Toxicologic Pathology, 38: 110-122, 2010

ISSN: 0192-6233 print / 1533-1601 online
DOI: 10.1177/0192623309354108

Estrogen and Xenoestrogens in Breast Cancer

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Abstract

There is growing concern that estrogenic environmental compounds that act as endocrine-disrupting chemicals might potentially have adverse effects on hormone-sensitive organs such as the breast. This concern is further fueled by evidence indicating that natural estrogens, specifically 17β-estradiol, are important factors in the initiation and progression of breast cancer. We have developed an in vitro–in vivo model in which we have demonstrated the carcinogenicity of E2 in human breast epithelial cells MCF-10F. Hypermethylation of NRG1, STXBP6, BMP6, CSS3, SPRY1, and SNIP were found at different progression stages in this model. The use of this powerful and unique model has provided a tool for exploring whether bisphenol A and butyl benzyl phthalate have relevance in the initiation of breast cancer. These studies provide firsthand evidence that the natural estrogen 17β-estradiol and xenoestrogenic substances like bisphenol A are able to induce neoplastic transformation in human breast epithelial cells.

Keywords: estrogen; BPA; BBP; cell transformation; breast cancer; epigenetic changes; neuregulin.

Introduction

Breast cancer is an estrogen-dependent malignancy whose incidence is steadily increasing in most Western societies and industrialized countries. Each year, 44,000 women die of breast cancer, making it the leading cause of cancer deaths among American women who do not smoke and among those aged forty to fifty-five years. Breast cancer is uncommon among women younger than thirty years of age, but the incidence increases sharply with age and slows somewhat between ages forty-five and fifty, around the time of menopause (Pike et al. 1983).

The elevated incidence of breast cancer in women has been associated with prolonged exposure to high levels of estrogens, as that occurring with early onset of menarche and late menopause (Bernstein and Ross 1993; Henderson, Ross, and Pike 1991). In obese menopausal women, adipose tissue becomes the major source of estrogens that contribute to increase the breast cancer risk (J. R. Harris et al. 1992; Z. Huang et al. 1997). The relationship between estrogen and breast cancer is supported by epidemiological data that demonstrated that women who receive hormone replacement therapy (HRT) are more likely to develop breast cancer than those who have never used HRT (Bakken et al. 2004; Beral 2003; Collaborative Group on Hormonal Factors in Breast Cancer 1997; Chlebowski et al. 2003; Colditz 2005; Li et al. 2003; Rossouw et al. 2002). Studies of blood estrogen levels among postmenopausal women have shown that higher levels are associated with increased risk of subsequent breast cancer (Colditz 1998; Friel, Hinchcliffe, and Wright 2005; Key et al. 2002). The Million Women Study (MWS) showed a significant increase in the RR of breast cancer in women taking estrogens (conjugated equine estrogens or estradiol orally) (Relative Risk = 1.30), although the increase was greater for women taking estrogens combined with synthetic progestins (RR = 2.00) (Beral 2003). The risk increased with duration of use of estrogen alone ($RR_{10\ years} = 1.37$) and estrogen plus progestin ($RR_{10\ years} = 2.31$).

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Xenoestrogens: Bisphenol A and Butyl Benzyl Phthalate

Xenoestrogens are part of a group of synthetic and naturally occurring agents termed endocrine disruptors because of their capacity to perturb normal hormonal actions. It has been suggested that some endocrine disrupters may contribute to the development of hormone-dependent cancers, such as breast and endometrial cancers (Jobling et al. 1995; Sonnenschein and Soto 1998).

Bisphenol A (BPA) is a xenoestrogen that has been widely used since the 1950s as a monomer that is polymerized to manufacture polycarbonate plastic and epoxy resins; it is also found as an environmental contaminant. Human exposure occurs when BPA leaches from common items such as plastic-lined food and beverage cans, as well as and from some dental sealants (Brotons et al. 1995; Olea et al. 1996). Bisphenol A thus leaches into food and beverages through the normal use of metal cans and polycarbonate plastic containers, and the rate of leaching increases when polycarbonate is scratched and discolored (Brede et al. 2003; Burridge 2003; Howdeshell et al. 2003). The amount of BPA released from polycarbonate plastics increases at high or low pH and at high temperatures. Bisphenol A also is used as an additive in many other products, with global capacity at more than six billion pounds per year (Burridge 2003). Evidence of the estrogenic effects of BPA has been reported in several studies showing that it activates estrogen receptors (ER) alpha and beta (Matthews, Twomey, and Zacharewski 2001; Routledge et al. 2000) and stimulates MCF-7 breast cancer cell growth (Krishnan et al. 1993). Although BPA mimics 17β-estradiol (E2) by competitively binding and activating endogenous ERs, its affinity is at least

ten thousand–fold less than E2 for both ERα and ERβ (Maruyama et al. 1999), suggesting that other mechanisms could be responsible for its biological effect.

