylin and eosin ×10.

Altogether these data clearly indicate that HBEC when treated with E_2 produces significant morphogenetic changes. The fact that the MCF-10F cells are both ER-α and ER-β negative, argue in favor of a metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. An increase in CE 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 and 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 [18–23]. Although this

Table 2
Phenotypic markers of cell transformation induced in MCF-10F cells by E_2, and BP

Cell type	No. of passages	Doubling time (DT) (h)[a]	Colony number (CN)[b]	Colony efficiency (%) (CE)[b]	Colony size (CS) (m)[b]
MCF-10F	113	93 ± 5.6	0.0	0.0	0.0
BP	4	42 ± 3.8	89	18 ± 4.5	670 ± 46
E_2	4	78 ± 16.0	24[c]	4.8 ± 0.9	170 ± 34
E_2-1[d]	4	81 ± 3.0	36	7.2 ± 3.7	180 ± 12
E_2-2[d]	4	68 ± 10	45	9.0 ± 2.0	150 ± 6
E_2-3	5	66 ± 8.0	39	7.9 ± 5.6	190 ± 9
E_2-4	3	82 ± 6.0	20	3.5 ± 1.1	134 ± 5
E_2-5	6	61 ± 5.6	63	12.6 ± 3.0	193 ± 12
E_2-6	4	73 ± 3.0	54	10.8 ± 4.9	189 ± 5

[a] DT was determined as described in [43]. DT was significantly different by Student's t-test between BP and all the other cells lines ($P < 0.001$).

[b] CN, CE, and CS were significantly different between MCF-10F and all other cell lines ($P = 0.00001$). CS of DES clones was significantly different from E_2 and BP cells ($P = 0.001$).

[c] From 24 colonies derived from E_2-treated cells, clones E_2-1, E_2-2, E_2-3, E_2-4, E_2-5 and E_2-6 were recovered and expanded.

[d] E_2-1 and E_2-2 cells have been used for detection o microsatellite DNA polymorphism.

pathway has not been demonstrated in the present work, the data are supporting but not definitively demonstrating the pathway. More studies in this subject are in progress in our laboratory to define this mechanism.

It was of great interest that by the fourth passage after four treatments during a 2-week period, clones derived from E_2-transformed cells exhibited LOH in chromosome 11, whereas during the same period of time, the chemical carcinogen BP did not induce genomic changes, even though we have previously reported that this carcinogen induces LOH in chromosome 17 [43], in addition to tumorigenesis in a heterologous host after a larger number of passages and a more prolonged selection process in vitro [44,45]. We have found that estrogen induces LOH in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2–25, respectively. It has been reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice [65]. Several genes, such as *HRAs*, *CTSD*, *ILK*, *TSG101* and *KI1* have been reported to be located on the short arm of chromosome 11 [53,54,65–71]. A region of deletion on 11q22–23 has been described on the long arm of chromosome 11in 40–60% of breast tumors [51,57,59,60,72–74]. The ataxia telangiectasia susceptibility gene (*ATM*) is the most widely studied candidate gene in this region [75]. *ATM* may act upstream of the *TP53* gene in cell cycle regulation [76,77] and its heterozygous mutation is associated with high incidence of early-onset breast cancer. This region has been reported to contain several tumor suppressor genes and genes involved in the metastatic process. In this latter group, the *MMP* genes encoding matrix metalloproteases involved in invasion, *ETS1* encoding a transcription factor involved in angiogenesis, and *VACM-1*, encoding a protein probably involved in cell cycle regulation have been identified [78]. Although some of these genes might be affected during the transformation of HBEC induced by estrogens, a more detailed allelotyping using multiple markers is required for better defining the significance of LOH in these cells.

Approximately 35% of breast cancers show LOH at the D11S29 and NCAM loci [79], and a higher frequency of LOH at this locus has also been found in melanomas [80]. LOH has been found at frequencies of 25 and 29% at the distal D11S968 (11-qter) and D11S29 (11q23.3 locus), slightly above the accepted baseline of 0–20% in colorectal cancer. The fact that breast cancer, melanoma, and colorectal cancer have been found to be influenced by estrogens [81], give relevance to our data that treatment of MCF-10F cells with estrogens induces LOH in this specific locus. LOH at 11q23-qter occurs frequently in ovarian and other cancers [82,83].

The most frequent allelic loss observed in breast cancer has been reported in chromosome 17p, suggesting that genes located in that chromosome arm, such as p53 oncogene, might be a likely target for this event. [33,80–100]. We have not been able up to now to demonstrate any LOH in chromosome 17 in estrogen transformed MCF-10F cells. However, we have used a small number of markers, and the possibility that LOH might be located at sites not tested yet cannot be ruled out. Therefore, the study of allelic imbalances at 17q and 17p, as well as in chromosome 16 [85,101,102] in estrogen transformed HBEC must be carried out to provide further understanding of the functional involvement of these chromosomes in the process of cell transformation by E_2.

The observations that E_2, and BP induce similar phenotypical, but different genomic alterations requires further investigation in order to elucidate the significance of timing of appearance of each type of changes with regards to cancer initiation and progression. There are several probable avenues for explaining these discrepancies. In this model, both estrogens and the chemical carcinogen as an early event induce phenotypic changes, whereas LOH is a rare event that is manifested in different chromosomes and only in few clones derived from E_2-treated cells. The rarity of the phenomenon is in agreement with the low frequency of LOH observed in BP transformed cells, in which the phenomenon is manifested at a more advanced stage of neoplastic progression [43,100]. Altogether these observations suggest that these two compounds might act through different genetic events for inducing similar transformation phenotypes.

Acknowledgements

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References

[1] M.C. Pike, D.V. Spicer, L. Dahmoush, M.F. Press, Estrogens, progesterone, normal breast cell proliferation and breast cancer risk, Epidemiol. Rev. 15 (1993) 17–35.
[2] J.L. Kelsey, M.D. Gammon, E.M. John, Reproductive factors and breast cancer, Epidemiol. Rev. 15 (1993) 36–47.
[3] L. Bernstein, R.K. Ross, Endogenous hormones and breast cancer risk, Epidemiol. Rev. 15 (1993) 48–65.
[4] B.E. Henderson, R. Ross, L. Bernstein, Estrogens as a cause of human cancer: the Richard, Estrogens as a cause of human cancer: the Richard & Hinda Rosenthal Foundation Award Lecture, Cancer Res. 48 (1988) 246–253.
[5] Y.J. Topper, L. Sankaran, P. Chomczynski, C. Prosser, P. Qasba, Three stages of responsiveness to hormones in the mammary cell, in: A. Angeli, H.L. Bradlow, L. Dogliotti (Eds.), Endocrinology of the Breast: Basic and Clinical Aspects, Ann. New York Acad. Sci. 464 (1986) 1–10.
[6] M.E. Lippman, K.K. Huff, R. Jakesz, T. Hecht, A. Kasid, S. Bates, R.B. Dickson, Estrogens regulate production of specific growth factors in hormone-dependent human breast cancer, in: A. Angeli, H.L. Bradlow, L. Dogliotti (Eds.), Endocrinology of the Breast: Basic and Clinical Aspects, Ann. New York Acad. Sci. 464 (1986) 11–6.
[7] W.D. Dupont, D.L. Page, Menopausal estrogen replacement therapy and breast cancer, Arch. Int. Med. 151 (1991) 67–72.

[8] M.A. Price, C.C. Tennant, R.C. Smith, S.J. Kennedy, P.N. Butow, M.B. Kossoff, S.M. Dunn, Predictors of breast cancer in women recall following screening, Aust. New Zealand J. Surg. 69 (1999) 639–646.

[9] J.F. Couse, K.S. Korach, Estrogen receptor null mice: what have we learned and where will they lead us? Endocr. Rev. 20 (1999) 358–417.

[10] A.K. Shiau, D. Barstad, P.M. Loria, L. Cheng, P.J. Kushner, D.A. Agard, G.L. Greene, The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen, Cell 95 (1998) 927–937.

[11] D.P. McDonnell, The molecular pharmacology of SERMs, TEM 10 (1999) 301–311.

[12] M.J. Tsai, B.W. O'Malley, Molecular mechanisms of steroid/thyroid receptor superfamily members, Annu. Rev. Biochem. 63 (1994) 451–486.

[13] B.S. Katzenellenbogen, Dynamics of steroid hormone receptor action, Annu. Rev. Physiol. 42 (1980) 17–35.

[14] S. Mosselman, J. Polma, R. Dijkema, ER-β: identification and characterization of a novel human estrogen receptor, FEBS Lett. 392 (1996) 49–53.

[15] G.G.J.M. Kuiper, B. Carlsson, K. Grandien, E. Enmark, et al., Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β, Endocrinology 138 (1997) 863–870.

[16] K. Paech, P. Webb, G.G. Kuiper, S. Nilsson, J. Gustafsson, P.J. Kushner, T.S. Scanlan, Differential ligand activation of estrogen receptors ER-alpha and ER-beta at AP1 sites, Science 277 (1997) 1508–1510.

[17] X. Chen, C. Danes, M. Lowe, T.W. Herliczek, K. Keyomarsi, Activation of the estrogen-signaling pathway by p21 WAF1/CIP1 in estrogen receptor negative breast cancer cells, J. Natl. Cancer Inst. 92P1403-13, 2000.

[18] J.G. Liehr, A.A. Ulubelen, H.W. Strobel, Cytochrome P-450-mediated redox cycling of estrogens, J. Biol. Chem. 261 (1986) 16865–16870.

[19] D. Roy, J.G. Liehr, Temporary decrease in renal quinone and reductase activity induced by chronic administration of estradiol to male Syrian hamsters-increased superoxide formation by redox cycling of estrogen, J. Biol. Chem. 263 (1988) 3646–3651.

[20] Z.-J. Yan, D. Roy, Mutations in DNA polymerase P mRNA of stilbene estrogen-induced kidney tumors in Syrian hamster, Biochem. Mol. Biol. Int. 37 (1997) 175–183.

[21] P. Ball, R. Knuppen, Catecholestrogens (2- and 4-hydroxy-oestrogens). Chemistry, biosynthesis, metabolism, occurrence and physiological significance, Acta Endocrinol. (Copenh) 232 (Suppl. 1) (1980) 127.

[22] B.T. Zhu, Q.D. Bui, J. Weisz, J.G. Liehr, Conversion of estrone to 2- and 4-hydroxyestrone by hamster kidney and liver microsomes: implications for the mechanism of estrogen-induced carcinogenesis, Endocrinology 135 (1994) 1772–1779.

[23] S.P. Ashburn, X. Han, J.G. Liehr, Microsomal hydroxylation of 2- and 4-fluoroestradiol to catechol metabolites and their conversion to methyl ethers: catechol estrogens as possible mediators of hormonal carcinogenesis, Mol. Pharmacol. 43 (1993) 534–541.

[24] D.J. Slamon, G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, W.L. McGuire, Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene, Science 235 (1987) 177–182.

[25] C. Escot, C. Theillet, R. Lidereau, F. Spyratos, M.-H. Champeme, J. Gest, R. Callahan, Genetic alteration of the *c-myc* proto-oncogene (*MYC*) in human primary breast carcinomas, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 4834–4838.

[26] I.U. Ali, G. Merio, R. Callahan, R. Lidereau, The amplification unit on chromosome 11q13 in aggressive primary human breast tumors entails the bcl-1, int-2 and hst loci, Oncogene 4 (1989) 89–92.

[27] C. Theillet, J. Adnane, P. Szepetowski, M.P. Simon, P. Jeanteur, D. Birnbaum, P. Gaudray, BCL-1 participates in the 11q13 amplification found in breast cancer, Oncogene 5 (1990) 147–149.

[28] C. Theillet, R. Lidereau, C. Escot, P. Hutzell, M. Brunet, J. Gest, J. Schlom, R. Callahan, Loss of a *c-H-ras-1* and aggressive human primary breast carcinomas, Cancer Res. 46 (1986) 4776–4781.

[29] C. Lundberg, L. Skoog, W.K. Cavenee, M. Nordenskjoid, Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 2372–2376.

[30] J. Mackay, C.M. Steel, P.A. Elder, A.P.M. Forrest, H.J. Evans, Allele loss on short arm of chromosome 17 in breast cancers, Lancet 2 (1988) 1384–1385.

[31] I.U. Ali, R. Lidereau, R. Callahan, Presence of two members of *c-erbAB* and *c-erbA2* in smallest region of somatic homiozygosity on chromosome 3p2l-p25 in human breast carcinoma, J. Natl. Cancer Inst. 81 (1989) 1815–1820.

[32] L.-C. Chen, C. Dolibaum, H. Smith, Loss of heterozygosity on chromosome lq in human breast cancer, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 7204–7207.

[33] R. Callahan, A. Campbell, Mutations in human breast cancer: an overview, J. Natl. Cancer. Inst. 81 (1989) 1780–1786.

[34] T. Sato, H. Saito, J. Swensen, A. Olifant, C. Wood, D. Danner, T. Sakamoto, K. Takita, F. Kasumi, Y. Miki, M. Skolnick, Y. Nakamura, The human prohibitin gene located on chromosome 17q2l is mutated in sporadic breast cancer, Cancer Res. 52 (1992) 1643–1646.

[35] L.C. Chen, W. Kurisu, B.M. Ljung, E.S. Goldman, D. Moore II, H.S. Smith, Heterogeneity for allelic loss in human breast cancer, J. Natl. Cancer Inst. 84 (1992) 506–510.

[36] M. Genuardi, N. Tsihira, D.E. Anderson, G.F. Saunders, Distal deletion of chromosome lq in ductal carcinoma of the breast, Am. J. Hum. Genet. 45 (1989) 73–89.

[37] C.S. Crop, R. Lidereau, G. Campbell, M.-H. Champene, R. Callahan, Loss of heterozygosity on chromosomes 17 and 18 in breast carcinoma: two additional regions identified, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 7737–7741.

[38] T. Sato, A. Tanigami, K. Yamakawa, F. Akiyama, F. Kasumi, G. Sakamoto, Y. Nakamura, Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer, Cancer Res. 50 (1990) 7184–7189.

[39] T. Sato, F. Akiyama, G. Sakamoto, F. Kasumi, Y. Nakamura, Accumulation of genetic alterations and progression of primary breast cancer, Cancer Res. 51 (1991) 5794–5799.

[40] H.D. Soule, T.M. Maloney, S.R. Wolman, W.D. Peterson Jr., R. Brenz, C.M. McGrath, J. Russo, R. Pauley, R.F. Jones, S.C. Brooks, Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10F, Cancer Res. 50 (1990) 6075–6086.

[41] L. Tait, H. Soule, J. Russo, Ultrastructural and immunocytochemical characterizations of an immortalized human breast epithelial cell line MCF-10F, Cancer Res. 50 (1990) 6087–6099.

[42] Y.F. Hu, K.M. Lau, S.M. Ho, J. Russo, Increased expression of estrogen receptor-β in chemically transformed human breast epithelial cells, Int. J. Oncol. 12 (1998) 1225–1228.

[43] J. Russo, G. Calaf, N. Sohi, Q. Tahin, P.L. Zhang, M.E. Alvarado, S. Estrada, I.H. Russo, Critical steps in breast carcinogenesis, N.Y. Acad. Sci. 698 (1993) 1–20.

[44] G. Calaf, J. Russo, Transformation of human breast epithelial cells by chemical carcinogens, Carcinogenesis 14 (1993) 483–492.

[45] J. Russo, G. Calaf, I.H. Russo, A critical approach to the malignant transformation of human breast epithelial cells, CRC Crit. Rev. Oncogen. 4 (1993) 403–417.

[46] G. Calaf, P.L. Zhang, M.V. Alvarado, S. Estrada, J. Russo, C-Ha ras enhances the neoplastic transformation of human breast epithelial cells treated with chemical carcinogens, Int. J. Oncol. 6 (1995) 5–11.

[47] L.-C. Chen, K. Matsumura, G. Deng, W. Kurisu, B.-M. Ljung, M.I. Lerman, F.M. Waldman, H.S. Smith, Deletion of two separate regions on chromosome 3p in breast cancers, Cancer Res. 54 (1994) 3021–3024.

[48] J.T. Bergthorsson, G. Eiriksdottir, R.B. Barkardottir, V. Egilsson, A. Arason, S. Ingvarsson, Linkage analysis and allelic imbalance in human breast cancer kindreds using microsatellite markers from the short arm of chromosome 3, Hum. Genet. 96 (1995) 437–443.

[49] F. Kerangueven, T. Noguchi, V. Wargniez, Multiple sites of loss of heterozygosity on chromosome arms 3p and 3q in human breast carcinomas, Oncol. Rep. 3 (1996) 313–316.

[50] N. Pandis, G. Bardi, F. Mitelman, S. Heim, Deletion of the short arm of chromosome 3 in breast tumors, Genes. Chrom. Cancer 18 (1997) 241–245.

[51] S. Man, I. Ellis, M. Sibbering, R. Blarney, J. Brook, Highs level of allele loss at the *FHIT* and *ATM* genes in non-comedo ductal carcinoma in situ and grade I tubular invasive breast cancers, Cancer Res. 56 (1996) 5484–5489.

[52] M. Negrini, C. Monaco, I. Vorechovsky, M. Ohta, T. Druck, R. Baffa, K. Huebner, C.M. Croce, The *FHIT* gene at 3pl4.2 is abnormal in breast carcinomas, Cancer Res. 56 (1996) 3173–3179.

[53] C. Theillet, R. Lidereau, C. Escot, P. Hutzell, M. Brunet, J. Gest, J. Schlom, R. Callahan, Loss of a c-H-*ras*-I allele and aggressive human primary breast carcinomas, Cancer Res. 46 (1986) 4776–4781.

[54] J. Mackay, P. Elder, D.I. Porteous, et al., Partial deletion of chromosome 11p in breast cancer correlates with size of primary turnout and estrogen receptor level, Br. J. Cancer 58 (1988) 710–714.

[55] K.-I. Takita, T. Sato, M. Miyagi, M. Watatani, F. Akiyama, G. Sakamoto, F. Kasumi, R. Abe, Y. Nakamura, Correlation of loss of alleles on the short arms of chromosomes 11 and 17 with metastasis of primary breast cancer to lymph nodes, Cancer Res. 52 (1992) 3914–3917.

[56] R. Winqvist, A. Mannermaa, M. Alavaikko, G. Blanco, P.J. Taskinen, H. Kiviniemi, I. Newsham, W. Cavenee, Refinement of regional loss of heterozygosity for chromosome 11pl5.5 in human breast tumors, Cancer Res. 53 (1993) 4486–4488.