There is some uncertainly as to the level of BPA exposure and the risk it presents, but evidence suggests that it can disrupt normal reproductive tract development in male and female rodents (Gupta 2000; Ramos et al. 2001; Suzuki et al. 2002; Takao et al. 1999). Bisphenol A exposure during perinatal periods has been shown to inhibit testosterone synthesis in adult rats (Akingbemi et al. 2004) and promote feminization of *Xenopus laevis* tadpoles (Levy et al. 2004). There is significant exposure of pregnant women and their fetuses to BPA, as indicated by its presence in maternal plasma (3.1 ng/mL), fetal plasma (2.3 ng/mL; approximately 10 nM), and placental tissues (1–104.9 ng/g) (Schonfelder et al. 2002). Bisphenol A levels were found to be even higher in amniotic fluid during fetal reproductive tract differentiation (8 ng/mL) (Ikezuki et al. 2002). Bisphenol A was detected in 95% of the urine samples tested in the United States at concentrations ≥0.1 μg/L (Calafat et al. 2005). Urinary levels of BPA and its conjugates have been found in various populations in Southeast Asia (Matsumoto et al. 2003; Ouchi and Watanabe 2002; M. Yang et al. 2003). In forty-eight female Japanese college students, BPA glucuronide was detected in all the urine samples at concentrations ranging from 0.2 to 19.1 ng/mL, with a median level of 1.2 μg/L (0.77 μg/g creatinine) (Ouchi and Watanabe 2002). The largest BPA non-occupational exposure assessment reported urinary levels of 9.54 μg/L (8.91 μg/g creatinine) in a group of seventy-three adult Koreans (53.4% female) (M. Yang et al. 2003).

Butyl benzyl phthalate (BBP) is another endocrine disruptor that has been reported to be an estrogenic compound and a partial agonist for ER (Andersen et al. 1999; Jobling et al. 1995; Zacharewski et al. 1998). It is used as a plasticizer and is widely used in food wraps and cosmetic formulations. The International Program of Chemical Safety (IPCS) concluded that BBP exposure to the general population is based almost entirely on food intake, because the concentrations of BBP in air, drinking water, and soil are very low, making intakes from these routes essentially negligible. Adult intake of BBP has been estimated at 2 μg/kg body weight (bw)/day, and exposure to infants and children could be up to threefold higher (IPCS 1999). The results of several in vivo studies indicate an antiandrogen–like activity of BBP or its major metabolite following in utero exposure. Studies in rats have described that exposure to 500 or 1000 mg/kg bw/day of BBP or 250 or 375 mg/kg bw/day of its major metabolite monobenzyl phthalate on days 15 to 17 of pregnancy induced significant alterations in the reproductive system of male offspring, including undescended testes and decreased anogenital distance (Ema and Miyawaki 2002; Ema et al. 2003). When administered during sexual differentiation, BBP also causes male reproductive tract malformation of the external genitalia, sex accessory glands, epididymides, and testes (Gray et al. 2000). Butyl benzyl phthalate estrogenic activity has been demonstrated by MCF-7 cell proliferation assays and its in vitro binding to the ER (Andersen et al. 1999; Harris et al. 1997; Hong et al. 2005; Jobling et al. 1995; Zacharewski et al. 1998). Butyl benzyl phthalate was found to be weakly estrogenic; it reduced in vitro binding of E2 to rainbow trout ER at a high concentration (0.01–10 mg/L) (Jobling et al. 1995).

In conclusion, BPA and BBP have estrogenic activity, although the concentrations required for the effects in vitro and in rodent models were extraordinarily greater than the estimated human exposure. The study of BPA and BBP effects in human breast epithelial cells might yield important information on whether these compounds have the potential to contribute to breast cancer initiation.

In Vitro–In Vivo Model of Estrogen Carcinogenicity in Human Breast Epithelial Cells

As indicated in the previous sections, there is overwhelming epidemiological, clinical, and experimental evidence that estrogens are initiators of breast cancer and that xenoestrogens may also influence the susceptibility or be involved in the initiation or progression of the disease. We have been pioneers in obtaining convincing experimental evidence that E2 and its catechol metabolites 4-hydroxyestradiol (4-OH-E2) and 2-hydroxyestradiol (2-OH-E2) are capable of transforming MCF-10F, a human breast epithelial cell (HBEC) line that is ERα negative and ERβ positive (Russo et al. 2002; Russo et al. 2003). The expression of phenotypes indicative of neoplastic transformation were accompanied by genomic alterations such as loss of heterozygosity and mutations at specific loci in chromosomes 13 and 17 (Fernandez, Russo and Russo 2006).