[57] J. Gudmundsson, R.B. Barkardottir, G. Eiriksdottir, T. Baldursson, A. Arason, V. Egilsson, S. Ingvarsson, Loss of heterozygosity at chromosome 11 in breast cancer: association of prognostic factors with genetic alterations, Br. J. Cancer 72 (1995) 696–701.

[58] M. Negrini, S. Sabbioni, M. Ohta, M.L. Veronese, S. Rattan, C. Junien, C.M. Croce, Seven-megabase yeast artificial chromosome contig at region 11pl5: identification of a yeast artificial chromosome spanning the breakpoint of a chromosomal translocation found in a case of Beckwith–Wiedmann syndrome, Cancer Res. 55 (1995) 2904–2909.

[59] S. Carter, M. Negrini, R. Baffa, D.R. Gillum, A.L. Rosenberg, G.F. Schwartz, C.M. Croce, Loss of heterozygosity at 11q22–q23 in breast cancer, Cancer Res. 54 (1994) 6270–6274.

[60] J. Koreth, C. Bakkenist, J.O.D. McGee, Allelic deletions at chromosome 11q22–q23.1 and 11q25–q term are frequent in sporadic breast but not colorectal, Cancers Oncogene 14 (1997) 431–437.

[61] J.L. Weber, Human DNA polymorphisms based on length variations in simple sequence tandem repeats, in: S. Tilghman, K. Davies (Eds.), Genome Analysis Series: Genetic and Physical Mapping, Vol. 1, Cold spring Harbor Laboratory Press, New York, 1990, pp. 159–181.

[62] M. Litt, PCR of TG microsatellites, in: M.C. McPherson, P. Quirke, G. Taylor (Eds.), PCR: A Practical Approach, Oxford University Press, Oxford, 1991, pp. 85–99.

[63] J.L. Weber, Informativeness of human (dC-dA)n (dG) n-polymorphisms, Genomic 7 (1990) 524–530.

[64] Y. Huang, B. Bove, Y. Wu, I.H. Russo, Q. Tahin, X. Yang, A. Zekri, J. Russo, Microsatellite instability during the immortalization and transformation of human breast epithelial cells in vitro, Mol. Carcinog. 24 (1999) 118–127.

[65] M. Negrini, S. Sabbioni, S. Haldar, L. Possati, A. Castagnoli, A. Corallini, G. Barbanti-Brodano, C.M. Croce, Tumor and growth suppression of breast cancer cells by chromosome 17-associated functions, Cancer Res. 54 (1994) 1818–1824.

[66] A.L. Borresen, T.I. Andersen, J. Garber, N. Barbier-Piraux, S. Thorlacius, J. Eyfjord, L. Ottestad, B. Smith-Sorensen, E. Hovig, D. Malkin, Screening for germ line *TP53* mutations in breast cancer patients, Cancer Res. 52 (1992) 3234–3236.

[67] A. Puech, I. Henry, C. Jeanpierre, C. Junien, A highly polymorphic probe on 11p15.5: L22.5.2 (D11S774), Nucleic Acids Res. 19 (1991) 5095–5099.

[68] G.E. Hannigan, J. Bayani, R. Weksberg, B. Beatty, A. Pandita, S. Dedhar, J. Squire, Mapping of the gene encoding the integrin-linked kinase, ILK, to human chromosome 11pl5.5–pl5.4, Genomics 42 (1997) 177–179.

[69] H. Wang, N. Shao, Q.M. Ding, J. Cui, E.S. Reddy, V.N. Rao, BRCA1 proteins arc transported to the nucleus in the absence of serum and splice variants BRCA1a, BRCA1b are tyrosine phosphoproteins that associate with E2F, cyclins and cyclin dependent kinases, Oncogene. 15 (1997) 143–157.

[70] J.-T. Dong, P.W. Lamb, C.W. Rinker-Schaeffer, J. Vukanovic, T. Ichikawa, J.T. Isaacs, J.C. Barrett, KA/1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2, Science 268 (1995) 884–886.

[71] Y. Wei, M. Lukashev, D. Simon, et al., Regulation of integrin function by the urokinase receptor, Science 273 (1996) 1551–1555.

[72] G.M. Hampton, A. Mannermaa, R. Winquist, M. Alavaikko, G. Blanco, P.G. Taskinen, H. Kiviniemi, I. Newsham, W.K. Cavenee, G.A. Evans, Losses of heterozygosity in sporadic human breast carcinoma: a common region between 11q22, Cancer Res. 54 (1994) 4586–4589.

[73] M. Negrini, D. Rasio, G.M. Hampton, S. Sabbioni, S. Rattan, S.M. Carter, A.L. Rosenberg, G.F. Schwartz, Y. Shiloh, W.K. Cavenee, C.M. Croce, Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11q23.3, Cancer Res. 55 (1995) 3003–3007.

[74] R. Winqvist, G.M. Hampton, A. Mannermaa, G. Blanco, M. Alavaiko, H. Kiviniemi, P.J. Taskinen, G.A. Evans, F.A. Wright, I. Newsham, W.K. Cavenee, Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis, Cancer Res. 55 (1995) 2660–2664.

[75] A. Elson, Y. Wang, C.J. Daugherty, C.C. Morton, F. Zhou, J. Campos-Torres, P. Leder, Pleiotropic defects in ataxia–telangiectasia protein-deficient mice, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 13084–13089.

[76] C.H. Westphal, C. Schmaltz, S. Rowan, A. Elson, D.E. Fisher, P. Leder, Genetic interactions between *atm* and *p53* influence cellular proliferation and irradiation-induced cell cycle checkpoints, Cancer Res. 57 (1997) 1664–1667.

[77] M. Swift, D. Morrel, R. Massey, C. Chase, Incidence of cancer in 161 families affected by ataxia–telangiectasia, N. Eng. J. Med. 325 (1991) 1831–1836.

[78] P.J. Byrd, T. Stankovic, C.M. McConville, A.D. Smith, P.R. Cooper, A.M. Taylor, Identification and analysis of expression of human *VACM-1* a cullin gene family member located on chromosome 11q22–23, Genome Res. 7 (1997) 71–75.

[79] I.P. Tomlinson, H. Nicolai, E. Solomon, W.F. Bodmer, The frequency and mechanism of loss of heterozygosity on chromosome 1lq in breast cancer, J. Pathol. 180 (1996) 38–43.

[80] I.P. Tomlinson, N.E. Beck, W.F. Bodmer, All ele loss on chromosome 1lq and microsatellite instability in malignant melanoma, Eur. J. Cancer 32A (1996) 1797–1802.

[81] K.C. Connolly, H. Gabra, C.J. Millwater, K.J. Taylor, G.J. Rabiasz, J.E. Watson, J.F. Smyth, A.H. Wvllie, D.I. Jodrell, Identification of a region of frequent loss of heterozygosity at 11q24 incolorectal cancer, Cancer Res. 59 (1999) 2806–2809.

[82] V. Launonen, F. Stenback, U. Puistola, R. Bloiu, P. Huusko, S. Kytola, A. Kauppila, R. Winqvist, Chromosome 11q22.3–q25 LOH in ovarian cancer: association with a more aggressive disease course and involved subregions, Gynecol. Oncol. 71 (1998) 299–304.

[83] R. Dahiva, J. McCarville, C. Lee, W. Hu, G. Kaur, P. Carroll, G. Deng, Deletion of chromosome 1 lpl5, pl2, q22, q23–24 loci in human prostate cancer, Int. J. Cancer 72 (1997) 283–288.

[84] B. Vogelstein, E.R. Fearon, S.E. Kern, S.R. Hamilton, A.C. Preisinger, Y. Nakamura, R. White, Allelotype of colorectal carcinomas, Science 244 (1989) 207–211.

[85] T. Sato, A. Tanigami, K. Yamakawa, F. Akiyama, F. Kasumi, G. Sakamoto, Y. Nakamura, Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer, Cancer Res. 50 (1990) 7184–7189.

[86] P.A. Futreal, P. Söderkvist, J.R. Marks, J.D. Iglehart, C. Cochran, J.C. Barrett, R.W. Wiseman, Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms, Cancer Res. 52 (1992) 2624–2627.

[87] P. Devilee, C.J. Cornelisse, Genetics of human breast cancer, Cancer Surv. 9 (1990) 605–630.

[88] M. Holistein, D. Sidransky, B. Vogelstein, C.C. Harris, *p53* mutations in human cancers, Science 253 (1991) 49–53.

[89] J. Prosser, A.M. Thompson, G. Cranston, H.J. Evans, Evidence that *p53* behaves as a tumor suppressor gene in sporadic breast tumors, Oncogene 5 (1990) 1573–1579.

[90] E. Hovig, B. Smith-Sorensen, A. Brogger, A.L. Borresen, Constant denaturant gel electrophoresis, a modification of denaturating gradient gel electrophoresis, in mutation detection, Mutat. Res. 262 (1991) 63–71.

[91] R.J. Osborne, G.R. Merlo, T. Mitsudomi, T. Venesio, D.S. Liscia, A.P.M. Cappa, I. Chiba, T. Takahashi, M.M. Nau, R. Callahan, J.D. Minna, Mutations in the *p53* gene in primary human breast cancers, Cancer Res. 51 (1991) 6194–6198.

[92] A.M. Thompson, T.J. Anderson, A. Condie, J. Prosser, U. Chetty, D.C. Carter, H.J. Evans, C.M. Steel, *p53* allele losses, mutations and expression in breast cancer and their relationship to clinico-pathological parameters, Int. J. Cancer 50 (1992) 528–532.

[93] J.M. Varley, W.J. Brammar, D.P. Lane, J.E. Swallow, C. Dolan, R.A. Walker, Loss of chromosome 17p13 sequences and mutation of *p53* in human breast carcinomas, Oncogene 6 (1991) 413–421.

[94] P.J. Biggs, N. Warren, S. Venitt, M.R. Stratton, Does a genotoxic carcinogen contribute to human breast cancer-7, Mutagenesis 8 (1993) 275–283.

[95] R. Kirchweger, R. Zeillinger, C. Schneeberger, P. Speiser, G. Louason, C. Theillet, Patterns of allele losses suggest the existence of five distinct regions of LOH on chromosome 17 in breast cancer, Int. J. Cancer 56 (1994) 193–199.

[96] I. Jantke, W. Jonat, H. Maass, H.W. Goedde, Human breast cancer: frequent p53 allele loss and protein overexpression, Hum. Genet. 90 (1993) 635–640.

[97] T. Anderson, A. Gaustad, L. Ottestad, G.W. Farrants, J.M. Nesland, K.M. Tveit, A.L. Borresen, Genetic alterations of the tumor suppressor gene regions 3p, 11p, 13q, 17p, and 17q in human breast carcinomas, Genes, Chromosomes & Cancer 4 (1992) 113–121.

[98] E.S. Goldman, D. More II, M. Balazs, V.E. Li, Loss of heterozygosity on the shortarm of chromosome 17 is associated with high proliferative capacity and DNA aneuploidy in primary human breast cancer, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 3847–3851.

[99] C. Coles, A.M. Thompson, P.A. Elder, B.B. Cohen, I.M. Mackenzie, G. Cranston, U. Chetty, J. Mackay, M. Macdonald, Y. Nakamura, Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis, Lancet 336 (1990) 761–763.

[100] J. Russo, Y.F. Hu, X. Yang, Y. Huang, I. Silva, B. Bove, N. Higgy, I.H. Russo, Breast cancer multistage progression, Front. Biosci. 3 (1998) 944–960.

[101] A. Lindblom, S. Rotstein, L. Skoog, M. Nordenskjöld, C. Larsson, Deletions on chromosome 16 in primary familial breast carcinomas are associated with development of distant metastases, Cancer Res. 53 (1993) 3707–3711.

[102] H. Tsuda, D.F. Callen, T. Fukutomi, Y. Nakamura, S. Hirohashi, Allele loss on chromosome 16q24.2-qter occurs frequently in breast cancers irrespectively of differences in phenotype and extent of spread, Cancer Res. 54 (1994) 513–517.

The role of estrogens in human breast cancer: a mechanistic view

4

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INTRODUCTION

Epidemiological and clinical evidence indicates that breast cancer risk is associated with prolonged exposure to female ovarian hormones, mainly since a greater incidence of this disease is associated with early onset of menarche and late menopause, two conditions directly regulated by ovarian function[1–4]. Although breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors[5,6], estrogens have been demonstrated to be of essential importance in this phenomenon. This postulate has been further supported by the greater cancer risk observed in postmenopausal hyperestrogenism resulting from the use of estrogenic hormone replacement therapy and obesity[7,8].

Estrogens, which are necessary for the normal development of both reproductive and non-reproductive organs, exert their physiological effects by binding to their specific receptors, the estrogen receptors (ER) α or β[9–15]. ERα resides in the nucleus of target cells in an inactive form associated with a large inhibitory protein complex. Both endogenous and exogenous estrogens, such as 17β-estradiol and the synthetic non-steroidal estrogen diethylstilbestrol (DES), bind to the C-terminal ligand-binding domain (LBD) of the ERα, activating the receptor, which undergoes a conformational change. The activated receptor undergoes dimerization, participating in the regulation of target gene transcription by one of two mechanisms: first, binding to transcription factors, such as AP-1, forming a complex that recruits transcriptional co-activators, i.e. the steroid receptor co-activator protein 1 (SRC-1)[16], or second, the ER can form a ternary complex with a co-activator protein after its direct interaction with specific regulatory sequences within target gene promoters[9–11]. Estrogens might also act through alternative non-receptor-mediated pathways. It has been recently found that overexpression of p21 in a p21-negative, ER-negative cell line induced both the ER and estrogen response element (ERE) promoters in an estrogen-responsive manner. Stable p21 clones that also lack the expression of wild-type ERE were responsive to the growth inhibitory effect of ICI-182 780, a potent antiestrogen, and the growth stimulatory effects of 17β-estradiol[17].

Estradiol, under the effect of 17β-oxidoreductase, is continuously interconverted to estrone, and both are hydroxylated at C-2, C-4, or C-16α positions by cytochrome P450 isoenzymes, i.e. CYP1A1, CYP1A2, or CYP1B1, to form catechol estrogens[18–23]. The demonstration that the catecholestrogen 4-hydroxyestradiol induces an estrogenic response in the uterus of ERα null mice, and the fact that this response is not inhibited by the antiestrogen ICI-182 780[9], indicate that this catecholestrogen does not exert its effect on the ER. There is also evidence that estrogen may not need to activate its nuclear receptors to initiate or promote breast carcinogenesis. The metabolic activation of estrogens can be mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct

genotoxic effects by increasing mutation rates. An increase in colony efficiency 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 via a Michael addition, and thus serve as the ultimate carcinogenic reactive intermediates in the peroxidatic activation of colony efficiency. Thus, estrogen and 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[18–23]. Although this pathway has been demonstrated in other systems, it still needs to be demonstrated in normal breast epithelial cells.

Breast cancers exhibit genomic alterations, such as DNA amplification and loss of genetic material that may represent tumor suppressor genes[24–39]. Although their role in the causation of the disease has not been clearly established, it is generally accepted that the accumulation of genetic alterations promotes tumor progression[38,39]. Specific types of genetic alterations, then, might identify essential steps in the initiation and/or progression of cancer. We postulate that if estrogens initiate the neoplastic process or are responsible for its progression, they would induce in the normal breast epithelium the same type of genomic alterations observed in spontaneous malignancies. In order to test this hypothesis we evaluated the transforming potential of estradiol and DES on human breast epithelial cells (HBEC) *in vitro*, utilizing the spontaneously immortalized HBEC MCF-10F[40,41]. This cell line lacks both ERα and ERβ, although this latter receptor is induced in cells transformed by chemical carcinogens[42]. The same phenotypes and characteristics that were expressed by MCF-10F cells transformed by the chemical carcinogen benz(a)pyrene (BP) and oncogenes[43–46] were evaluated in estradiol- and DES-treated cells: anchorage-independent growth, colony formation in agar methocel, ductulogenic capacity in collagen gel and invasiveness index in Matrigel. In addition, DNA of treated cells was analyzed for specific genomic alterations such as loss of heterozygosity (LOH) at chromosomal loci known to be affected in spontaneously occurring breast lesions, such as ductal hyperplasia, carcinoma *in situ* and invasive carcinoma[47–60].

PROOF OF PRINCIPLE

In order to determine the optimal doses for the expression of the cell transformation phenotype, we treated the immortalized HBEC MCF-10F with 17β-estradiol for testing whether they expressed colony formation in agar methocel, or colony efficiency, and loss of ductulogenesis in collagen matrix, phenotypes also induced by the carcinogen BP[44,45]. MCF-10F cells were treated with 0.0, 0.007 nmol/l. 70 nmol/l, or 0.25 mmol/l 17β-estradiol twice a week for 2 weeks. Colony efficiency increased from 0 in controls to 6.1, 9.2 and 8.7 with increasing estradiol doses (Figure 1). Ductulogenesis was 75 ± 4.9 in control cells; it decreased to 63.7 ± 28.8, 41.3 ± 12.4 and 17.8 ± 5.0 in estradiol-treated cells (Figures 2 and 3a), which also formed solid masses (Figure 3b), whose numbers increased from 0 in controls to 18.5 ± 6.7, 107 ± 11.8 and 130 ± 10.0 for each estradiol dose (Figure 2). Based upon these results, a dose of 3.7 μmol/l (1 μg/ml) was selected for testing the effect of estradiol or DES.

EVALUATION OF THE EFFECT OF ESTROGENS ON THE EXPRESSION OF CELL TRANSFORMATION PHENOTYPES

The spontaneously immortalized MCF-10F cells were treated with 1.0 μg/ml 17β-estradiol or DES; control cells were treated with dimethyl sulfoxide (DMSO). MCF-10F cells treated with 1.0 μg/ml BP served as positive controls for cell transformation assays. In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were first treated with 17β-estradiol, DES, or BP at 72 h and 120 h post-plating. At the end of the first week of treatment, the cells were divided for evaluation of specific phenotypic charac-

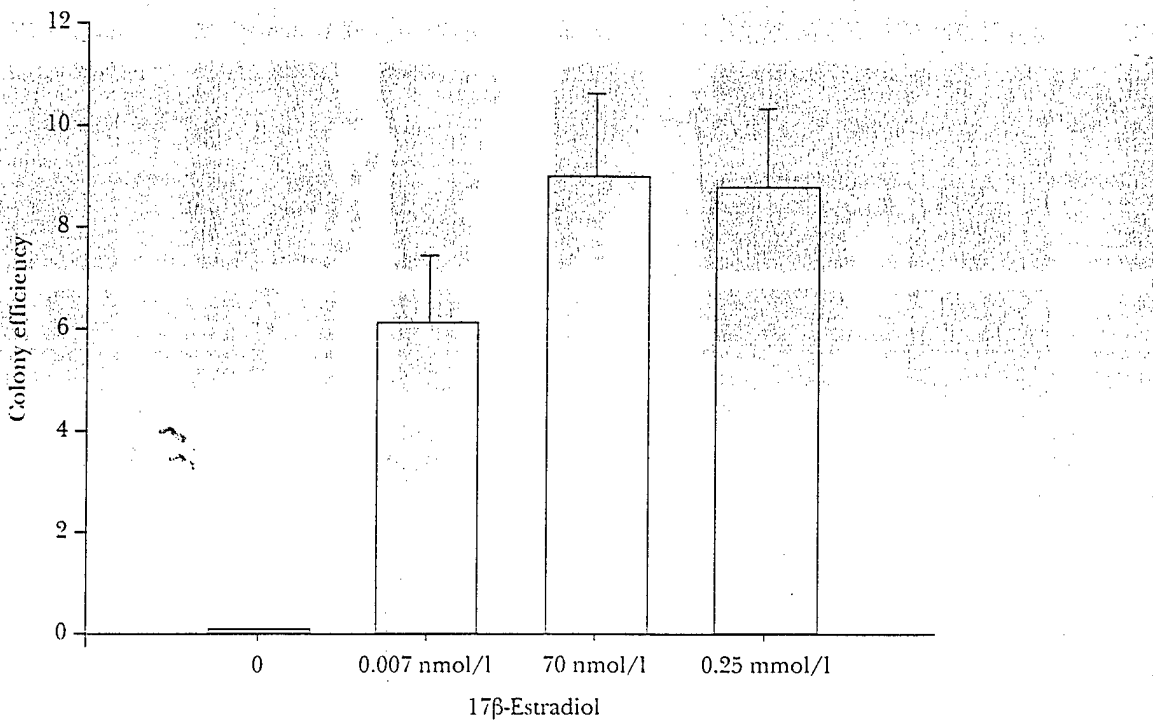

Figure 1 Histogram showing the colony efficiency in agar–methocel of MCF-10F cells treated with different concentrations of 17β-estradiol

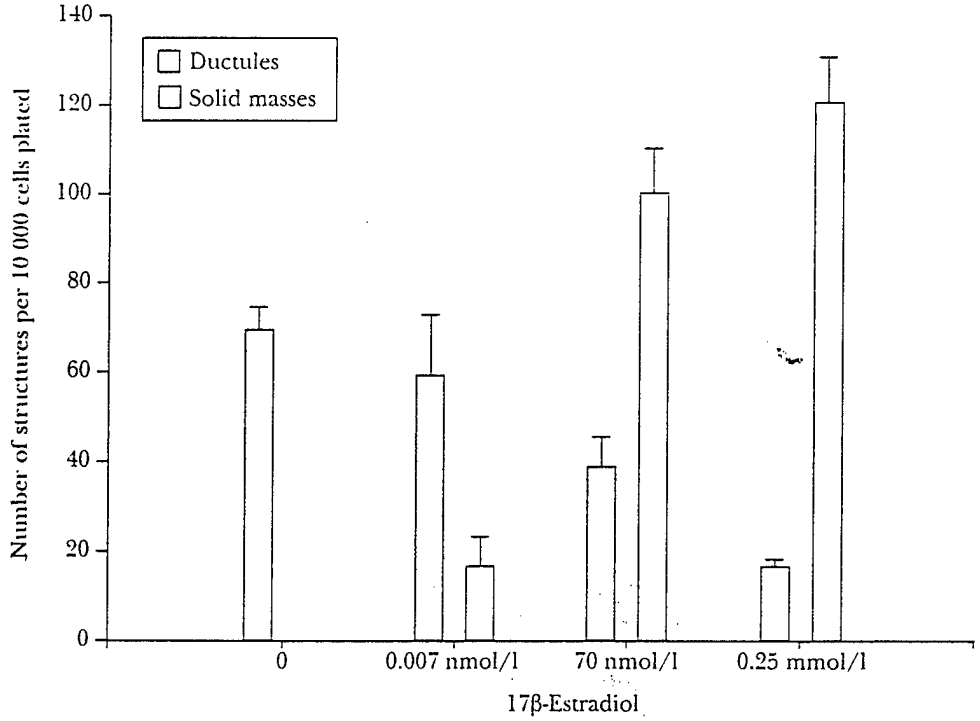

Figure 2 Histogram showing the decrease in ductule formation in a collagen matrix and the increase in the formation of solid masses at increasing concentrations of 17β-estradiol

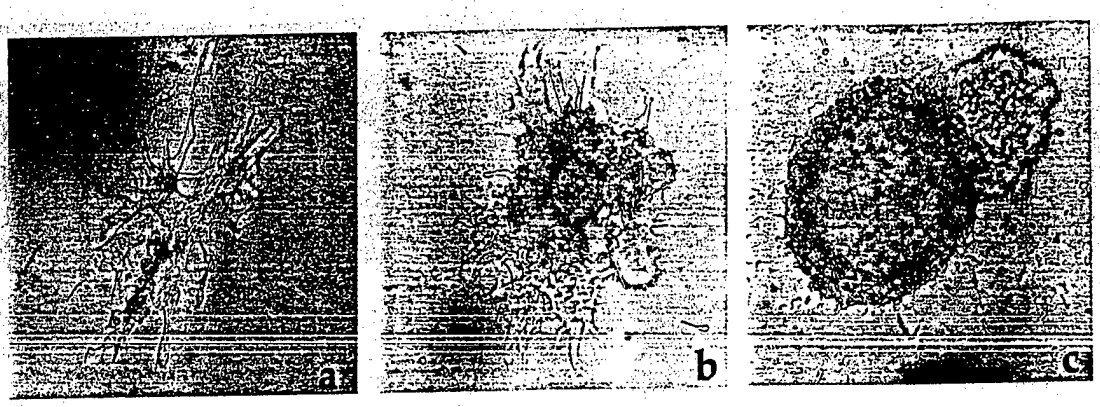

Figure 3 (a) Ductules formed in a collagen matrix by MCF-10F cells; (b) the ductular pattern is distorted by 0.007 nmol/l of 17β-estradiol; (c) formation of solid masses by MCF-10F cells treated with 70 nmol/l of 17β-estradiol

teristics or they were passaged for administration of another two periods of hormonal treatment. Treatments were repeated during the second week, and cells were collected on the 14th 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 doubling time, survival efficiency, colony efficiency, colony size and ductulogenic capacity.

The doubling time of MCF-10F cells was 93 ± 5.6 h. It was decreased, but not significantly, in estradiol- and DES-treated cells and their derived clones. A significant decrease, greater than 50%, was observed in BP-treated cells (Table 1). Evaluation of colony formation at the end of the second week of treatment revealed that MCF-10F cells treated with estradiol, DES, or BP, formed colonies in agar–methocel, whereas MCF-10F control cells treated with DMSO did not (Table 1, Figure 4). Estradiol-treated cells formed 24 colonies, from which six clones were expanded and maintained in culture. These clones were designated E_2-1 to E_2-6 (Table 1). DES-treated MCF-10F cells formed 151 colonies, from which 24 colonies were isolated and seven clones survived. They were expanded and maintained in culture, being designated DES-1 to DES-7 (Table 1). BP-treated cells formed 89 colonies, which had an average size of 670 ± 46 μm in diameter. Those colonies formed by DES- and estradiol-treated cells and their respective derived clones were significantly smaller than those formed by BP-transformed cells. However, DES-treated cell colonies were larger than those formed by estradiol-treated cells and their derived clones (Table 1, Figure 4).

Ductulogenesis was qualitatively evaluated by estimating the ability of the cells plated in collagen to form tubular and ductular structures. It was maximal (++) in MCF-10F cells, and completely negative (–) in BP-treated cells, which grew as a solid or cystic mass (Table 1). Estradiol- and DES-treated cells, and E_2-4 and DES-1 clones, exhibited a moderately decreased ability to form ductules (+). It was interesting to observe that all the other clones derived from estradiol- and DES-treated cells exhibited an overall decrease of ductulogenic capacity. Clones E_2-3, E_2-6 and DES-5 had completely lost this property, being in this sense similar to BP-treated cells (Table 1).

GENOMIC CHANGES INDUCED IN ESTRADIOL- AND DIETHYLSTILBESTROL-TRANSFORMED MCF-10 CELLS

Looking for allelic losses, we evaluated the regions of chromosomes 1, 2, 3, 6, 8, 9, 11, 12, 13, 16, 17 and 18 most frequently reported to exhibit LOH in spontaneous breast tumors.

Table 1 Phenotypic markers of cell transformation induced in MCF-10F cells by 17β-estradiol (E_2), diethylstilbestrol (DES) and benz(a)pyrene (BP). Reprinted from reference 117 with permission

Cell type	*No. of passages*	*Doubling time* (h)	*Colony number*	*Colony efficiency* (%)	*Colony size* (μm)	*Ductulogenesis*
MCF-10F	113	93 ± 5.6	0.0	0.0	0.0	++
BP	4	42 ± 3.8	89	18 ± 4.5	670 ± 46	–
E_2	4	78 ± 16.0	24	4.8 ± 0.9	170 ± 34	+
DES	4	73 ± 13	151	30.20 ± 8.9	190 ± 23	+
E_2-1*	4	81 ± 3.0	36	7.2 ± 3.7	180 ± 12	+/–
E_2-2*	4	68 ± 10	45	9.0 ± 2.0	150 ± 6	+/–
E_2-3	5	66 ± 8.0	39	7.9 ± 5.6	190 ± 9	–
E_2-4	3	82 ± 6.0	20	3.5 ± 1.1	134 ± 5	+
E_2-5	6	61 ± 5.6	63	12.6 ± 3.0	193 ± 12	+/–
E_2-6	4	73 ± 3.0	54	10.8 ± 4.9	189 ± 5	–
DES-1*	4	73 ± 9	167	33.17 ± 6.3	278 ± 40	+
DES-2	4	74 ± 10	148	29.4 ± 10.0	189 ± 23	+/–
DES-3*	3	78 ± 5	130	25.8 ± 3.9	278 ± 12	+/–
DES-4*	3	75 ± 8	189	37.5 ± 7.3	239 ± 34	+/–
DES-5*	6	68 ± 10	167	33.17 ± 5.9	360 ± 60	–
DES-6	5	67 ± 3	150	29.8 ± 10	290 ± 32	+/–
DES-7	5	78 ± 4	99	19.6 ± 6.9	207 ± 28	+/–

Doubling time was determined as described in reference 43. Doubling time was significantly different by Student's t test between BP and all the other cells lines($p < 0.001$). Colony number, colony efficiency and colony size were significantly different between MCF-10F and all other cell lines ($p = 0.00001$). Colony size of DES clones was significantly different from E_2 and BP cells ($p = 0.001$). From 24 colonies derived from E_2-treated cells, clones E_2-1, -2, -3, -4, -5 and -6 were recovered and expanded. From 151 colonies derived from DES-treated cells, 24 colonies were isolated clones DES-1, -2, -3, -4, -5, -6 and -7 were recovered and expanded. Ductulogenesis, duct-like formation in collagen gel. *These cells have been used for detection of microsatellite DNA polymorphism

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 base pair) runs of tandem repeated di- to tetranucleotide sequence motifs[61–63]. 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 proved 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[64]. LOH was defined as a total loss, or more than 50% reduction, of 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.

Among 67 markers tested, which were selected on the basis of chromosomal changes reported to be present in breast and other cancers, only clones DES-5, and E_2-1 and E_2-2, exhibited LOH in chromosomes 3 and 11, respectively. LOH in chromosome 3 was detected at three different loci, which were detected with five different markers: 3p21.3-21.2 (markers D3S1478 and D3S2384), 3p21.1-14.2 (marker D3S1450) and 3p21 (marker D3S1217 and D3S1447) (Figure 5). It

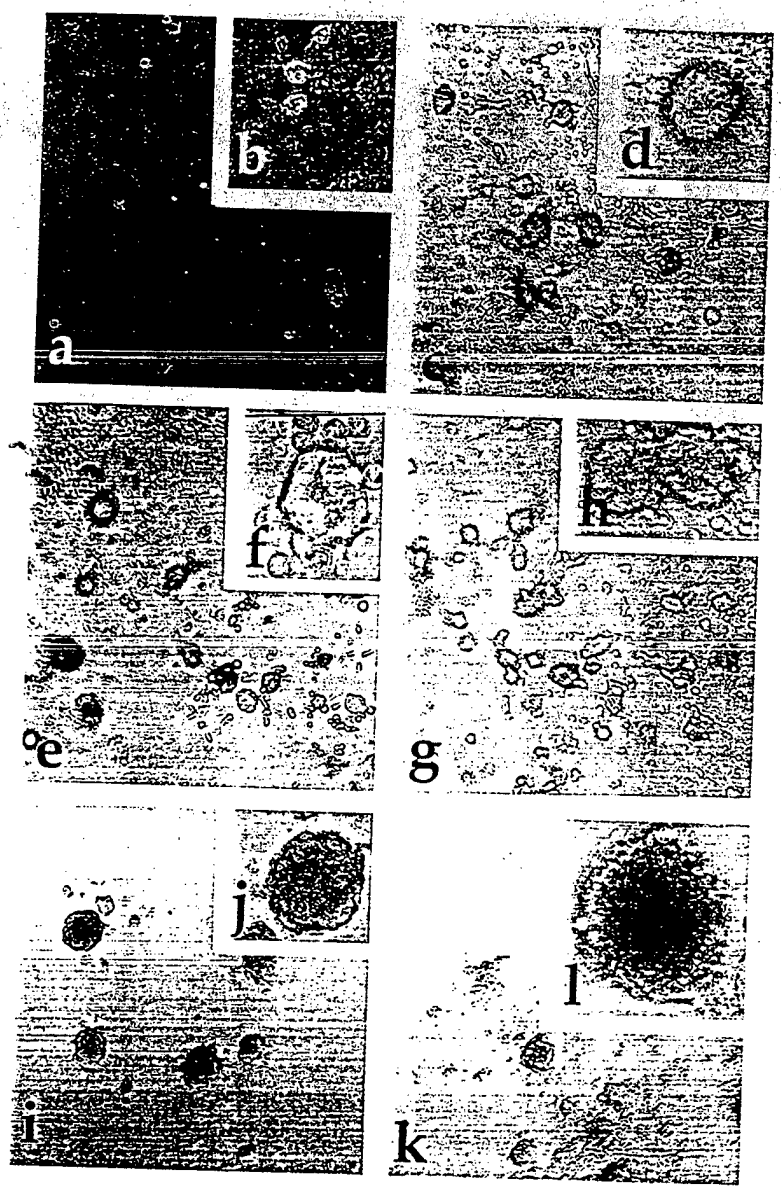

Figure 4 MCF-10F cells plated in agar–methocel for colony assay. Control cells did not form colonies. Only isolated cells are present (a, 4×; b, 10×). (c–k) Colonies formed by 17β-estradiol (E_2)-, diethylstilbestrol (DES)- and benzo(a)pyrene (BP)-treated MCF-10F cells. E_2-induced colonies (c, 4×; d, 10×); DES-induced colonies (e, 4×; f, 10×); colonies of E_2-2 clone (g, 4×; h, 10×), colonies of DES-3 clone (i, 4×; j, 10×); colonies of BP-treated cells (k, 4×; l, 10×). Phase contrast. Reprinted with permission from reference 117

was of interest that clone DES-5, in addition to exhibiting LOH in chromosome 3, was the one exhibiting the most marked expression of transformation phenotypes, i.e. larger colony size and absent ductulogenic ability in collagen gel (Table 1). Clones E_2-1 and E_2-2 identically expressed LOH in chromosome 11 at 11q23.3 (marker D11S29), and 11q24.2-11q25 (marker D11S912). BP-treated cells did not exhibit LOH at any of the loci tested (Figure 6). Interestingly, we have found that all the clones of the cells transformed with either estradiol, DES or BP presented microsatellite instability (MSI), expressed as an allelic expansion at

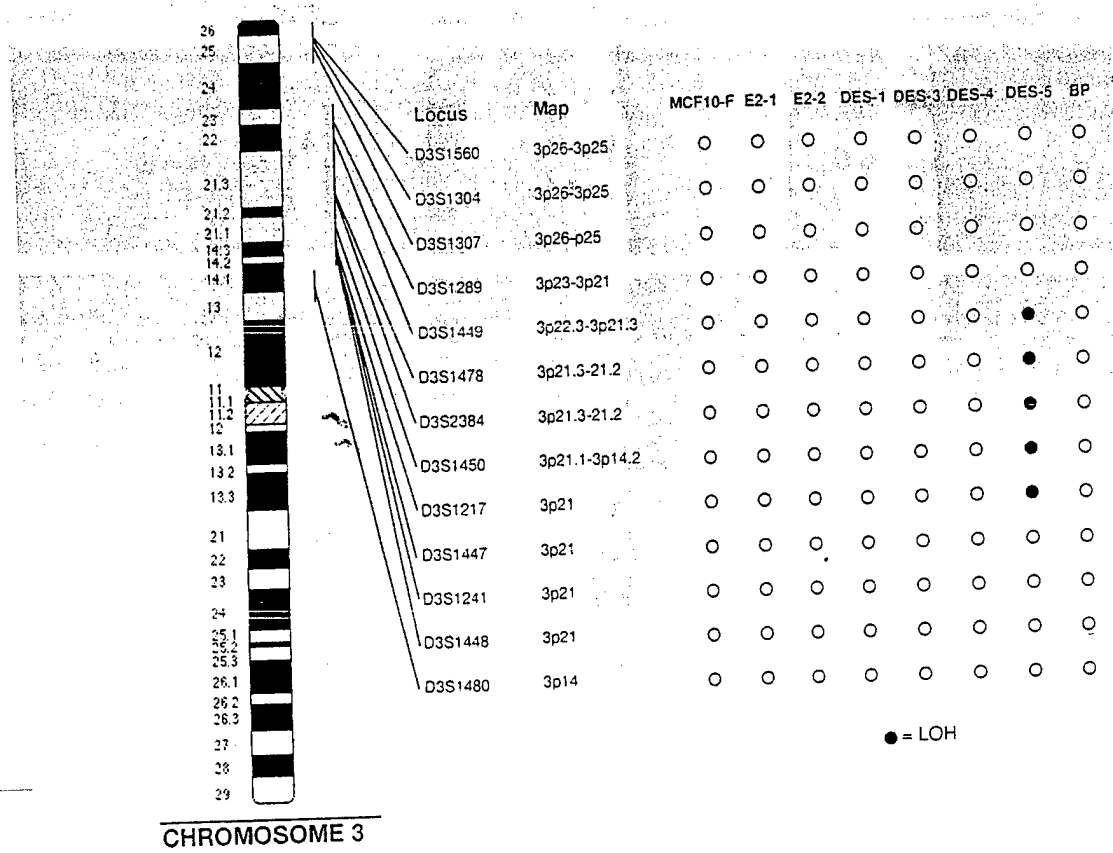

Figure 5 Ideogram of chromosome 3, showing the loss of heterozygosity (LOH) in clones E_2-1 and E_2-2 derived from 17β-estradiol-transformed MCF-10F cells

3p21 locus (marker D3S1447) (data not shown). In order to determine whether these MSIs were related to alterations in mismatch repair genes, we performed microsatellite DNA analysis in loci 1p13.1, with marker BAT40, 2p16, with marker D2S123, and 18q22.3-23, with marker D18S58, which are related to mismatch repair genes. However, none of these markers showed alterations with this technique.