MCF-10F cells were treated with 0.007 nM, 70 nM or 3.6 μM E2 for twenty-four hours, twice a week for two weeks. At the end of the second week, the cells were assayed for survival efficiency, ductulogenic capacity, and colony efficiency (CE) in agar methocel, all phenotypes indicative of cell transformation (Russo et al. 2003). The cells' ductulogenic capacity, which expresses the ability of the cells to grow forming three-dimensional structures, was quantitatively evaluated in cells plated in a collagen matrix. Control MCF-10F cells formed ductlike structures that were lined by a single or double layer of low cubical epithelium in collagen and did not form colonies in agar methocel. Estradiol-treated cells lost their ability to produce ductules in a dose-dependent manner, and the number of solid masses increased parallel to the formation of colonies in agar methocel, expressing phenotypes similar to those induced by the carcinogen benzo[a]pyrene (BP) (Russo et al. 2003). The cells treated with either dose of E2 formed colonies in agar methocel that did not differ in size among groups; however, the CE in agar methocel increased with increasing doses of E2. These phenotypes were not abrogated when the cells were treated with tamoxifen or the pure antiestrogen ICI182780 (Russo et al. 2003). Cells were analyzed by flow cytometry, which revealed that the percentage of cells in the S phase was increased in E2 treated cells in a dose-dependent fashion. The effect of E2 on the invasive capabilities of MCF-10F cells was evaluated using Boyden chambers (Lareef et al. 2005). These

chambers are fitted with an 8-μm pore size membrane coated with a thin layer of matrigel basement membrane matrix that occludes the pores, blocking noninvasive cells from migrating. Invasive cells are able to detach from and invade through the matrigel matrix to pass through the pores. MCF-10F cells treated with 70 nM E2 (also called trMCF) in their ninth passage were placed in the chamber wells and the cells that crossed the basement membrane were collected, expanded, and designed as bsMCF (Figure 1A). When the bsMCF cells were injected in the mammary fat pad of severe combined immunodeficient (SCID) mice (Russo et al. 2006), they formed tumors in nine of ten animals within forty-five days of injection. The tumors induced by bsMCF cells were poorly differentiated adenocarcinomas; they were ERα and progesterone receptor (PR) negative and expressed basic keratins of high molecular weight, E-cadherin, CAM5.2, and vimentin. Cells were isolated from the tumors and grown in vitro, which yielded caMCF cells. The caMCF1, caMCF2, caMCF3, and caMCF4 cell lines were isolated from four single xenograft tumors grown in four animals, and all of them produced tumors when they were injected to SCID mice (Y. Huang et al. 2007; Russo et al. 2006) (Figure 1A). This in vitro–in vivo model of estrogen-transformed human breast epithelial cells developed in our laboratory represent different stages of tumor initiation and progression; MCF-10F represented the normal stage; trMCF cells, the transformed stage; bsMCF cells, the invasive stage; and caMCF cells, the tumorigenic stage (Figure 1B).

The transforming potential of E2 was also evaluated on primary breast epithelial cells. Primary breast cells form ductlike structures in collagen matrix, contrary to tumor cell lines, which form spherical masses. Primary breast epithelial cells were isolated from tissues obtained from three reduction mammoplasties performed for cosmetic reasons. The mammary tissues were digested with collagenase and hyaluronidase, and the organoids were maintained in low-Ca^{2+} medium, as described by Soule et al. (1990). The three primary cell cultures were treated with 70 nM E2 twice a week for two weeks, and as a control, cells were treated with dimethyl sulfoxide (DMSO), the vehicle in which E2 was dissolved. After the treatments, the ductulogenic capacity of the cells was evaluated: 5,000 cells were suspended in 400 μL 89.3% (PureCol) collagen matrix (Inamed Biomaterials, Fremont, CA, USA) and plated on a 400 μL precoated collagen well using a twenty-four-well chamber plate. After three weeks in collagen, the E2-treated cells formed some spherical masses, whereas no solid masses were observed in the controls (Table 1). This is the first report indicating that E2 is able to induce phenotypes of cell transformation in primary breast epithelial cells.

Bisphenol A and BBP Induce Neoplastic Transformation of Human Breast Epithelial Cells In Vitro