CORRELATION BETWEEN GENOTYPE AND PHENOTYPE

In the present work we have capitalized on the availability in our laboratory of an *in vitro* model of transformation of immortalized HBEC by the chemical carcinogen BP for comparison with phenotypic and genomic changes induced by the natural estrogen 17β-estradiol and the synthetic estrogen DES in the same cells[44,45]. The immortalized human breast epithelial cells MCF-10F are negative for both ERα and ERβ[42]. Short-term treatments of these cells with these two estrogenic compounds induce 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. It was of great interest that by the fourth passage after four treatments during a 2-week period, clones derived from DES- and estradiol-transformed cells exhibited loss of heterozygosity in chromosomes 3 and 11, respectively, whereas during the same period of time the chemical carcinogen BP did not

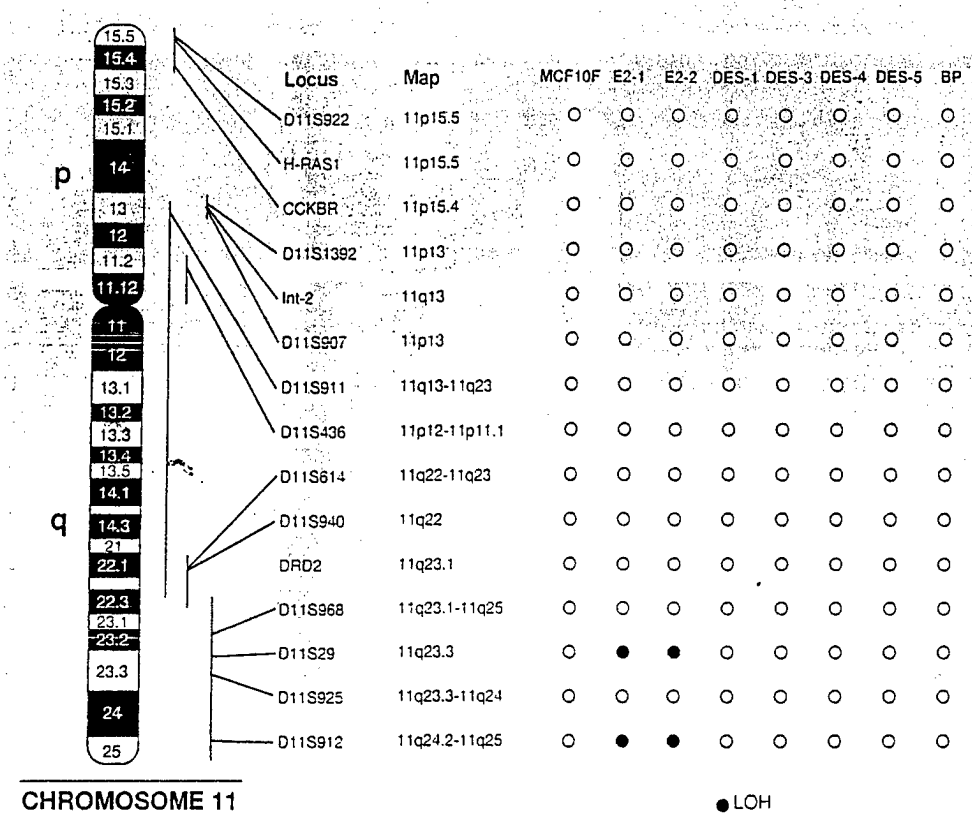

Figure 6 Ideogram of chromosome 11 showing the loss of heterozygosity (LOH) in the DES-5 clone derived from diethylstilbestrol-transformed MCF-10F cells

induce genomic changes, even though we have previously reported that this carcinogen induces LOH in chromosome 17[43], in addition to tumorigenesis in a heterologous host after a larger number of passages and a more prolonged selection process *in vitro*[44,45]. The expression of LOH in chromosome 3 in DES-transformed breast epithelial cells acquired relevance in view of the reports that frequent homozygous deletions, rearrangements and hypermethylation at 3p21 loci have been found to be present in spontaneously occurring breast lesions, such as ductal hyperplasia, carcinoma *in situ* and invasive carcinoma[31,47–50,65–68]. The existence of suppressor genes on 3p has also been suggested by transfection studies in which 3p DNA fragments inhibited tumorigenesis in nude mice[68,69]. We have observed LOH at 3p21 using markers D3S1217 and D3S1447, and in the region 3p21.1-p21.2 with markers D3S1478 and D3S2384. LOH in this region has been reported in nearly all small-cell lung carcinomas[70]. Even though deletion in these regions is not considered to be specific for breast cancer, our observations might indicate that they represent a genetic event triggered by estrogens, which could play a key role in the development and progression of tumors originated from this type of epithelium. LOH in 3p21.3 has been found more frequently in breast cancer metastases than in primary tumors. Several putative 'metastasis-related genes' are located in this region, such as 37LRP[71–74], CTNNB1, which encodes β catenin[75], and the αRLC gene, which encodes a new integrin subunit, identified by positional cloning, that shows homology with the α1 integrin involved in the metastatic process[70]. We have also found LOH in the 3p21.1-14.2 (marker D3S1450). This region has been found to be associated with dysregulated cell

proliferation rather than with tumor progression[50]. It is also frequently deleted in *in situ* carcinoma, benign tumors and familial breast cancers[51,76,77]. The 3p14.2 region contains a fragile site known as FRA3B, from which the FHIT gene has recently been cloned. It encodes a protein showing homology with a yeast hydrolase, and its transcripts show rearrangements in different cell lines and tumors[52,77]. Recently, telomerase-regulating genes have been located in 3p21.3-p22 and 3p12-21.1 using the microcell monochromosome transfer technique[78]. We have also found that estrogen induces LOH in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2-25, respectively. It has been reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice[79]. Several genes, such as HRAs, CTSD, ILK, TSG101 and KI1, have been reported to be located on the short arm of chromosome 11[53–54,79–85]. A region of deletion on 11q22-23 has been described on the long arm of chromosome 11 in 40–60% of breast tumors[51,57,59,60,86,88]. The ataxia telangiectasia susceptibility gene (ATM) is the most widely studied candidate gene in this region[89]. ATM may act upstream of the TP53 gene in cell cycle regulation[90,91] and its heterozygous mutation is associated with a high incidence of early-onset breast cancer. This region has been reported to contain several tumor suppressor genes and genes involved in the metastatic process. In this latter group, the MMP genes encoding matrix metalloproteases involved in invasion, ETS1 encoding a transcription factor involved in angiogenesis, and VACM-1 encoding a protein probably involved in cell cycle regulation, have been identified[92]. Although some of these genes might be affected during the transformation of HBEC induced by estrogens, a more detailed allelotyping using multiple markers is required for better definition of the significance of LOH in these cells.

Approximately 35% of breast cancers show LOH at the D11S29 and NCAM loci[93], and a higher frequency of LOH at this locus has also been found in melanomas[94]. LOH has been found at frequencies of 25% and 29% at the distal D11S968 (11qter) and D11S29 (11q23.3 locus), slightly above the accepted baseline of 0–20% in colorectal cancer. The fact that breast cancer, melanoma and colorectal cancer have been found to be influenced by estrogens[95] gives relevance to our data that treatment of MCF-10F cells with estrogens induces LOH in this specific locus. LOH at 11q23-qter occurs frequently in ovarian and other cancers[96,97].

The most frequent allelic loss observed in breast cancer has been reported in chromosome 17p, suggesting that genes located in that chromosome arm, such as the p53 oncogene, might be a likely target for this event[33,93–114]. To date, we have not been able to demonstrate any LOH in chromosome 17 in estrogen-transformed MCF-10F cells. However, we have used a small number of markers, and the possibility that LOH might be located at sites not yet tested cannot be ruled out. Therefore, the study of allelic imbalances at 17q and 17p, as well as in chromosome 16[99,115,116] in estrogen-transformed HBEC must be carried out to provide further understanding of the functional involvement of these chromosomes in the process of cell transformation by 17β-estradiol and DES.

The observations that 17β-estradiol, DES and BP induce similar phenotypical, but different genomic, alterations requires further investigation in order to elucidate the significance of the timing of the appearance of each type of change with regards to cancer initiation and progression. There are several probable avenues for explaining these discrepancies. In this model, phenotypic changes were induced by both estrogens and the chemical carcinogen as an early event, whereas LOH was a rare event that was manifested in different chromosomes and in only a few clones derived from estradiol- and DES-treated cells. The rarity of the phenomenon is in agreement with the low frequency of LOH observed in BP-transformed cells, in which the phenomenon was manifested at a more advanced stage of neoplastic progres-

sion[43,114]. Altogether these observations suggest that these three compounds might act through different genetic events for inducing similar transformation phenotypes.

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References

1. Pike MC, Spicer DV, Dahmoush L, Press MF. Estrogens, progesterone, normal breast cell proliferation, and breast cancer risk. *Epidemiol Rev* 1993;15:17–35
2. Kelsey JL, Gammon MD, John EM. Reproductive factors and breast cancer. *Epidemiol Rev* 1993;15:36–47
3. Bernstein L, Ross RK. Endogenous hormones and breast cancer risk. *Epidemiol Rev* 1993;15: 48–65
4. Henderson BE, Ross R, Bernstein L. Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res* 1988;48:246–53
5. Topper YJ, Sankaran L, Chomczynski P, Prosser C, Qasba P. Three stages of responsiveness to hormones in the mammary cell. In Angeli A, Bradlow HL, Dogliotti L, eds. *Endocrinology of the Breast: Basic and Clinical Aspects. Annals of the New York Academy of Sciences* 1986;464:1–10
6. Lippman ME, Huff KK, Jakesz R, *et al.* Estrogens regulate production of specific growth factors in hormone-dependent human breast cancer. In Angeli A, Bradlow HL, Dogliotti L, eds. *Endocrinology of the Breast: Basic and Clinical Aspects. Annals of the New York Academy of Sciences* 1986;464:11–16
7. Dupont WD, Page DL. Menopausal estrogen replacement therapy and breast cancer. *Arch Intern Med* 1991;151:67–72
8. Price MA, Tennant CC, Smith RC, *et al.* Predictors of breast cancer in women recalled following screening. *Aust NZ J Surg* 1999;69: 639–46
9. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* 1999;20:358–417
10. Shiau AK, Barstad D, Loria PM, *et al.* The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998;95: 927–37
11. McDonnell DP. The molecular pharmacology of SERMs. *Trends Endocrinol Metab* 1999;10: 301–11
12. Tsai MJ, O'Malley BW. Molecular mechanisms of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 1994;63:451–86
13. Katzenellenbogen BS. Dynamics of steroid hormone receptor action. *Annu Rev Physiol* 1980;42:17–35
14. Mosselman S, Polma J, Dijkema R. ER β: identification and characterization of a novel human estrogen receptor. *FEBS Lett* 1996;392: 49–53
15. Kuiper GGJM, Carlsson B, Grandien K, *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* 1997;138: 863–70
16. Paech K, Webb P, Kuiper GG, *et al.* Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 1997;277: 1508–10
17. Chen X, Danes C, Lowe M, Herliczek TW, Keyomarsi K. Activation of the estrogen-signaling pathway by p21 WAF1/CIP1 in estrogen receptor negative breast cancer cells. *J Natl Cancer Inst* 2000;92:1403–13
18. Liehr JG, Ulubelen AA, Strobel HW. Cytochrome P-450-mediated redox cycling of estrogens. *J Biol Chem* 1986;261:16865–70
19. Roy D, Liehr JG. Temporary decrease in renal quinone and reductase activity induced by chronic administration of estradiol to male Syrian hamsters – increased superoxide formation by redox cycling of estrogen. *J Biol Chem* 1988;263:3646–51
20. Yan Z-J, Roy D. Mutations in DNA polymerase P mRNA of stilbene estrogen-induced kidney tumors in Syrian hamster. *Biochem Mol Biol Int* 1997;37:175–83
21. Ball P, Knuppen R. Catecholestrogens (2- and 4-hydroxy-oestrogens). Chemistry, biosynthesis, metabolism, occurrence and physiological significance. *Acta Endocrinol (Copenh)* 1980;232 (Suppl):127
22. Zhu BT, Bui QD, Weisz J, Liehr JG. Conversion of estrone to 2- and 4-hydroxyestrone by hamster kidney and liver microsomes: implications for the mechanism of estrogen-induced carcinogenesis. *Endocrinology* 1994;135:1772–9
23. Ashburn SP, Han X, Liehr JG. Microsomal hydroxylation of 2- and 4-fluoroestradiol to catechol metabolites and their conversion to methyl ethers: catechol estrogens as possible

mediators of hormonal carcinogenesis. *Mol Pharmacol* 1993;43:534–41
24. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. *Science* 1987;235:177–82
25. Escot C, Theillet C, Lidereau R, *et al.* Genetic alteration of the *c-myc* proto-oncogene (*MYC*) in human primary breast carcinomas. *Proc Natl Acad Sci USA* 1986;83:4834–8
26. Ali IU, Merio G, Callahan R, Lidereau R. The amplification unit on chromosome 11 q13 in aggressive primary human breast tumors entails the *bcl*-1, *int*-2 and *hst* loci. *Oncogene* 1989;4: 89–92
27. Theillet C, Adnane J, Szepetowski P, *et al.* BCL-1 participates in the 11q13 amplification found in breast cancer. *Oncogene* 1990;5:147–9
28. Theillet C, Lidereau R, Escot C, *et al.* Loss of a c-H-*ras*-1 and aggressive human primary breast carcinomas. *Cancer Res* 1986;46:4776-81
29. Lundberg C, Skoog L, Cavenee WK, Nordenskjoid M. Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. *Proc Natl Acad Sci USA* 1987;84:2372–6
30. Mackay J, Steel CM, Elder PA, Forrest APM, Evans HJ. Allele loss on short arm of chromosome 17 in breast cancers. *Lancet* 1988;2:1384–5
31. Ali IU, Lidereau R, Callahan R. Presence of two members of *c-erbAB* and *c-erbA2* in smallest region of somatic homiozygosity on chromosome 3p2l-p25 in human breast carcinoma. *J Natl Cancer Inst* 1989;81:1815–20
32. Chen L-C, Dolibaum C, Smith H. Loss of heterozygosity on chromosome lq in human breast cancer. *Proc Natl Acad Sci USA* 1989;86: 7204–7
33. Callahan R, Campbell A. Mutations in human breast cancer: an overview. *J Natl Cancer Inst* 1989;81:1780–6
34. Sato T, Saito H, Swensen J, *et al.* The human prohibitin gene located on chromosome 17q2l is mutated in sporadic breast cancer. *Cancer Res* 1992;52:1643–6
35. Chen LC, Kurisu W, Ljung BM, Goldman ES, Moore D II, Smith HS. Heterogeneity for allelic loss in human breast cancer. *J Natl Cancer Inst* 1992;84:506–10
36. Genuardi M, Tsihira N, Anderson DE, Saunders GF. Distal deletion of chromosome lq in ductal carcinoma of the breast. *Am J Hum Genet* 1989;45:73–89
37. Crop CS, Lidereau R, Campbell G, Champene M-H, Callahan R. Loss of heterozygosity on chromosomes 17 and 18 in breast carcinoma: two additional regions identified. *Proc Natl Acad Sci USA* 1990;87:7737–41
38. Sato T, Tanigami A, Yamakawa K, *et al.* Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res* 1990;50:7184–9
39. Sato T, Akiyama F, Sakamoto G, Kasumi F, Nakamura Y. Accumulation of genetic alterations and progression of primary breast cancer. *Cancer Res* 1991;51:5794–9
40. Soule HD, Maloney TM, Wolman SR, *et al.* Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res* 1990;50:6075–86
41. Tait L, Soule H, Russo J. Ultrastructural and immunocytochemical characterizations of an immortalized human breast epithelial cell line MCF-10. *Cancer Res* 1990;50:6087–99
42. Hu YF, Lau KM, Ho SM, Russo J. Increased expression of estrogen receptor-β in chemically transformed human breast epithelial cells. *Int J Oncol* 1998;12:1225–8
43. Russo J, Calaf G, Sohi N, *et al.* Critical steps in breast carcinogenesis. *NY Acad Sci* 1993;698: 1–20
44. Calaf G, Russo J. Transformation of human breast epithelial cells by chemical carcinogens. *Carcinogenesis* 1993;14:483–92
45. Russo J, Calaf G, Russo IH. A critical approach to the malignant transformation of human breast epithelial cells. *CRC Crit Rev Oncogenesis* 1993;4:403–17
46. Calaf G, Zhang PL, Alvarado MV, Estrada S, Russo J. C-Ha *ras* enhances the neoplastic transformation of human breast epithelial cells treated with chemical carcinogens. *Int J Oncol* 1995;6:5–11
47. Chen L-C, Matsumura K, Deng G, *et al.* Deletion of two separate regions on chromosome 3p in breast cancers. *Cancer Res* 1994;54:3021-4
48. Bergthorsson JT, Eiriksdottir G, Barkardottir RB, Egilsson V, Arason A, Ingvarsson S. Linkage analysis and allelic imbalance in human breast cancer kindreds using microsatellite markers from the short arm of chromosome 3. *Hum Genet* 1995;96:437–43
49. Kerangueven F, Noguchi T, Wargniez V. Multiple sites of loss of heterozygosity on chromosome arms 3p and 3q in human breast carcinomas. *Oncol Rep* 1996;3:313–16
50. Pandis N, Bardi G, Mitelman F, Heim S. Deletion of the short arm of chromosome 3 in breast tumors. *Genes Chrom Cancer* 1997;18: 241–5
51. Man S, Ellis I, Sibbering M, Blarney R, Brook J. Highs level of allele loss at the FHIT and ATM genes in non-comedo ductal carcinoma *in situ* and grade I tubular invasive breast cancers. *Cancer Res* 1996;56:5484–9
52. Negrini M, Monaco C, Vorechovsky I, *et al.* The FHIT gene at 3pl4.2 is abnormal in breast carcinomas. *Cancer Res* 1996;56:3173-9