The human breast epithelial cells MCF-10F were treated with 10^{-3}M, 10^{-4} M, 10^{-5} M and 10^{-6} M BPA or BBP continuously for two weeks, with fresh medium added every day. These doses have already been tested by other authors in HBEC (Hong et al. 2005). As controls, MCF-10F cells were not treated and maintained in the regular medium (control group) or treated with 0.284% DMSO (vehicle). The cells treated with 10^{-3} M BPA and BBP died on the second day of treatment. The concentration of 10^{-4} M BPA and BBP was also toxic for MCF-10F cells: the cells died on the fourth and sixth day of treatment, respectively. These data indicated that 10^{-3} M and 10^{-4} M BPA and BBP are toxic for MCF-10F cells, and BPA is more toxic than BBP. When the treatments finished, the cells were tested for the expression of transformed phenotypes using the collagen assay. For this assay 3,000 cells were plated per well, and at the end of the observation period (eight days), the ductlike structures and solid masses formed on 3-D culture were counted (Figure 2). The cells formed a high percentage of ductlike structures in collagen, although there was a significant decrease of ducts in the cells treated with BPA (10^{-5} M) compared to the control and DMSO groups. MCF-10F treated with 10^{-5} M and 10^{-6} M BPA formed a high percentage of solid masses (27% and 20%, respectively), and differences were statistically significant between 10^{-5} M BPA and control/DMSO groups. Butyl benzyl phthalate did not increase the number of solid masses. Interestingly, the number of solid masses after 10^{-5} M BPA treatment was even higher than in cells treated with 70 nM E2, and the differences were significant ($p < .01$) (Figure 2). The ductules formed by the cells treated with 10^{-5} M BPA were wider and shorter, similar to the ones formed by the cells treated with 70 nM E2 (Figure 3). Evidence for the estrogenic effects of BPA has been reported in several studies showing that BPA activates estrogen receptors alpha (ERα) and beta (ERβ), although with at least ten-thousand–fold less affinity than E2 (Maruyama et al. 1999). Ten passages after treatment, the invasive capacity of the cells treated with BPA and BBP was evaluated using Boyden chambers (Figure 4). The cells that invaded through the Matrigel membrane were counted under a light microscope, and values of chemo-invasion were expressed as the number of cells that migrated to the lower chamber. The cells treated with BBP (10^{-5} M and 10^{-6} M) showed a higher invasive capacity compared to the control, and the differences were statistically significant ($p \leq .01$) (Figure 4). MCF-10F cells treated with E2 at different concentrations (0.007 nM, 70 nM, and 3.6 μM) had higher invasion capacity compared to the control (Figure 4). Furthermore, the invasion capacity of MCF-10F cells treated with 10^{-6} M BBP was similar to that of cells treated with the 0.007 nM E2; the differences were not significant between these groups ($p > .05$) (Figure 4).

Also, we have treated primary human breast epithelial cells with BPA continuously for two weeks, and preliminary data indicated that the cells treated with 10^{-6}M BPA express alterations in the ductulogenic pattern in the collagen matrix.

Genomic and Epigenetic Changes in the In Vitro–In Vivo Model of Cell Transformation Induced by E2

The in vitro–in vivo model described in the previous section represents different stages of cancer progression; MCF-10F

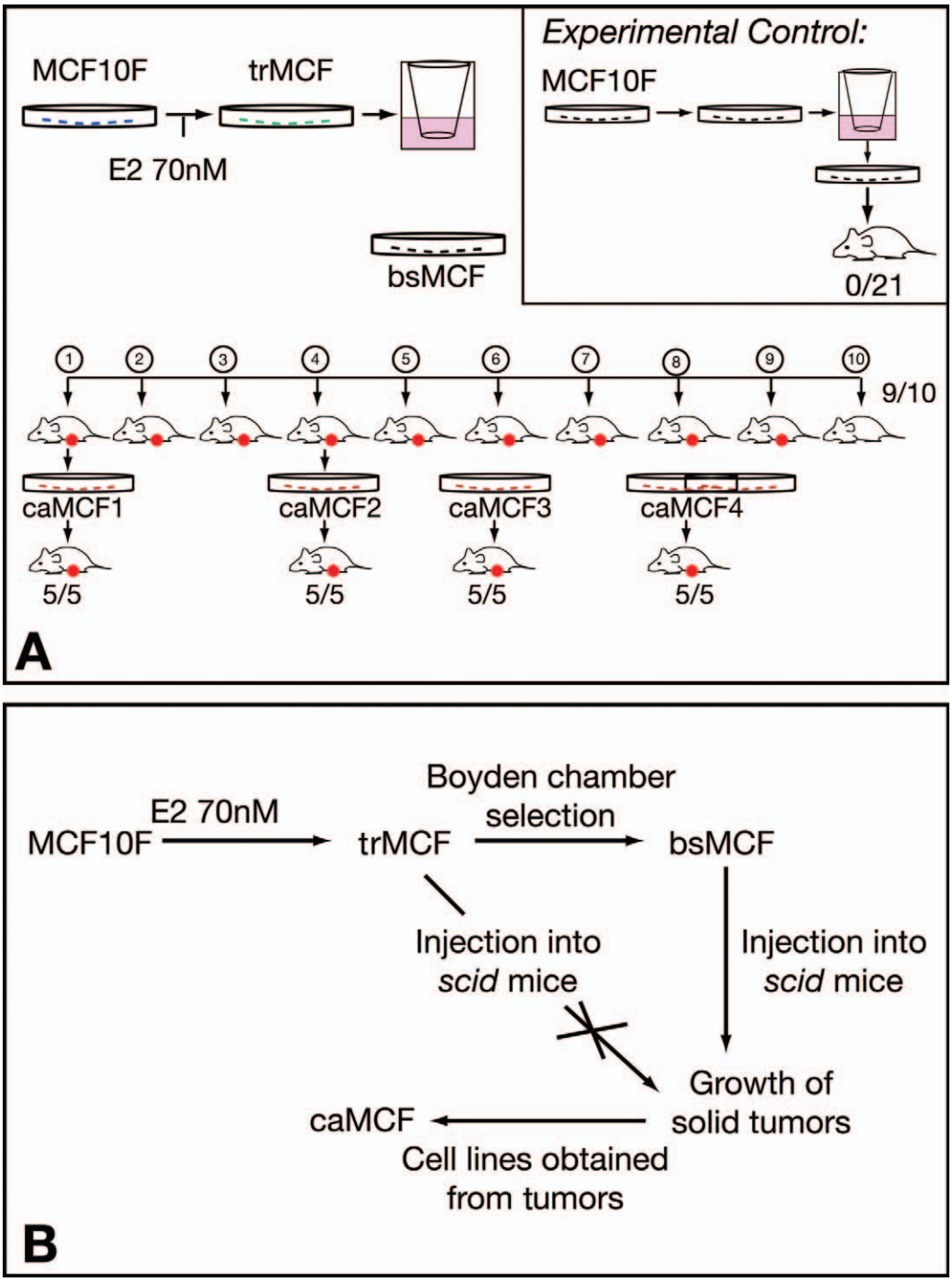

FIGURE 1.—In vitro–in vivo model of estrogen-induced transformation of the human breast epithelial cell MCF-10F. (A) Schematic representation of the experiments performed in SCID mice. MCF-10F was treated with 70 nM E2, which yielded trMCF cells; the trMCF cells were placed on matrigel Boyden chambers, and the cells that passed through the membrane were collected, expanded, and injected into the fat mammary pad of SCID mice; these cells were designated bsMCF. The bsMCF cells produced tumors in nine out of ten mice. Cells were isolated from the tumors originating from caMCF1, caMCF2, caMCF3, and caMCF4, and all of these cells produced tumors when they were injected into the mammary gland of SCID mice. Experimental control: MCF-10F cells were placed in Boyden chambers, and the cells that passed through the membranes were collected, expanded, and injected into SCID mice; none of these cells produced tumors (modified from Russo et al. 2006). (B) Different stages in the in vitro–in vivo model of cell transformation: MCF-10F (normal stage), trMCF (cells transformed by estradiol), bsMCF (invasive stage), and caMCF (tumorigenic stage). The trMCF cells did not form tumors in SCID mice, although the invasive bsMCF cells formed tumors that gave origin to caMCF cell lines (modified from Y. Huang et al. 2007).

TABLE 1.—Primary HBEC treated with E2 plated on collagen. Primary cells were plated in collagen after treatment with E2. As control, the cells were treated with DMSO (vehicle in which E2 was dissolved). The means of spherical masses ($\pm$ *SD*) in four wells are indicated.

Primary cell designation	Treatment	Number of spherical masses on collagen
HSM3 left	DMSO	0
	70 nM E2	2.75 $\pm$ 0.5
308 Left	DMSO	0
	70 nM E2	15.5 $\pm$ 1.7
307 left	DMSO	0
	70 nM E2	4.25 $\pm$ 1.2

Abbreviations: DMSO, dimethyl sulfoxide; E2, 17β-estradiol; HBEC, human breast epithelial cells; *SD*, standard deviation.

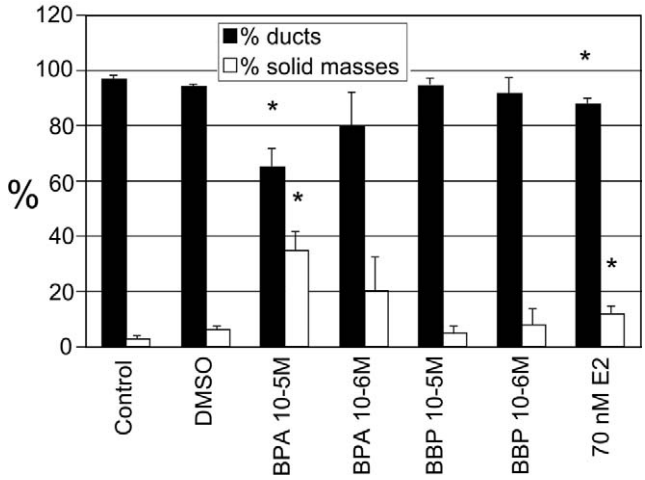

FIGURE 2.—Percentage of ducts and solid masses in collagen matrix after bisphenol A (BPA), butyl benzyl phthalate (BBP), and 17β-estradiol (E2) treatments. MCF-10F cells were treated with 10^{-5} M and 10^{-6} M BPA or BPA continuously for fifteen days. As controls, the cells were treated with dimethyl sulfoxide (DMSO; vehicle) or grown in regular media. Also, MCF-10F cells were treated with 70 nM 17β-estradiol for twenty-four hours, twice a week, for two weeks. Student *t*-test was used for comparison between treatments and control, and $p \leq .01$ was accepted as statistically significant. The number of tubules and solid masses grown in four wells were counted. The mean ($\pm$*SD*) and the statistically significant differences from the control (*) are indicated.