53. Theillet C, Lidereau R, Escot C, *et al.* Loss of a c-H-*ras*-I allele and aggressive human primary breast carcinomas. *Cancer Res* 1986;46:4776-81
54. Mackay J, Elder P, Porteous DI, *et al.* Partial deletion of chromosome 11p in breast cancer correlates with size of primary turnout and estrogen receptor level. *Br J Cancer* 1988;58: 710–14
55. Takita K-I, Sato T, Miyagi M, *et al.* Correlation of loss of alleles on the short arms of chromosomes 11 and 17 with metastasis of primary breast cancer to lymph nodes. *Cancer Res* 1992;52:3914–17
56. Winqvist R, Mannermaa A, Alavaikko M, *et al.* Refinement of regional loss of heterozygosity for chromosome 11pl5.5 in human breast tumors. *Cancer Res* 1993;53:4486–8
57. Gudmundsson J, Barkardottir RB, Eiriksdottir G, *et al.* Loss of heterozygosity at chromosome 11 in breast cancer: association of prognostic factors with genetic alterations. *Br J Cancer* 1995;72:696–701
58. Negrini M, Sabbioni S, Ohta M, *et al.* Seven-megabase yeast artificial chromosome contig at region 11pl5: identification of a yeast artificial chromosome spanning the breakpoint of a chromosomal translocation found in a case of Beckwith–Wiedmann syndrome. *Cancer Res* 1995;55:2904–9
59. Carter S, Negrini M, Baffa R, *et al.* Loss of heterozygosity at 11q22-q23 in breast cancer. *Cancer Res* 1994;54:6270–4
60. Koreth J, Bakkenist C, McGee JOD. Allelic deletions at chromosome 11q22-q23.1 and 11q25-q term are frequent in sporadic breast but not colorectal. *Cancers Oncogene* 1997;14: 431–7
61. Weber JL. Human DNA polymorphisms based on length variations in simple sequence tandem repeats. In Tilghman, S, Davies K, eds. *Genetic and Physical Mapping, Genome Analysis Series.* New York: Cold Spring Harbor Laboratory Press, 1990;1:159–81
62. Litt M. PCR of TG microsatellites. In McPherson MC, Quirke P, Taylor G, eds. *PCR: A Practical Approach.* Oxford: Oxford University Press, 1991:85–99
63. Weber JL. Informativeness of human (dC-dA)n (dG)n polymorphisms. *Genomics* 1990;7:524–30
64. Huang Y, Bove B, Wu Y, *et al.* Microsatellite instability during the immortalization and transformation of human breast epithelial cells *in vitro. Mol Carcinog* 1999;24:118–27
65. Ben Cheickh M, Rouanet P, Louason G, Jeanteur P, Theillet C. An attempt to define sets of cooperating genetic alterations in human breast cancer. *Int J Cancer* 1992;51:542–7
66. Sato T, Akivama F, Sakamoto G, Kasumi F, Nakamura Y. Accumulation of genetic alterations and progression of primary breast cancer. *Cancer Res* 1991;51:5794–9
67. Deng G, Chen LC, Schott DR, *et al.* Loss of heterozygosity and p53 gene mutations in breast cancer. *Cancer Res* 1994;54:499–505
68. Sanchez Y, el-Naggar A, Pathak S, Killary AM. A tumor suppressor locus within 3pl4-pl2 mediates rapid cell death of renal cell carcinoma *in vivo. Proc Natl Acad Sci USA* 1994; 91:3383–7
69. Killary A, Wolf M, Giambernardi T, Naylor S. Definition of a tumor suppressor locus within human chromosome 3p21 –p22. *Proc Natl Acad Sci USA* 1992;89:10877–81
70. Hibi Y, Yamakawa IC, Ueda R, Horio Y. Aberrant upregulation of a novel integrin α subunit gene at 3p21.3 in small cell lung cancer. *Oncogene* 1994;9:611–19
71. Jackers P, Minoletti F, Belotti D, *et al.* Isolation from a multigene family of the active human gene of the metastasis-associated multifunctional protein 37LRP/p4O at chromosome 3p21.3. *Oncogene* 1996;13:495–503
72. Wewer UM, Taraboletti G, Sobel ME, Albrechtsen R, Liotta LA. Role of laminin receptor in tumor cell migration. *Cancer Res* 1987;47:5691–8
73. Martignone S, Menard S, Bufalino R, *et al.* Prognostic significance of the 67-kilodalton laminin receptor expression in human breast carcinomas. *J Natl Cancer Inst* 1993;85:398–402
74. Maemura M, Dickson RB. Are cellular adhesion molecules involved in metastasis of breast cancer? *Breast Cancer Res Treat* 1994;32:239–60
75. Trent JM, Wiltshire R, Su L, Nicolaides NC, Vogelstein B, Kinzler KW. The gene for the APC-binding protein beta-catenin (CTNNB1) maps to chromosome 3p22, a region frequently altered in human malignancies. *Cytogenet Cell Genet* 1995;71:343–4
76. Dietrich CU, Pandis N, Teixeira MR, *et al.* Chromosome abnormalities in benign hyperproliferative disorders of epithelial and stromal breast tissue. *Int J Cancer* 1995;60:49–53
77. Pennisi E. New gene forges link between fragile site and many cancers. *Science* 1996;272:649
78. Cuthbert AP, Bond J, Trott DA, *et al.* Telomerase repressor sequences on chromosome 3 and induction of permanent growth arrest in human breast cancer cells. *J Natl Cancer Inst* 1999;91:37–45
79. Negrini M, Sabbioni S, Haldar S, *et al.* Tumor and growth suppression of breast cancer cells by chromosome 17-associated functions. *Cancer Res* 1994;54:1818-24
80. Borresen AL, Andersen TI, Garber J, *et al.* Screening for germ line TP53 mutations in breast cancer patients. *Cancer Res* 1992;52:3234–6
81. Puech A, Henry I, Jeanpierre C, Junien C. A

highly polymorphic probe on 11p15.5: L22.5.2 (D11S774). *Nucleic Acids Res* 1991;19:5095–9
82. Hannigan GE, Bayani J, Weksberg R, *et al.* Mapping of the gene encoding the integrin-linked kinase, ILK, to human chromosome 11pl5.5-pl5.4. *Genomics* 1997;42:177–9
83. Wang H, Shao N, Ding QM, Cui J, Reddy ES, Rao VN. BRCA1 proteins are transported to the nucleus in the absence of serum and splice variants BRCA1a, BRCA1b are tyrosine phosphoproteins that associate with E2F, cyclins and cyclin dependent kinases. *Oncogene* 1997;15: 143–57
84. Dong J-T, Lamb PW, Rinker-Schaeffer CW, *et al.* KA/1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. *Science* 1995;268:884–6
85. Wei Y, Lukashev M, Simon D, *et al.* Regulation of integrin function by the urokinase receptor. *Science* 1996;273:1551–5
86. Hampton GM, Mannermaa A, Winquist R, *et al.* Losses of heterozygosity in sporadic human breast carcinoma: a common region between 11q22 and 11q23.3. *Cancer Res* 1994;54:4586–9
87. Negrini M, Rasio D, Hampton GM, *et al.* Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11 q23.3. *Cancer Res* 1995;55:3003–7
88. Winqvist R, Hampton GM, Mannermaa A, *et al.* Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. *Cancer Res* 1995; 55:2660–4
89. Elson A, Wang Y, Daugherty CJ, *et al.* Pleiotropic defects in ataxia-telangiectasia protein-deficient mice. *Proc Natl Acad Sci USA* 1996;93:13084–9
90. Westphal CH, Schmaltz C, Rowan S, Elson A, Fisher DE, Leder P. Genetic interactions between *atm* and *p53* influence cellular proliferation and irradiation-induced cell cycle checkpoints. *Cancer Res* 1997;57:1664–7
91. Swift M, Morrel D, Massey R, Chase C. Incidence of cancer in 161 families affected by ataxia-telangiectasia. *N Engl J Med* 1991;325: 1831–6
92. Byrd PJ, Stankovic T, McConville CM, Smith AD, Cooper PR, Taylor AM. Identification and analysis of expression of human VACM-1, a cullin gene family member located on chromosome 11 q22-23. *Genome Res* 1997;7:71–5
93. Tomlinson IP, Nicolai H, Solomon E, Bodmer WF. The frequency and mechanism of loss of heterozygosity on chromosome 11q in breast cancer. *J Path* 1996;180:38–43
94. Tomlinson IP, Beck NE, Bodmer WF. Allele loss on chromosome 11q and microsatellite instability in malignant melanoma. *Eur J Cancer* 1996;32A:1797–802
95. Connolly KC, Gabra H, Millwater CJ, *et al.* Identification of a region of frequent loss of heterozygosity at 11 q24 incolorectal cancer. *Cancer Res* 1999;59:2806–9
96. Launonen V, Stenback F, Puistola U, *et al.* Chromosome 11q22.3-q25 LOH in ovarian cancer: association with a more aggressive disease course and involved subregions. *Gynecol Oncol* 1998;71:299–304
97. Dahiva R, McCarville J, Lee C, *et al.* Deletion of chromosome 1 lpl5, pl2, q22, q23-24 loci in human prostate cancer. *Intl J Cancer* 1997; 72:283–8
98. Vogelstein B, Fearon ER, Kern SE, *et al.* Allelotype of colorectal carcinomas. *Science* 1989;244:207–11
99. Sato T, Tanigami A, Yamakawa K, *et al.* Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res* 1990;50:7184–9
100. Futreal PA, Söderkvist P, Marks JR, *et al.* Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. *Cancer Res* 1992;52:2624–7
101. Devilee P, Cornelisse CJ. Genetics of human breast cancer. *Cancer Surv* 1990;9:605–30
102. Holistein M, Sidransky D, Vogelstein B, Harris CC. P53 mutations in human cancers. *Science* 1991;253:49–53
103. Prosser J, Thompson AM, Cranston G, Evans HJ. Evidence that p53 behaves as a tumor suppressor gene in sporadic breast tumors. *Oncogene* 1990;5:1573–9
104. Hovig E, Smith-Sorensen B, Brogger A, Borresen AL. Constant denaturant gel electrophoresis, a modification of denaturating gradient gel electrophoresis, in mutation detection. *Mutation Res* 1991;262:63–71
105. Osborne RJ, Merlo GR, Mitsudomi T, *et al.* Mutations in the p53 gene in primary human breast cancers. *Cancer Res* 1991;51:6194–8
106. Thompson AM, Anderson TJ, Condie A, *et al.* P53 allele losses, mutations and expression in breast cancer and their relationship to clinicopathological parameters. *Int J Cancer* 1992;50: 528–32
107. Varley JM, Brammar WJ, Lane DP, Swallow JE, Dolan C, Walker RA. Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinomas. *Oncogene* 1991;6: 413–21
108. Biggs PJ, Warren N, Venitt S, Stratton MR. Does a genotoxic carcinogen contribute to human breast cancer-7? *Mutagenesis* 1993;8: 275–83
109. Kirchweger R, Zeillinger R, Schneeberger C, Speiser P, Louason G, Theillet C. Patterns of allele losses suggest the existence of five

distinct regions of LOH on chromosome 17 in breast cancer. *Int J Cancer* 1994;56:193–9
110. Jantke I, Jonat W, Maass H, Goedde HW. Human breast cancer: frequent p53 allele loss and protein overexpression. *Hum Genet* 1993; 90:635–40
111. Anderson T, Gaustad A, Ottestad L, *et al.* Genetic alterations of the tumor suppressor gene regions 3p, 11p, 13q, 17p, and 17q in human breast carcinomas. *Genes Chromosomes Cancer* 1992;4:113–21
112. Goldman ES, More D II, Balazs M, Li VE. Loss of heterozygosity on the short arm of chromosome 17 is associated with high proliferative capacity and DNA aneuploidy in primary human breast cancer. *Proc Natl Acad Sci* 1991;88:3847–51
113. Coles C, Thompson AM, Elder PA, *et al.* Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis. *Lancet* 1990;336:761–3
114. Russo J, Hu YF, Yang X, *et al.* Breast cancer multistage progression. *Frontiers Biosci* 1998;3: 944–60
115. Lindblom A, Rotstein S, Skoog L, Nordenskjöld M, Larsson C. Deletions on chromosome 16 in primary familial breast carcinomas are asso-ciated with development of distant metastases. *Cancer Res* 1993;53: 3707–11
116. Tsuda H, Callen DF, Fukutomi T, Nakamura Y, Hirohashi S. Allele loss on chromosome 16q24.2-qter occurs frequently in breast cancers irrespectively of differences in phenotype and extent of spread. *Cancer Res* 1994;54:513–17
117. Russo J, Hu YF, Tahin Q, *et al.* Carcinogenicity of estrogens in human breast epithelial cells. *Acta Path Microb Immunol Scand* 2001;109: 39–52

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Neoplastic Transformation of Human Breast Epithelial Cells by Estrogens and Chemical Carcinogens

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Sporadic breast cancer, the most common cancer diagnosed in American and Northern European women, is gradually increasing in incidence in most Western countries. Prevention would be the most efficient way of eradicating this disease. This goal, however, cannot be accomplished until the specific agent(s) or mechanisms that initiate the neoplastic process are identified. Experimental studies have demonstrated that mammary cancer is a hormone-dependent multistep process that can be induced by a variety of compounds and mechanisms, that is, hormones, chemicals, radiation, and viruses, in addition to or in combination with genetic factors. Although estrogens have been shown to play a central role in breast cancer development, their carcinogenicity on human breast epithelial cells (HBECs) has not yet been clearly demonstrated. Breast cancer initiates in the undifferentiated lobules type 1, which are composed of three cell types: highly proliferating cells that are estrogen-receptor negative (ER−), nonproliferating cells that are ER positive (ER+), and very few (<1%) ER+ cells that proliferate. Interestingly, endogenous 17β-estradiol (E_2) is metabolized by the cytochrome P450 enzyme isoforms CYP1A1 and CYP1B1, which also activate benzo[a]pyrene (B[a]P), a carcinogen contained in cigarette smoke. We postulate that if estrogens are carcinogenic in HBECs, they should induce the same transformation phenotypes induced by chemical carcinogens and ultimately genomic changes observed in spontaneously developing primary breast cancers. To test this hypothesis we compared the transforming potential of E_2 on the HBEC MCF-10F with that of B[a]P. Both E_2 and B[a]P induced anchorage-independent growth, colony formation in agar methocel, and loss of ductulogenic capacity in collagen gel, all parameters indicative of cell transformation. In addition, the DNA of E_2-transformed cells expressed LOH in chromosome 11 at 11q23.3, 11q24.2–q25, and LOH at 13q12–q13. B[a]P-induced cell transformation was also associated with LOH at 13q12–q13 and at 17p13.2. The relevance of these findings is highlighted by the observation that E_2- and B[a]P-induced genomic alterations in the same loci found in ductal hyperplasia, ductal carcinoma in situ, and invasive ductal carcinoma of the breast. Environ. Mol. Mutagen. 39:254–263, 2002.

Key words: breast cancer; neoplastic transformation; epithelial cells; estrogens; chemical carcinogens

INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy in American women, with 182,800 cases detected and 40,800 deaths in the United States during the year 2000 [Greenlee et al., 2000]. Epidemiological and clinical evidence indicate that breast cancer risk is associated with prolonged exposure to female ovarian hormones [Bernstein and Ross, 1993; Kelsey et al., 1993; Pike et al., 1993; Greenlee et al., 2000]. Breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors [Topper et al., 1986; Henderson et al., 1988]. Estrogens have been demonstrated to be of essential importance in this disease because it is observed in postmenopausal hyperestrogenism resulting from the use of estrogenic hormone replacement therapy and obesity [Lippman et al., 1986; Russo et al., 2000]. Estrogens, which are necessary for the normal development of both reproductive and nonreproductive organs, exert their physiological effects by binding to their specific receptors, the estrogen receptors ERα or ERβ. Estrogens might act as well through alternate nonreceptor mediated pathways [Chen et al., 2000]. Passive and active exposure to tobacco smoke, which contains benzo[a]pyrene (B[a]P), is also considered an etiologic factor for breast cancer [Bennett et al., 2000; Wells, 2000]. The link between 17β-estradiol (E_2) and B[a]P is that both are metabolically

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activated by xenobiotic metabolizing enzymes such as those of the cytochrome P450 family, which have been identified in lung and breast tissues [Hellmold, 1998].

Enzymatic activation of carcinogens yields intermediate metabolites that are chemically more reactive than the initial compound [Hellmold, 1998; Bennett et al., 2000; Cavalieri et al., 2000]. These enzymes contribute to polycyclic aromatic hydrocarbon (PAH)-dependent carcinogenesis by promoting production of DNA-reactive intermediates, such as diol epoxides [Hellmold, 1998; Cavalieri et al., 2000; Russo et al., 2000]. Among the P450 isoforms, it is known that both human and rat CYP1A1 metabolize endogenous E_2 and also activate the carcinogen B[a]P. Another isoform, CYP1B1, metabolizes estradiol to its carcinogenic metabolite 4-hydroxyestradiol and activates the polycyclic hydrocarbon 7,12-dimethylbenz[a]anthracene (DMBA). In addition, these two isoforms also activate other PAHs and heterocyclic amines [Hellmold, 1998; Jefcoate et al., 2000]. Other mechanisms that have been reported to play a role in determining the carcinogenic potential are alterations in the detoxification of carcinogenic compounds, which are mediated by the conjugation of the compounds or their metabolites to glutathione by glutathione *S*-transferase (GST). GSTs are class II detoxification enzymes that are encoded by four classes of polymorphic genes [Bennett et al., 2000].

Breast cancers exhibit genomic alterations, such as DNA amplification and loss of genetic material, that may represent tumor-suppressor genes [Chen et al., 1989, 1992, 1994; Genuardi et al., 1989; Sato et al., 1991, 1992, 1994; Russo et al., 1993a,b; Calaf et al., 1995], although the role of these genomic alterations in the causation of the disease has not yet been clearly established. Specific types of genetic alterations, then, might identify essential steps in the initiation and/or progression of cancer. We postulate that, if estrogens and chemical carcinogens such as B[a]P initiate the neoplastic process or are responsible for its progression, they would induce in the normal breast epithelium the same type of genomic alterations observed in spontaneous malignancies. To test this hypothesis we evaluated the transforming potential of E_2 and B[a]P on human breast epithelial cells (HBECs) in vitro, utilizing the spontaneously immortalized HBEC MCF-10F [Soule et al., 1990; Tait et al., 1990]. This cell line lacks both ERα and ERβ, although this latter receptor is induced in cells transformed by chemical carcinogens [Hu et al., 1998]. The same phenotypes and characteristics that were expressed by MCF-10F cells transformed by the chemical carcinogens and oncogenes [Calaf and Russo, 1993; Russo et al., 1993a; Calaf et al., 1995] were evaluated in E_2- and B[a]P-treated cells: anchorage-independent growth, colony formation in agar methocel, and ductulogenic capacity in collagen gel. In addition, the DNA of treated cells was analyzed for specific genomic alterations such as loss of heterozygosity (LOH) at chromosomal loci known to be affected in spontaneously occurring breast lesions, such as ductal hyperplasia, ductal carcinoma in situ, and invasive carcinoma [Theillet et al., 1986, 1990; Mackay et al., 1988; Takita et al., 1992; Winqvist et al., 1993, 1995; Carter et al., 1994; Chen et al., 1994; Bergthorsson et al., 1995; Gudmundsson et al., 1995; Negrini et al., 1995a, 1996; Kerangueven et al., 1996; Man et al., 1996; Koreth et al., 1997; Pandis et al., 1997].