represents the normal stage; trMCF, the transformed stage; bsMCF, the invasive stage; and a more advanced stage is represented by caMCF (Figure 1B). These phenotypes correlated with gene dysregulation during the progression of the transformation. The highest number of dysregulated genes was observed in caMCF, was slightly lower in bsMCF, and lowest in trMCF. This order was consistent with the extent of chromosome aberrations (caMCF > bsMCF >>> trMCF) that were studied using comparative genomic hybridization (CGH) (Figure 5). For CGH, the DNA hybridization and digital image analysis were performed as previously described (Fernandez et al. 2005). Gain or losses are progressive at the different stages of the in vitro–in vivo model; there are few gains or losses in trMCF, and more in the invasive bsMCF and tumorigenic caMCF cells (Figure 5). Only small chromosome gains were observed in trMCF, and most alterations were observed in bsMCF and caMCF cells (Figure 5). Chromosomal amplifications were found in 1p36.12-pter, 5q21.1-qter in both bsMCF and caMCF. Losses of the complete chromosome 4 and 8p11.21-23.1 were found in bsMCF and caMCF cells (Figure 5). In caMCF cells, additional losses were found in 3p12.1-14.1, 9p22.1-pter, and 18q11.21-qter. Also, a chromosomal amplification in 13q21.31-qter was found in caMCF using high-density SNP arrays (Y. Huang et al. 2007). Functional profiling of dysregulated genes revealed progressive changes in the integrin signaling pathway, inhibition of apoptosis, acquisition of tumorigenic cell surface markers, and epithelial-mesenchymal transition (EMT). In tumorigenic cells, the levels of E-cadherin, EMA, and various keratins were low and CD44E/CD24 was negative, whereas SNAI2, vimentin, S100A4, FN1, HRAS, TGFβ1, and CD44H levels were high.

Global CpG island methylation at the different stages in the in vitro–in vivo model of cell transformation was studied using restriction landmark genomic scanning (RLGS) (Fernandez et al. 2006). Restriction landmark genomic scanning is based on DNA digestion with the restriction enzyme NotI, which is methylation sensitive because it is able to cut only unmethylated DNA (Figure 6). The sites of differential methylation in trMCF, bsMCF, and caMCF cells were compared with MCF-10F methylation profile. A total of thirty-eight genes were identified as hypermethylated, and four genes were hypomethylated when trMCF, bsMCF and caMCF were compared with the control MCF-10F cells (Table 2). The data revealed that the methylation pattern of different genes related to ductulogenesis and branching, estrogen metabolism, apoptosis, and proliferation was altered (Table 2). This is the first demonstration that changes in DNA methylation are involved in the early and late stages of breast cancer progression, indicating that from the thirty-eight hypermethylated genes, seventeen of them were potentially involved in the branching process: HOXA9, HOXB5, EPB49, FGF14, SYNE1, FOXD1, BMP6, SPRY1, TM4SF9, SNIP, STXBP6, TLL1, CSS3, OTP, TBR1, NRG1, and ITGA11 (Table 2). Real-time reverse transcriptase polymerase chain reaction (RT-PCR) to study gene expression was important in corroborating that hypermethylation is associated with the silencing of these genes. By using this validation method, differences in expression were confirmed for SPRY1, NRG1, STXBP6, BMP6, CSS3, and SNIP among cells in different stages (Table 3). SPRY1, NRG1, STXBP6, BMP6, CSS3, and SNIP expressions were down regulated at different stages in the in vitro–in vivo model when compared to the expression in MCF-10F cells (Table 3). trMCF showed low expression of NRG1, STXBP6, and BMP6 compared to MCF-10F, and increased CSS3 (4.79-fold) and SNIP (1.89-fold) expressions. The invasive bsMCF cells showed low expression of SPRY1, NRG1, STXBP6, BMP6, CSS3, and SNIP. In caMCF, no expression of NRG1 and CSS3 was detected, and these cells had low expression of BMP6, SPRY1,