THE TARGET CELL OF MAMMARY CARCINOGENESIS

The breast is a hormone-responsive organ *par excellence.* Its development is influenced by a myriad of hormones and growth factors, responding selectively to given hormonal stimuli with either cell proliferation or differentiation. Among all the complex hormonal influences, estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium. 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 [Russo et al., 2000]. The progressive differentiation of Lob 1 into Lob 2 and Lob 3, occurring under the hormonal influences of the menstrual cycle, and the full differentiation into Lob 4, as the result of pregnancy, lead to a concomitant reduction in the proliferative activity of the mammary epithelium [Russo et al., 2000]. Of interest is the fact that the content of ERα and PgR in the lobular structures of the breast is directly proportional to the rate of cell proliferation. These three parameters are maximal in the undifferentiated Lob 1, decreasing progressively in Lob 2, Lob 3, and Lob 4. ERα- and PgR-positive cells are found exclusively in the epithelium; the myoepithelium and the stroma are totally devoid of steroid receptor-containing cells. The highest number of cells positive for both receptors is found in Lob 1, decreasing progressively in Lob 2 and Lob 3 [Russo et al., 2000].

To clarify the relationship between steroid receptor-positive cells and proliferating cells we utilized a double-staining procedure, combining in the same tissue section anti-Ki67 and ERα, Ki67 and PgR, or ERα and PgR antibodies. It was found that a higher percentage of cells reacted simultaneously with both ERα and PgR, appearing purplish red in color, whereas the number of cells positive for both ERα and Ki67 or PgR and Ki67 was very low. The highest percentage of ERα-, PgR-, and Ki67-positive cells was observed in Lob 1. The percentages of Ki67-, ERα-, and PgR-positive cells were reduced in Lob 2 and became negligible in Lob 3. The utilization of a double-labeling immunocytochemical technique has allowed us to demonstrate that the expression of the receptors occurs in cells other than the proliferating cells, confirming results reported by others. The findings that proliferating cells are different from those that are ERα- and PgR-positive support data indicating that estrogen controls cell proliferation by an indirect mechanism. This phenomenon has been demonstrated using the supernatant of estrogen-treated ERα-pos-

itive 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. ERα-positive cells treated with antiestrogens secrete TGFβ that inhibits the proliferation of ERα-negative cells. The fact that the highest proliferative activity and the highest percentages of ERα- and PgR-positive cells are present in Lob 1 provides a mechanistic explanation for the higher susceptibility of these structures to be transformed by chemical carcinogens in vitro, supporting as well the observation that Lob 1 is the site of origin of ductal carcinomas [Russo et al., 2000].

IN VITRO MODEL OF CELL TRANSFORMATION

General Concepts

It is generally accepted that malignant transformation involves genetic and epigenetic changes that derail common regulatory mechanisms, resulting in uncontrolled cellular proliferation and/or aberrant programmed cell death or apoptosis [Russo et al., 1988, 1993a,b, 1996, 1998; Holliday, 1996]. These cellular abnormalities, hallmarks of a carcinogenic process, are frequently associated with molecular alterations involving activation of protooncogenes and inactivation of tumor-suppressor genes as a result of genetic predisposition and/or exposure to physical (e.g., radiation), chemical (e.g., carcinogens, dietary components), and biological (e.g., viruses) environmental factors [Briand et al., 1987; Russo et al., 1988, 1993a,b, 1996, 1998; Band et al., 1990; Bartek et al., 1990; Soule et al., 1990; Tait et al., 1990; Garcia et al., 1991; Calaf and Russo, 1993; Couch 1996; Holliday, 1996; Hu et al., 1997]. A central challenge to cancer biology is the understanding of the cellular and molecular processes that drive a normal human breast epithelial cell to neoplastic growth. In vitro models have proven to be useful for testing whether chemical carcinogens can be causative agents of breast cancer and whether genomic changes play a functional role in the initiation and progression of this disease. HBECs are susceptible to undergoing neoplastic transformation when treated with estrogens or B[a]P. Transformation of HBECs in vitro, however, requires that specific conditions be met by the target cells, similar to what has been observed in in vivo experimental models [Russo et al., 1993a,b, 1996]. Only under optimal conditions of susceptibility will specific carcinogens initiate a cascade of changes in HBECs that recapitulate the phenotypic stages of tumor initiation and progression, culminating in the expression of tumorigenesis in a heterologous host [Calaf and Russo, 1993].

Transformation of Breast Epithelial Cells

Normal HBECs senesce after 10–20 passages in vitro when cultured in standard culture medium containing 1.05 mM calcium (Ca^{++}) [Soule et al., 1990; Tait et al., 1990]. We have developed an in vitro system that has allowed us to determine that the ability of HBECs to grow in culture greatly reflects their in vivo characteristics, that is, the degree of lobular development of the breast tissues from which they were obtained. Lobular development, which in turn determines the epithelium's rate of proliferation, is the final result of life-time influences, such as aging, endocrine, reproductive, and environmental factors [Russo et al., 1988, 1996]. These characteristics influence as well the response of the cells to the transforming potential of chemical carcinogens known to be of etiologic importance in various experimental models of mammary cancer [Russo et al., 1993a,b]. When primary cultures of HBECs obtained from Lob 1, which represent the most undifferentiated structure present in the breast of young nulliparous females, and from the more differentiated Lob 3 of parous women were placed in culture, it was observed that cells obtained from Lob 1 retained in vitro a higher rate of cell proliferation and exhibited greater survival efficiency than cells obtained from Lob 3 [Russo et al., 1988, 1993a,b, 1996].

Treatment of the cells with the chemical carcinogens 7,12-dimethylbenz[a]anthracene (DMBA), 1-methyl-3-nitro-1-nitroso-guanidine (MNNG), *N*-methyl-*N*-nitrosourea (NMU), or B[a]P increased survival efficiency in cells from Lob 1, but full transformation was not achieved. These results indicated that the susceptibility of HBECs in vitro was influenced by the differentiation status of the breast in vivo [Russo et al., 1988, 1993a]. However, the fact that primary cell cultures only expressed increased survival efficiency, an early phenotypic marker of neoplastic transformation, indicated that further elucidation of the conditions required for inducing cell transformation with chemicals was needed. The spontaneous immortalization of the HBEC line MCF-10M, derived from the human breast tissue sample number 130 (S130), provided such conditions [Soule et al., 1990; Tait et al., 1990]. After 2 years of continuous culture of the mortal MCF-10M cells in medium containing a 0.04 mM Ca^{++} (low Ca^{++}), they spontaneously gave rise to an immortal cell line, MCF-10, which grew either as attached (MCF-10A) or as floating (MCF-10F) cells [Soule et al., 1990; Tait et al., 1990]. Immortalization of these cells was characterized by their continuous growth in culture medium containing the conventional 1.05 mM calcium concentration (that for our purposes was called "high Ca^{++} medium") without entering into senescence [Soule et al., 1990; Tait et al., 1990]. The analysis of the growth characteristics of these primary cells and the cell lines derived from them revealed that the growth curves of MCF-10M, MCF-10A, and MCF-10F cells were similar, independent of the calcium concentration in the culture medium. Mortal MCF-10M cells, however, were unable to continue growing in high Ca^{++} medium after the 20th passage, whereas the immortal cells MCF-10A and MCF-10F continued growing indefinitely. The human breast epithelial origin of the mor-

tal and the immortalized cells was confirmed by their genetic, cytogenetic, ultrastructural, and phenotypic characteristics [Soule et al., 1990; Tait et al., 1990]. The immortal MCF-10F cells were identical to the mortal MCF-10M cells from which they were derived in all the aspects for which they were evaluated. The only difference found to date is that MCF-10F cells are pseudodiploid and express minimal chromosomal alterations (46XX,1p+,t[3;9][p13:p22]) [Soule et al., 1990; Tait et al., 1990; Russo et al., 1996].

Dose-Response Effect of E_2 in HBECs

To determine the optimal doses for the expression of the cell transformation phenotype, we treated the immortalized HBEC MCF-10 F with E_2 for testing the survival efficiency (SE), colony-forming ability in agar methocel, or colony efficiency (CE) and loss of ductulogenesis in collagen matrix. MCF-10F cells were treated with 0.0, 0.007 nM, 70 nM, or 0.25 mM of E_2 twice a week for 2 weeks [Russo et al., 2001]. The SE was increased with 0.007 and 70 nM of E_2 and decreased with 0.25 mM. The cells treated with either doses of E_2 formed colonies in agar methocel that did not differ in size; however, the CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses. Ductulogenesis, or the number of ductules per 10,000 cells plated, was 75 ± 4.9 in control cells; it decreased to 63.7 ± 28.8, 41.3 ± 12.4, and 17.8 ± 5.0 in E_2-treated cells, which also formed spherical-like structures or solid masses, whose numbers increased from 0 in controls to 18.5 ± 6.7, 107 ± 11.8, and 130 ± 10.0 for each E_2 dose.

Estrogen and Chemical Carcinogens Induce Transformation Phenotypes

Evaluation of colony formation at the end of the second week of E_2 and B[a]P treatment revealed that MCF-10F cells formed colonies in agar methocel over 60 microns in diameter. MCF-10F control cells treated with DMSO did not form colonies. The total CE was significantly increased by E_2 and B[a]P (Figs. 1 and 2).

Ductulogenesis was qualitatively evaluated by estimating the ability of the cells plated in collagen to form ductular structures (cells lining a lumen). It was maximal in MCF-10F cells (Fig. 3a) and completely negative (−) in B[a]P-treated cells, which grew as a solid or cystic mass. All the cells treated with E_2 exhibited decreased ability to form ductules (Fig. 3b–e). Progesterone did not significantly affect the ductulogenic capacity. The collagen matrix embedded in paraffin and cross-sectioned for determination of cell morphology showed that MCF-10F cells form a well-defined ductule lined by a monolayer of cuboidal epithelial cells (Fig. 4a), whereas with those treated with E_2, the number of layers increase and in some cases the whole lumen is obliterated (Fig. 4b–d). B[a]P also forms structures similar to those induced by estrogen, whereas the ductules

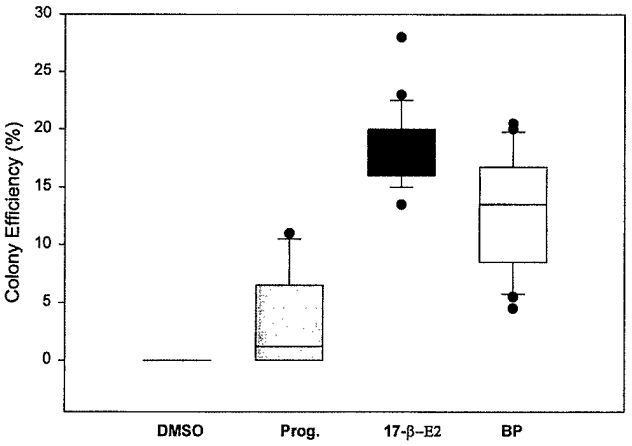

Fig. 1. Box plot showing the effect of different compounds on MCF-10F cell colony efficiency in agar methocel.

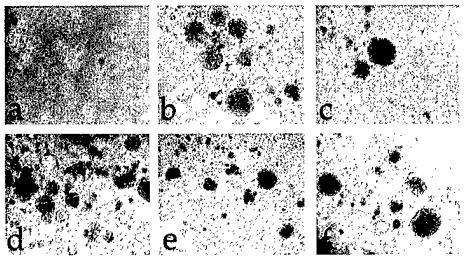

Fig. 2. MCF-10F cells plated in agar methocel for colony assay. **a:** Control cells do not form colonies, only isolated cells are present. **b–d:** Colonies formed by E_2-treated MCF-10F cells at the doses of 0.007 nM (b), 70 nM (c), and 1 μg (d). **e:** Progesterone-treated cells. **f:** B[a]P-treated cells induce slightly larger colonies. Phase-contrast microscope: 4 × magnification.

formed by progesterone treatment are smaller, with a reduced luminal size lined by a monolayer of cuboidal epithelial cells.

Genomic Changes Induced in E_2-Transformed Cells

From the E_2-treated cells, six clones out of 24 colonies were expanded and maintained in culture. These clones, designated E_2-1 to E_2-6 (Table I), were selected for genomic analysis. DNA fingerprint analysis of parent E_2- and B[a]P-treated cells and their derived clones revealed that their allelic pattern was identical in all the cell lines analyzed. These results confirmed that all the cells tested had the same HBEC origin and that they were free of contamination from other cell lines maintained in our laboratory. Among 67 markers tested, which were selected based on chromosomal changes reported to be present in breast and other cancers, only clones E_2-1 and E_2-2 identically exhibited LOH in chromosome 11 at 11q23.3 (marker *D11S29*) and 11q24.2–q25 (marker *D11S912*). B[a]P-treated cells did not exhibit LOH at any of the loci tested for chromosome 11. Interestingly, we have found that all the clones of the cells trans-

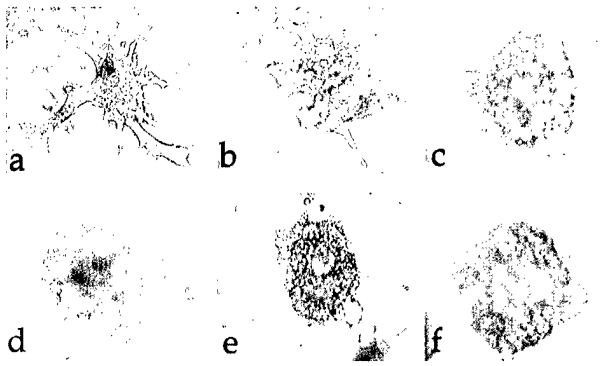

Fig. 3. **a:** MCF 10F cells treated with solvent (DMSO) forming well-defined ductular structures in collagen matrix; (**b**) 0.007 nM of E_2 induces alteration in the ductular pattern; (**c, d**) 70 nM of E_2 induces the loss of ductular formation in collagen matrix; (**e, f**) 1 μg of E_2 or B[a]P, respectively, induces the formation of spherical masses in collagen matrix. Phase-contrast microscope: 10 × magnification.

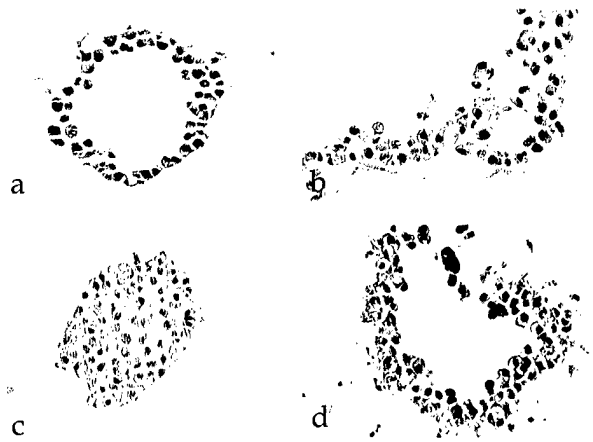

Fig. 4. Histological sections of cells growing in collagen gel. The cells have been fixed in buffered formalin, embedded in paraffin and the sections stained with hematoxylin and eosin. **a:** MCF 10F cells treated with solvent (DMSO) forming well-defined ductular structures lined by a single cuboidal layer of cells; (**b**) 0.007 nM of E_2 induces alteration in the ductular pattern forming spherical masses lined by two to three layers of cells; (**c**) 70 nM of E_2 induces the loss of ductular formation in collagen matrix and the solid spherical masses are composed of large cuboidal cells; (**d**) 1 μg of E_2 or B[a]P induces the formation of spherical masses lined by multiple layers of cells. Bright field: 10 × magnification.

formed with either E_2 or B[a]P had microsatellite instability (MSI), expressed as an allelic expansion at the 3p21 locus (marker *D3S1447*) (data not shown). It was of great interest that by the fourth passage after four treatments during a 2-week period, clones derived from E_2-transformed cells exhibited LOH in chromosome 11. It was previously reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice [Negrini et al., 1995].

Several genes, such as *HRAs, CTSD, ILK, TSG101,* and *KI1* have been located to the short arm of chromosome 11 [Theillet et al., 1986; Mackay et al., 1988; Puech et al., 1991; Borresen et al., 1992; Negrini et al., 1994; Dong et al., 1995; Hannigan et al., 1997; Wang et al., 1997]. A region of deletion on 11q22–23 has been described on the long arm of chromosome 11 in 40–60% of breast tumors [Carter et al., 1994; Hampton et al., 1994; Negrini et al., 1995; Winqvist et al., 1995; Man et al., 1996; Hannigan et al., 1997; Koreth et al., 1997]. The ataxia telangiectasia susceptibility gene (*ATM*) is the most widely studied candidate gene in this region [Elson et al., 1996]. *ATM* may act upstream of the *TP53* gene in cell cycle regulation [Swift et al., 1991; Westphal et al., 1997], and its heterozygous mutation is associated with a high incidence of early-onset breast cancer. This region has been reported to contain several tumor-suppressor genes and genes involved in the metastatic process. In this latter group, the *MMP* genes encoding matrix metalloproteases involved in invasion, *ETS1,* encoding a transcription factor involved in angiogenesis, and *VACM-1,* encoding a protein probably involved in cell cycle regulation, have been identified [Swift et al., 1991; Byrd et al., 1997]. Although some of these genes might be affected during the transformation of HBECs by estrogens, a more detailed allelotyping using multiple markers is required for better defining the size of the region exhibiting LOH in these cells. Approximately 35% of breast cancers have LOH at the *D11S29* and *NCAM* loci [Tomlinson et al., 1996a], and a higher frequency of LOH at this locus has also been found in melanomas [Tomlinson et al., 1996b]. LOH has been found at frequencies of 25% and 29% at the distal *D11S968* (11qter) and *D11S29* (11q23.3 locus), slightly above the accepted baseline of 0–20% in colorectal cancer. The fact that breast cancer, melanoma, and colorectal cancer are influenced by estrogens [Connolly et al., 1999] gives relevance to our data that treatment of MCF-10F cells with estrogens induces LOH in this specific locus. LOH at 11q23–qter occurs frequently in ovarian and other cancers [Dahiva et al., 1997; Launonen et al., 1998].

Genomic Changes Induced in Chemically Transformed Cells

We utilized DNA amplification of microsatellite length polymorphisms for detecting whether either allelic loss or microsatellite instability was present in MCF-10M, MCF-10F, BP1, BP1E, D3, and D3-1 cells at different passages [Huang et al., 1999; Yang et al., 1999]. Of all the cell lines and clones tested, only BP1E cells exhibited LOH in chromosome 17p13, which was detected first with the *P144D6* marker [Wu et al., 1997; Russo et al., 1998] and later on narrowed using the marker *D17S796* [Lareef et al., 2001].

The expression of transformation phenotypes was preceded by microsatellite instability, as revealed by the observation that the transformed BP1 and BP1E cells, which expressed anchorage independence, loss of ductule-like for-

TABLE I. Phenotypic Markers of Cell Transformation Induced in MCF-10F Cells by 17β-Estradiol (E_2) and Benzo[a]pyrene (B[a]P)

Cell type	No. of passages	Doubling time (DT)[a]	Colony number (CN)[b]	Colony efficiency (%) (CE)[c]	Colony size (CS) (μm)[d]
MCF-10F	113	93 ± 5.6	0.0	0.0	0.0
B[a]P-derived	4	42 ± 3.8	89	18 ± 4.5	670 ± 46
E_2-derived	4	78 ± 16.0	24[e]	4.8 ± 0.9	170 ± 34
E_2-1[f]	4	81 ± 3.0	36	7.2 ± 3.7	180 ± 12
E_2-2[f]	4	68 ± 10	45	9.0 ± 2.0	150 ± 6
E_2-3	5	66 ± 8.0	39	7.9 ± 5.6	190 ± 9
E_2-4	3	82 ± 6.0	20	3.5 ± 1.1	134 ± 5
E_2-5	6	61 ± 5.6	63	12.6 ± 3.0	193 ± 12
E_2-6	4	73 ± 3.0	54	10.8 ± 4.9	189 ± 5

[a]Doubling time (DT) in hr (expressed as mean ± SD) was determined as described in Calaf and Russo [1993]. DT was significantly different by Students' t-test between B[a]P-treated and all other cell lines ($P < 0.001$).
[b]Colony number (CN) was significantly different between MCF-10F and all other cell lines ($P = 0.00001$).
[c]Colony efficiency (CE) (expressed as mean ± SD) was significantly different between MCF-10F and all other cell lines ($P = 0.00001$).
[d]Colony size (CS) (expressed as mean ± SD) was significantly different between MCF-10F and all other cell lines ($P = 0.00001$). CS of DES clones was significantly different from E_2- and B[a]P-treated cells ($P = 0.001$).
[e]From 24 colonies derived from E_2-treated cells, clones E_2-1, E_2-2, E_2-3, E_2-4, E_2-5, and E_2-6 were recovered and expanded.
[f]These cells have been used for detection of microsatellite DNA polymorphism.

mation in collagen gel, and increased chemotactic and invasive properties, also expressed MSI on chromosome 11 at loci represented by *D11S912* at q25 and on chromosome 13 at 13q12–13 (flanking the *BRCA2* locus), as detected with markers *D13S260* and *D13S289* [Huang et al., 1999]. MSI in chromosome 11 at 11p13 and chromosome 17 at 17p13.3 and 17p13.1 was also present, although they were retained from the parent immortalized MCF-10F cells, as was a variant band in one of the heterozygous alleles of *TP53* of MCF-10F cells, which was detected using single-strand conformational polymorphism [Huang et al., 1999]. D3 and D3-1 cells, which expressed an early stage of transformed phenotype equivalent to that of the BP1 cells, also exhibited MSI at locus *D13S260* on chromosome 13q12–13 [Huang et al., 1999] and in *D16S285* on chromosome 16q12.1 [Wu et al., 1997]. D3 and D3-1 cells did not exhibit as many genetic alterations as the B[a]P-derived cell lines [Huang et al., 1999]. Neoplastic progression was also associated with mutations and/or amplification of *c-H-ras*, *int-2*, *c-neu*, *c-myc,* and *MDM2* genes [Wu et al., 1997; Russo et al., 1998], in addition to MSI at 11q25 and 13q12–q13 and LOH at 17p13.2. Detection of MSI in our in vitro model served as an indicator of the progression of normal HBECs from immortalization to transformation, a phenomenon initiated and driven by the carcinogen. The relevance of these findings is highlighted by the observation that the loci in which MSI and LOH were detected in the HBECs transformed with chemical carcinogens are the same that are found in primary breast cancers, as described below.

Genomic Changes in Human Breast Lesions

Microsatellites, which are short, repetitive sequences of DNA scattered throughout the genome [Weber and May, 1989; Boyer et al., 1995] have been used to detect genomic alterations in various human cancers through the identification of LOH or MSI [Ionov et al., 1993; Wooster et al., 1994]. Studies using this approach provided direct evidence for the involvement of chromosome 13 in human breast carcinomas and led to the identification of the well-known tumor-suppressor gene *RB1* (13q14) and breast cancer susceptibility gene *BRCA2* (13q12–13) [Wooster et al., 1998]. We previously observed MSI at the chromosomal regions 13q12–13, 11q25, and 16q12.1 in the early stages of chemical transformation of HBECs [Wu et al., 1997; Huang et al., 1999]. We tested the hypothesis that, if MSI represents an early event in breast carcinogenesis in vivo, a relationship exists between microsatellite alteration and the progression of cancer. Thus, we performed polymorphic studies on ductal carcinoma in situ (DCIS) of the breast using three microsatellite DNA polymorphic markers, *D13S289, D13S260,* and *D13S267,* that flank the *BRCA2* region at 13q12–13.

Paraffin-embedded breast tumors were obtained from the patient sample depository at the Breast Cancer Research Laboratory, Fox Chase Cancer Center (Philadelphia, PA). Microscopically identifiable populations of epithelial cells from normal ducts and DCIS were collected by microdissection from 10 μm-thick serial paraffin sections [Aldaz et al., 1995; Radford et al., 1995]. Paraffin sections were deparaffinized with xylene, rehydrated, and digested with 0.15 μg of proteinase K followed by organic extraction. The DNA was precipitated with ethanol, resuspended in TE, and stored at 4°C until use. Microsatellite DNA was amplified by PCR [Huang et al., 1999]. MSI was defined as an increase or decrease in the number of bands and/or in the size of one or both alleles in relation to the normal alleles [Aaltone et al., 1993; Thibodeau et al., 1993]. Of 35 infor-

TABLE II. Expression of Microsatellite Instability (MSI) in Human Breast Epithelial Cells Treated With Chemical Carcinogens in Vitro

	Cell line					
Sites of MSI	MCF-10M	MCF-10F	BP1	BP1-E	D3	D3-1
Marker (locus)		*D11S1392* (11p13)	*D11S1392 (11p13)*	*D11S1392* (11p13)		
Marker (locus)			*D11S912* (11q25)	*D11S912* (11q25)		
Marker (locus)			*D13S260* (13q12–13)		*D13S260* (13q12–13)	*D13S260* (13q12–13)
Marker (locus)			*D13S289* (13q12–13)			
Marker (locus)		*D17S849* (17p13.3)	*D17S849* (17p13.3)	*D17S849* (17p13.3)	*D17S849* (17p13.3)	*D17S849* (17p13.3)
Marker (locus)		*D17S796* (17p13.1)	*D17S796* (17p13.1)	*D17S796* (17p13.1)	*D17S796* (17p13.1)	*D17S796* (17p13.1)
Marker (locus)		*D17S513* (17p13.1)	*D17S513* (17p13.1)	*D17S513* (17p13.1)	*D17S513* (17p13.1)	*D17S513* (17p13.1)
Marker (locus)		*Tp53* (17p13.1)	*Tp53 (17p13.1)*	*Tp53* (17p13.1)	*Tp53* (17p13.1)	*Tp53* (17p13.1)
Marker (locus)		*D17S786* (17p13.1)	*D17S786* (17p13.1)	*D17S786* (17p13.1)	*D17S786* (17p13.1)	*D17S786* (17p13.1)

mative DCIS, MSI was found to be present in 5 (14%) and LOH in 4 (11%) of the cases with marker *D13S260.* At locus *D13S267,* which is more distal to *D13S260,* MSI was seen in 3 (9%) and LOH in 8 (24%) of a total of 33 informative cases. Marker *D13S289,* the most distal of those flanking the *BRCA2* locus had 23 informative cases, 5 of which were affected by MSI (22%) and 2 by LOH (9%) (Table II). Nine (30%) of these cases exhibited MSI and LOH at loci *D13S260,* 9 (26%) at *D13S267,* and 7 (30%) at *D13S289,* indicating that these three markers reflected approximately the same incidence of genomic alterations at the *BRCA2* region.

The application of microsatellite DNA polymorphic analysis provided evidence that MSI and LOH in chromosomal region 13q12–13 are associated with the development of preinvasive breast cancers, thus indicating the involvement of DNA repair defects and/or gene inactivation in breast carcinogenesis. To further test the pattern of expression of genomic changes in the progression of breast cancer, we performed microsatellite polymorphism analysis in genomic DNA extracted by microdissection from breast tissues of 21 breast cancer patients that contained three different types of breast lesions: ductal hyperplasia (DHP), DCIS, and invasive ductal carcinoma (INV) (Table III). We analyzed specific loci on chromosomes 11, 13, 16, and 17 using an array of 7 markers, *D11S912, D11S940, D13S260, D13S289, D13S267, D16S285,* and *D17S855.* Among these markers, *D11S912* showed MSI in 10/19 (53%) of all samples including 2/8 of the preneoplastic lesion DHP, 8/15 DCIS, and 3/5 of INV (Table III). MSI was detected in chromosome 13 with marker *D13S260* in 5/16 (31%) DCIS and 3/4 (75%) INV (Table III), but was absent in DHP, suggesting a correlation with the progression of the disease, and consistent with its alteration in more advances phases of in vitro transformation, such as in BP1E cells. In addition, the high MSI incidence in all samples for markers *D11S912* (53%), *D13S260* (23%), *D13S289* (36%), and *D13S267* (45%), compared to lower rates of *D11S940* (10%), *D16S285* (6%), and *D17S855* (9%), may suggest that instability preferentially occurs in specific loci during breast carcinogenesis, also in good agreement with the data from the transformed HBEC system (except for *D13S267*).

TABLE III. Microsatellite Instability (MSI) and Loss of Heterozygosity (LOH) in Ductal Carcinoma in Situ of the Breast

Marker	Map Position	Total cases[a]	MSI (%)	LOH (%)	MSI and LOH (%)
D13S260	13q12–13	35	5 (14)	4 (11)	9 (26)
D13S267	13q12–13	33	3 (9)	8 (24)	11 (33)
D13S289	13q12–13	23	5 (22)	2 (9)	7 (30)

[a]Includes both informative and uninformative cases.

In conclusion, the observation that E_2, B[a]P, and other chemical carcinogens induce similar phenotypic changes, but different genomic alterations in vitro, could be an indication of different genomic pathways for transformation. Interestingly, the data show that in both cases the genomic alterations detected are the ones that are also observed in breast lesions. These observations support the premise that substances that are carcinogenic to the HBECs and responsible for the initiation of the neoplastic process induce the same type of genomic alterations in the normal breast epithelium that are observed in spontaneous malignancies.

TABLE IV. Microsatellite Instability (MSI) and Loss of Heterozygosity (LOH) in Ductal Hyperplasia (DHP), Ductal Carcinoma in Situ (DCIS), and Invasive Carcinoma (INV)

		Histopathological type of breast lesions					
		DHP[a]		DCIS[b]		INV[c]	
Marker	Map Position	MSI	LOH	MSI	LOH	MSI	LOH
Int2	11q13.3	2/24 (8.3)	0/24 (0)	4/35 (11.4)	2/35 (5.7)	2/14 (14.3)	0/14 (0)
D11S614	11q21–23.3	2/33 (6.1)	0/30 (0)	3/38 (7.9)	0/38 (0)	2/16 (12.5)	0/16 (0)
D11S912	11q25	3/37 (5.4)[b]	1/33 (3.03)[c]	15/54 (27.8)[b]	5/54 (9.2)[c]	3/22 (13.6)[b]	8/22 (36.4)[c]
D11S940	11q21–23.3	1/27 (3.7)	0/37 (0)	2/39 (5.1)	1/39 (2.6)	2/16 (12.5)	0/16 (0)
D13S260	13q12–13	0/14 (0)	0/14 (0)	3/23 (13)	3/23 (13)	2/13 (15)	4/13 (31)
D13S267	13q12–13	0/11 (0)	0/11 (0)	3/21 (14)	7/21 (33)	1/12 (8)	2/12 (17)
D13S289	13q12–13	0/7 (0)	0/7 (0)	3/11 (27)	2/11 (18)	1/5 (20)	3/5 (60)

[a]No. lesions affected/no. informative cases (%).
[b]Fisher's exact test, $P < 0.05$.
[c]Fisher's exact test, $P < 0.01$.

REFERENCES

Aldaz CM, Chen T, Sahin A, Cunningham J, Bondy M. 1995. Comparative allelotype of *in situ* and invasive human breast cancer: high frequency of microsatellite instability in lobular breast carcinomas. Cancer Res 55:3976–3981.

Band V, Zagetowski D, Kulesa V, Sager R. 1990. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. Proc Natl Acad Sci USA 87:463–467.

Bartek J, Durban EM, Hallowes R, Taylor-Papadimitriou J. 1990. Selective immortalization of a phenotypically distinct epithelial cell type by microinjection of SV40 DNA into cultured human milk cells. Int J Cancer 45:1105–1112.

Bennett WP, Alavanja MCR, Blomeke B, Vähäkangas KH, Castrén K, Welsh JA, Bowman ED, Khan MA, Flieder DB, Harris C. 2000. Environmental tobacco smoke, genetic susceptibility, and risk of lung cancer in never-smoking women. J Natl Cancer Inst 91:2009–2014.

Bergthorsson JT, Eiriksdottir G, Barkardottir RB, Egilsson V, Arason A, Ingvarsson S. 1995. Linkage analysis and allelic imbalance in human breast cancer kindreds using microsatellite markers from the short arm of chromosome 3. Hum Genet 96:437–443.

Bernstein L, Ross RK. 1993. Endogenous hormones and breast cancer risk. Epidemiol Rev 15:48–65.

Borresen AL, Andersen TI, Garber J, Barbier-Piraux N, Thorlacius S, Eyfjord J, Ottestad L, Smith-Sorensen B, Hovig E, Malkin D. 1992. Screening for germ line TP53 mutations in breast cancer patients. Cancer Res 52:3234–3236.

Boyer JC, Umar A, Risinger JI, Lipford JR, Kane M, Yin S, Barrett JC, Kolodner RD, Kunkel TA. 1995. Microsatellite instability, mismatch repair deficiency, and genetic defects in human cancer cell lines. Cancer Res 55:6063–6070.

Briand P, Petersen OW, van Deurs B. 1987. A new diploid non-tumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. In Vitro Cell Dev Biol 23:186–188.

Byrd PJ, Stankovic T, McConville CM, Smith AD, Cooper PR, Taylor AM. 1997. Identification and analysis of expression of human VACM-1, a cullin gene family member located on chromosome 11q22–23. Genome Res 7:71–75.

Calaf G, Russo J. 1993. Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis 14:483–492.

Calaf G, Zhang PL, Alvarado MV, Estrada S, Russo J. 1995. C-Ha ras enhances the neoplastic transformation of human breast epithelial cells treated with chemical carcinogens. Int J Oncol 6:5–11.

Carter S, Negrini M, Baffa R, Gillum DR, Rosenberg AL, Schwartz GF, Croce CM. 1994. Loss of heterozygosity at 11q22–q23 in breast cancer. Cancer Res 54:6270–6274.

Cavalieri E, Frenkel K, Liehr JG, Rogan E, Roy D. 2000. Estrogens as endogenous genotoxic agents-DNA adducts and mutations. J Natl Cancer Inst Monogr 27:75–94.

Chen L-C, Dolibaum C, Smith H. 1989. Loss of heterozygosity on chromosome lq in human breast cancer. Proc Natl Acad Sci USA 86:7204–7207.

Chen L-C, Kurisu W, Ljung BM, Goldman ES, Moore D 2d, Smith HS. 1992. Heterogeneity for allelic loss in human breast cancer. J Natl Cancer Inst 84:506–510.

Chen L-C, Matsumura K, Deng G, Kurisu W, Ljung B-M, Lerman MI, Waldman FM, Smith HS. 1994. Deletion of two separate regions on chromosome 3p in breast cancers. Cancer Res 54:3021–3024.

Chen X, Danes C, Lowe M, Herliczek TW, Keyomarsi K. 2000. Activation of the estrogen-signaling pathway by p21 WAF1/CIP1 in estrogen receptor negative breast cancer cells. J Natl Cancer Inst 92:1403–1413.

Connolly KC, Gabra H, Millwater CJ, Taylor KJ, Rabiasz GJ, Watson JE, Smyth JF, Wvllie AH, Jodrell DI. 1999. Identification of a region of frequent loss of heterozygosity at 11q24 in colorectal cancer. Cancer Res 59:2806–2809.

Couch DB. 1996. Carcinogenesis: basic principles. Drug Chem Toxicol 19:133–148.

Dahiva R, McCarville J, Lee C, Hu W, Kaur G, Carroll P, Deng G. 1997. Deletion of chromosome 11p15, p12, q22, q23–24 loci in human prostate cancer. Int J Cancer 72:283–288.

Dong J-T, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT, Barrett JC. 1995. KA/1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science 268:884–886.

Elson A, Wang Y, Daugherty CJ, Morton CC, Zhou F, Campos-Torres J, Leder P. 1996. Pleiotropic defects in ataxia-telangiectasia protein-deficient mice. Proc Natl Acad Sci USA 93:13084–13089.

Garcia I, Brandt D, Weintraub J, Zhou W, Aapro M. 1991. Loss of heterozygosity for the short arm of chromosome 11 (11p15) in human milk epithelial cells immortalized by microinjection of SV40 DNA. Cancer Res 51:294–300.

Genuardi M, Tsihira N, Anderson DE, Saunders GF. 1989. Distal deletion of chromosome 1q in ductal carcinoma of the breast. Am J Hum Genet 45:73–89.