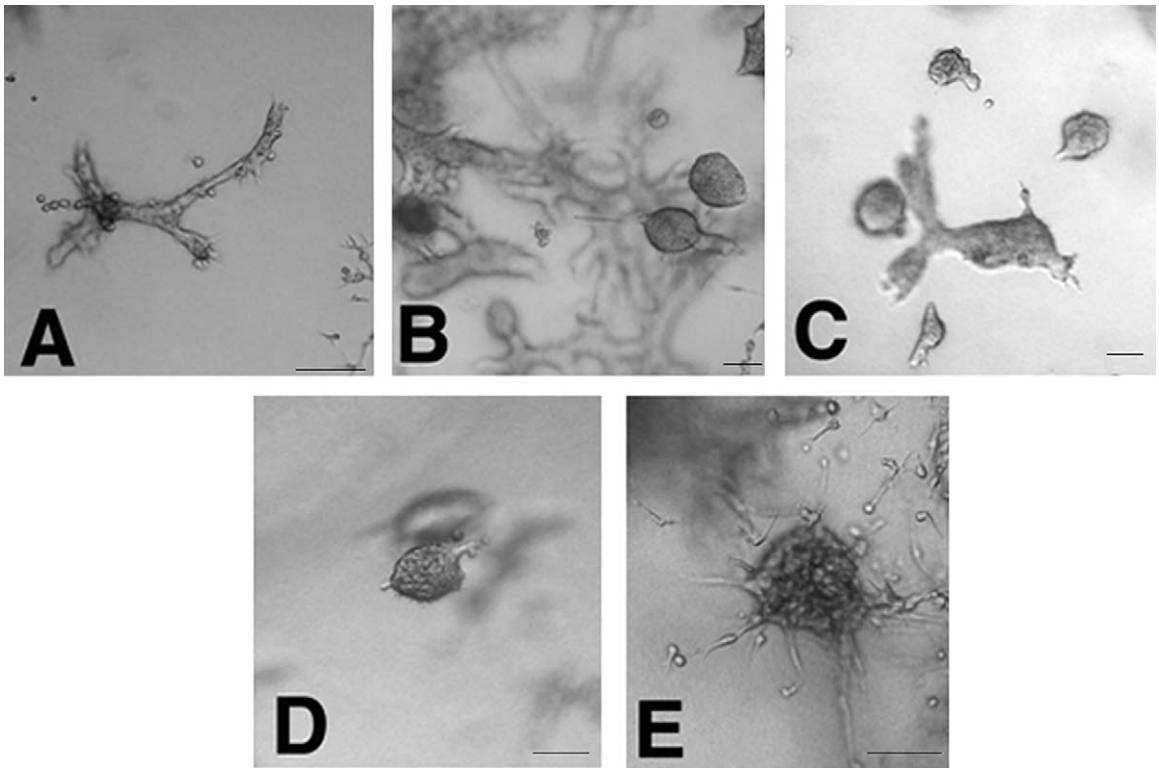

FIGURE 3.—Ducts and solid masses formed by MCF-10F cells treated with bisphenol A (BPA) or butyl benzyl phthalate (BBP). MCF-10F treated with (A) DMSO; (B) BPA 10^{-5}M; (C) BPA 10^{-6}M; (D) BBP10^{-5}M; (E) BBP 10^{-6}M. The bars at the bottom right corners of each picture represent 100 μm.

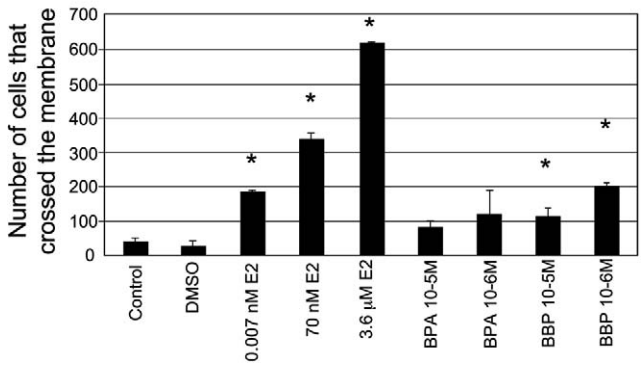

FIGURE 4.—Invasion assay. The cells that migrated through the membrane were counted after the different treatments: MCF-10F cells treated with 10-5M bisphenol A (BPA); 10-6M BPA; 10-5M butyl benzyl phthalate (BBP), 10-6M BBP, and MCF-10F treated with 17β-estradiol (E2) (0.007 nM, 70 nM, or 3.6 μM). As controls, cells were grown in regular media or media with dimethyl sulfoxide. Student *t*-test was used for comparison between treatments and control, and $p \leq .01$ was accepted as statistically significant. Three replicates were made for each treatment. The mean ($\pm SD$) and the statistically significant differences from the control (*) are indicated.

and SNIP; however, they showed higher expressions of STXBP6 (Table 3). The expression studies did not correlate with the RLGS results for HOXA9, HOXB5, EPB49, FGF14, SYNE1, FOXD1, TLL1, OTP, TBR1, TM4SF9, and ITGA11; these genes showed the same level of expression at the different stages.

There are three active DNA methyltransferases (DNMTs), and DNA hypermethylation was correlated with their high expressions in tumors. Increased expression of DNMT1 was found in colon tumors and leukemia (Smiraglia and Plass 2002), and its expression appears to increase progressively with advancing stages of both colon and lung cancers (Lee et al. 1996). DNMT3A and DNMT3B expressions have been reported to be elevated in acute myeloid leukemia (Melki et al. 1998) and in solid tumors (Belinsky et al. 1996). DNMT expressions were studied in the in vitro–in vivo model of cell transformation using real-time RT-PCR. The trMCF and caMCF4 cells showed higher expression of DNMT3B and DNMT3A, respectively, compared to MCF-10F ($p < .01$) (Figure 7). DNMT1 did not show significant differences at the different stages of the in vitro–in vivo model.