Greenlee RT, Murray T, Bolden S, Wingo PA. 2000. Cancer statistics 2000. CA Cancer J Clin 50:7–33.

Gudmundsson J, Barkardottir RB, Eiriksdottir G, Baldursson T, Arason A, Egilsson V, Ingvarsson S. 1995. Loss of heterozygosity at chromo-

some 11 in breast cancer: association of prognostic factors with genetic alterations. Br J Cancer 72:696–701.

Hampton GM, Mannermaa A, Winqvist R, Alavaikko M, Blanco G, Taskinen PG, Kiviniemi H, Newsham I, Cavenee WK, Evans GA. 1994. Losses of heterozygosity in sporadic human breast carcinoma: a common region between 11q22 and 11q23.3. Cancer Res 54:4586–4589.

Hannigan GE, Bayani J, Weksberg R, Beatty B, Pandita A, Dedhar S, Squire J. 1997. Mapping of the gene encoding the integrin-linked kinase, ILK, to human chromosome 11p15.5–p15.4. Genomics 42:177–179.

Hellmold H. 1998. Toxicological and endocrinological aspects of cytochrome P450 in breast and lung. PhD Thesis, Stockholm.

Henderson BE, Ross R, Bernstein L. 1988. Estrogens as a cause of human cancer [The Richard and Hinda Rosenthal Foundation Award Lecture]. Cancer Res 48:246–253.

Holliday R. 1996. Neoplastic transformation: the contrasting stability of human and mouse cells. Cancer Surv 28:103–115.

Hu YF, Russo IH, Zalipsky U, Lynch HT, Russo J. 1997. Environmental chemical carcinogens induce transformation of breast epithelial cells from women with familial history of breast cancer. In Vitro Cell Dev Biol 33:495–498.

Hu YF, Lau KM, Ho SM, Russo J. 1998. Increased expression of estrogen receptor-β in chemically transformed human breast epithelial cells. Int J Oncol 12:1225–1228.

Huang Y, Bove B, Wu YL, Russo IH, Yang X, Zekri A, Russo J. 1999. Microsatellite instability during immortalization and transformation of human breast epithelial cells *in vitro*. Mol Carcinog 24:118–127.

Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. 1993. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363:558–561.

Jefcoate CR, Liehr JG, Santen RJ, Sutter TR, Yager JD, Yue W, Santner SJ, Tekmal R, Demers L, Pauley R, Naftolin F, Mor G, Berstein L. 2000. Tissue-specific synthesis and oxidative metabolism of estrogens. J Natl Cancer Inst Monogr 27:95–112.

Kelsey JL, Gammon MD, John EM. 1993. Reproductive factors and breast cancer. Epidemiol Rev 15:36–47.

Kerangueven F, Noguchi T, Wargniez V. 1996. Multiple sites of loss of heterozygosity on chromosome arms 3p and 3q in human breast carcinomas. Oncol Rep 3:313–316.

Koreth J, Bakkenist C, McGee JOD. 1997. Allelic deletions at chromosome 11q22–q23.1 and 11q25–q term are frequent in sporadic breast but not colorectal. Cancers Oncogene 14:431–437.

Lareef MH, Tahin Q, Russo IH, Mor G, Song J, Mihaila D, Slater CM, Russo J. 2001. Transfer of chromosome 17(p13.1) to chemically transformed human breast epithelial cells induces Fas-mediated apoptosis. Proc Am Assoc Cancer Res 42:1475a.

Launonen V, Stenback F, Puistola U, Bloigu R, Huusko P, Kytola S, Kauppila A, Winqvist R. 1998. Chromosome 11q22.3–q25 LOH in ovarian cancer: association with a more aggressive disease course and involved subregions. Gynecol Oncol 71:299–304.

Lippman ME, Huff KK, Jakesz R, Hecht T, Kasid A, Bates S, Dickson RB. 1986. Estrogens regulate production of specific growth factors in hormone-dependent human breast cancer. In: Angeli A, Bradlow HL, Dogliotti L, editors. Endocrinology of the breast: basic and clinical aspects. New York: New York Academy of Sciences. p 11–16.

Mackay J, Elder P, Porteous DI, Steel CM, Hawkins RA, Going JJ, Chetty U. 1988. Partial deletion of chromosome 11p in breast cancer correlates with size of primary tumour and estrogen receptor level. Br J Cancer 58:710–714.

Man S, Ellis I, Sibbering M, Blarney R, Brook J. 1996. Highs level of allele loss at the FHIT and ATM genes in non-comedo ductal carcinoma in situ and grade I tubular invasive breast cancers. Cancer Res 56:5484–5489.

Negrini M, Rasio D, Hampton GM, Sabbioni S, Rattan S, Carter SM, Rosenberg AL, Schwartz GF, Shiloh Y, Cavenee WK, Croce CM. 1995a. Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11q23.3. Cancer Res 55:3003–3007.

Negrini M, Sabbioni S, Ohta M, Veronese ML, Rattan S, Junien C, Croce CM. 1995b. Seven-megabase yeast artificial chromosome contig at region 11p15: identification of a yeast artificial chromosome spanning the breakpoint of a chromosomal translocation found in a case of Beckwith–Wiedmann syndrome. Cancer Res 55:2904–2909.

Negrini M, Monaco C, Vorechovsky I, Ohta M, Druck T, Baffa R, Huebner K, Croce CM. 1996. The FHIT gene at 3p14.2 is abnormal in breast carcinomas. Cancer Res 56:3173–3179.

Pandis N, Bardi G, Mitelman F, Heim S. 1997. Deletion of the short arm of chromosome 3 in breast tumors. Genes Chromosomes Cancer 18:241–245.

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

Puech A, Henry I, Jeanpierre C, Junien C. 1991. A highly polymorphic probe on 11p15.5: L22.5.2 (D11S774). Nucleic Acids Res 19: 5095–5099.

Radford DM, Fair KL, Phillips NJ, Ritter JH, Steinbrueck T, Holt MS, Donis-Keller H. 1995. Allelotyping of ductal carcinoma *in situ* of the breast: deletion of loci on 8p, 13q, 16p,17p and 17q. Cancer Res 55:3399–3405.

Russo J, Reina D, Frederick J, Russo IH. 1988. Expression of phenotypical changes by human breast epithelial cells treated with carcinogens *in vitro*. Cancer Res 48:2837–2857.

Russo J, Calaf G, Russo IH. 1993a. A critical approach to the malignant transformation of human breast epithelial cells with chemical carcinogens. Crit Rev Oncog 44:403–417.

Russo J, Calaf G, Sohi N, Tahin Q, Zhang PL, Alvarado ME, Estrada S, Russo IH. 1993b. Critical steps in breast carcinogenesis. Ann N Y Acad Sci 698:1–20.

Russo J, Barnabas N, Higgy N, Salicioni AM, Wu YL, Russo IH. 1996. Molecular basis of human breast epithelial cell transformation. In: Calvo F, Crepin M, Magdelenat H, editors. Breast cancer advances in biology and therapeutics. Paris: John Libbey Eurotext. p 33–43.

Russo J, Hu YF, Yang X, Huang Y, Silva I, Bove B, Higgy N, Russo IH. 1998. Breast cancer multistage progression. Front Biosci 3:944–960.

Russo J, Hu YF, Yang X, Russo IH. 2000. Developmental, cellular, and molecular basis of breast cancer. J Natl Cancer Inst Monogr 27: 17–38.

Russo J, Hu YF, Tahin Q, Mihaila, D, Slater C, Lareef HM, Russo IH. 2001. Carcinogenicity of estrogens in human breast epithelial cells. APMIS 109:39–52.

Sato T, Tanigami A, Yamakawa K, Akiyama F, Kasumi F, Sakamoto G, Nakamura Y. 1990. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res 50:7184–7189.

Sato T, Akiyama F, Sakamoto G, Kasumi F, Nakamura Y. 1991. Accumulation of genetic alterations and progression of primary breast cancer. Cancer Res 51:5794–5799.

Sato T, Saito H, Swensen J, Olifant A, Wood C, Danner D, Sakamoto T, Takita K, Kasumi F, Miki Y, Skolnick M, Nakamura Y. 1992. The human prohibitin gene located on chromosome 17q21 is mutated in sporadic breast cancer. Cancer Res 52:1643–1646.

Soule HD, Maloney TM, Wolman SR, Peterson WD Jr, Brenz R, McGrath CM, Russo J, Pauley R, Jones RF, Brooks SC. 1990. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50:6075–6086.

Swift M, Morrel D, Massey R, Chase C. 1991. Incidence of cancer in 161 families affected by ataxia-telangiectasia. N Engl J Med 325:1831–1836.

Tait L, Soule H, Russo J. 1990. Ultrastructural and immunocytochemical

characterizations of an immortalized human breast epithelial cell line MCF-10. Cancer Res 50:6087–6094.

Takita K-I, Sato T, Miyagi M, Watatani M, Akiyama F, Sakamoto G, Kasumi F, Abe R, Nakamura Y. 1992. Correlation of loss of alleles on the short arms of chromosomes 11 and 17 with metastasis of primary breast cancer to lymph nodes. Cancer Res 52:3914–3917.

Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J, Callahan R. 1986. Loss of a *c-H-ras-1* and aggressive human primary breast carcinomas. Cancer Res 46:4776–4781.

Theillet C, Adnane J, Szepetowski P, Simon MP, Jeanteur P, Birnbaum D, Gaudray P. 1990. BCL-1 participates in the 11q13 amplification found in breast cancer. Oncogene 5:147–149.

Thibodeau SN, Bren G, Schaid D. 1993. Microsatellite instability in cancer of the proximal colon. Science 260:816–819.

Tomlinson IP, Nicolai H, Solomon E, Bodmer WF. 1996a. The frequency and mechanism of loss of heterozygosity on chromosome 11q in breast cancer. J Pathol 180:38–43.

Tomlinson IP, Beck NE, Bodmer WF. 1996b. Allele loss on chromosome 11q and microsatellite instability in malignant melanoma. Eur J Cancer 32A:1797–1802.

Topper YJ, Sankaran L, Chomczynski P, Prosser C, Qasba P. 1986. Three stages of responsiveness to hormones in the mammary cell. In: Angeli A, Bradlow HL, Dogliotti L, editors. Endocrinology of the breast: basic and clinical aspects. New York: New York Academy of Sciences. p 1–10.

Wang H, Shao N, Ding QM, Cui J, Reddy ES, Rao VN. 1997. BRCA1 proteins are transported to the nucleus in the absence of serum and splice variants BRCA1a, BRCA1b are tyrosine phosphoproteins that associate with E2F, cyclins and cyclin dependent kinases. Oncogene 15:143–157.

Weber JL, May PE. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 44:388–396.

Wells AJ. 2000. Smoking and cancer in women. J Women's Cancer 2:55–66.

Westphal CH, Schmaltz C, Rowan S, Elson A, Fisher DE, Leder P. 1997. Genetic interactions between *atm* and *p53* influence cellular proliferation and irradiation-induced cell cycle checkpoints. Cancer Res 57:1664–1667.

Winqvist R, Mannermaa A, Alavaikko M, Blanco G, Taskinen PJ, Kiviniemi H, Newsham I, Cavenee W. 1993. Refinement of regional loss of heterozygosity for chromosome 11p15.5 in human breast tumors. Cancer Res 53:4486–4488.

Winqvist R, Hampton GM, Mannermaa A, Blanco G, Alavaiko M, Kiviniemi H, Taskinen PJ, Evans GA, Wright FA, Newsham I, Cavenee WK. 1995. Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. Cancer Res 55:2660–2664.

Wooster R, Cleton-Jansen AM, Collins N, Mangion J, Cornelis RS, Cooper CS, Gusterson BA, Ponder BA, von Deimling A, Wiestler OD, Cornelisse CJ, Devilee P, Stratton MR. 1994. Instability of short tandem repeats (microsatellites) in human cancers. Nat Genet 6:152–156.

Wu Y, Barnabas N, Russo IH, Yang X, Russo J. 1997. Microsatellite instability and loss of heterozygosity in chromosomes 9 and 16 in human breast epithelial cells transformed by chemical carcinogens. Carcinogenesis 18:1069–1074.

Yang X, Russo IH, Huang Y, Russo J. 1997. Microsatellite instability on chromosome 17 is associated with progression of breast cancer. Int J Oncol 11:41–46.

Yee CJ, Roodi N, Verrier CS, Parl FF. 1994. Microsatellite instability and loss of heterozygosity in breast cancer. Cancer Res 54:1641–1644.

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#4704 ESTROGENS INDUCED LOSS OF HETEROZYGOSITY IN CHROMOSOMES 3 AND 11 IN HUMAN BREAST EPITHELIAL CELLS. Jose Russo, Q Tahin, D Mihaila, Y F Hu, and I H Russo, *Fox Chase Cancer Ctr, Philadelphia, PA*

Breast cancers exhibit genomic alterations in multiple chromosomes. Although it is not known whether these genomic alterations are responsible of cancer initiation, they have been observed in human breast epithelial cells (HBEC) transformed *in vitro* by chemical carcinogens (Mol. Carcinog. 24:118, 1999). Estrogens are considered to be involved in the genesis of breast cancer. Although there is no direct evidence that natural or synthetic steroids induce neoplastic transformation in the human breast, we have demonstrated that 17-β estradiol (E_2) and diethylstilbestrol (DES) induce in MCF-10F cells the expression of phenotypes indicative of neoplastic transformation, i.e., anchorage independent growth and colony formation in agar methocel. Amplification of DNA of E_2- and DES-transformed HBEC revealed that these cells presented loss of heterozygosity (LOH) in chromosome 11, at 11q23.3 and 11q23.1–25 regions, and chromosome 3, at 3p21; 3p21–21.2; 3p21.1–14.2 and 3p14.2–14.1 regions, respectively. Our observations that estrogens induce genotypic changes in HBEC at loci also affected in spontaneously occurring breast malignancies suggest that these hormones have the potential to initiate the neoplastic process in the human breast epithelium. (Supported by grant RO1 CA67238 from NIH).

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#4743 **Estrogen Induces Transformation Phenotypes in the Estrogen Receptor Negative MCF-10F Cells.** M. H. Lareef, C. M. Slater, I. H. Russo, A. Rogatko, and J. Russo. *Fox Chase Cancer Center, Philadelphia, PA.*

The association found between breast cancer development and prolonged exposure to estrogens suggests that this hormone is of etiologic importance in the causation of this disease. In order to prove this postulate we treated the immortalized human breast epithelial cells (HBEC) MCF-10 F with 17β-estradiol ($E22222222_{2222\ 22}$) for testing whether they express colony formation in agar methocel, or colony efficiency (CE), and loss of ductulogenesis in collagen matrix, phenotypes also induced by the carcinogen benz (a) pyrene (BP). MCF-10F cells were treated with 0.0, 0.07 nM, 70 nM, or 0.25 mM of E_2 twice a week for two

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weeks. CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses. Ductulogenesis was 75±4.9 in control cells; it decreased to 63.7±28.8, 41.3±12.4, and 17.8±5.0 in E_2 treated cells, which also formed solid masses, whose numbers increased from 0 in controls to 18.5±6.7, 107±11.8 and 130±10.0 for each E_2 dose. MCF-10F cells were also treated with 3.7 μM E_2, the antiestrogen ICI 182,780 alone or with E_2, progesterone (P), and BP. CE was similarly increased in E_2- (CE=21.3±2.8) and BP- (CE=17.5±4.0) treated cells; it was reduced by ICI 182,780 alone (4.9± 1.4), and less by addition of E_2 (CE=13.2 ± 1.9) ($p<0.001$). The CE of P-treated cells was 8.7± 1.3. These results indicate that estrogen, like BP, induces in HBEC phenotypes indicative of neoplastic transformation. (This work was supported by NCI Grant R01 CA67238 and DAMD 17-00-1-0249).

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#5276 Transformation of human breast epithelial cells by estrogen metabolites. Mohamed H. Lareef, Irma H. Russo, Fathima S. Sheriff, Carolyn Slater, and Jose Russo. *Fox Chase Cancer Center, Philadelphia, PA.*

Breast cancer is a hormone-dependent malignancy whose development is predominantly influenced by estrogens. Although it is well known that estrogens regulate normal development of reproductive and non-reproductive organs, an effect mediated by activation of intracellular receptors, the mechanisms by which estrogens stimulate the formation of breast cancer remain poorly understood. Because it has been postulated that estrogens act as endogenous carcinogens through the formation of oxidative metabolites, and we have been able to induce transformation of human breast epithelial cells (HBEC) with 17b-estradiol (E2), the present work was designed with the purpose of testing whether estrogen metabolites induce transformation of MCF-10F cells, which are estrogen receptor (ER) negative. MCF-10F cells were treated with the estrogen metabolites 4-hydroxyestradiol (4-OHE2), 2-OHE2, and 16-a-OHE2. In addition, 4-OHE2 was tested in combination with the antiestrogen ICI-182-7802. Controls consisted of non-treated MCF-10F and cells subjected to the following treatments: E2, progesterone (P), diethylstilbestrol (DES), ICI-182-780, and tamoxifen (TAM), E2+P, E2+ICI-182-780, E2+TAM, the carcinogen benz(a)pyrene (BP), and cholesterol. All hormones, BP and cholesterol were added in triplicate to the culture medium at the following concentrations: 0.007nM, 70nM or 1mg per ml. Treatments were applied twice a week for two weeks and all experiments were repeated three or more times. Cell transformation was assessed by evaluating the following parameters: colony formation in agar-methocel, or colony efficiency (CE), growth in collagen matrix with reduction of ductule formation, expressed as ductulogenic

index (DI), at the expense of solid mass (SM) formation, DI:SM ratio, invasiveness in Matrigel chambers, or invasive index (INVI), and S-phase determination by flow cytometry, parameters that have been validated in BP-transformed MCF-10F cells. Treatment with 4-OHE2 at 70nM resulted in 13.3% CE, 6.75 DI, 106 SM, 0.06 DI:SM ratio, 533 INVI, 12.5% S-phase. Cells treated with 2OHE2 and 16-a-OHE2, showed similar results, except that CE and S-phase were lower in the former. Values were also similar in cells treated with BP, E2, P, and DES. Addition of ICI-182-780 to the 4-OHE2 treatment did not modify any of the parameters tested, whereas its addition to E2-treated cells reverted the phenomenon, with reduction in CE, increased both DI and DI:SM ratio and decreased INVI. These observations demonstrated that estrogen metabolites, and more remarkably 4-OHE2 induce the expression of phenotypes indicative of neoplastic transformation in MCF-10F cells. These results indicate that the transformation of HBEC by estrogen metabolites occurs through estrogen receptor independent pathway. (Supported by grants NIH-RO1-CA 67238, DAMD17-00-1-0247, and DAMD17-00-1-249