CONTROLLING THE EPIGENETIC MECHANISM OF GENE SILENCING

In contrast to mutations, which are essentially irreversible, methylation changes are reversible, raising the possibility of developing therapeutics based on restoring the normal methylation state to cancer-associated genes. The drugs 5-aza-2′-cytidine (5-aza-C) and 5-aza-2′-deoxycytidine (5-aza-dC) have proved valuable for basic studies of DNA methylation and transcriptional silencing. Using these compounds and the histone

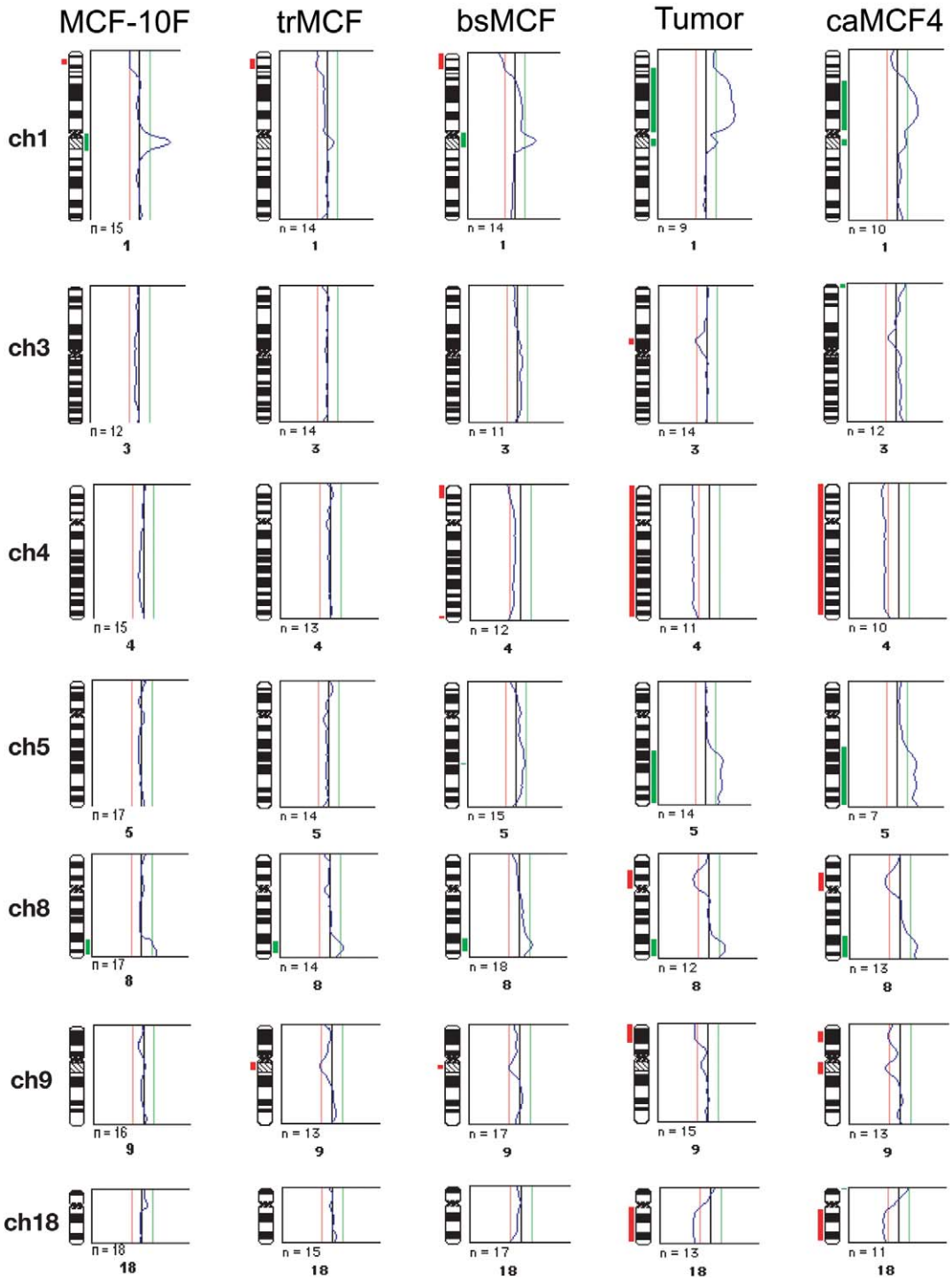

FIGURE 5.—Comparative genomic hybridization (CGH) analysis of the in vitro–in vivo model of cell transformation induced by 17β-estradiol (E2). The threshold was set at 0.8 and 1.2 for losses and gains, respectively; the mean values of individual ratio profiles were calculated from at least seven metaphase spreads, and averaged values were plotted as profiles alongside individual chromosome ideograms. The chromosomal imbalances are shown in a histogram, which represents the DNA gains (in green) and losses (in red) as an incidence curve along each chromosome. Only chromosomes with changes are represented. Also, the gains and losses found in the tumor (Tumor) that gave origin to caMCF4 are represented.

deacetylase inhibitor trichostatin A (TSA), we have confirmed that the genes identified by RLGS were epigenetically silenced. SPRY1, NRG1, STXBP6, BMP6, CSS3, and SNIP showed increased expression after 5-aza-dC and/or TSA treatments, indicating that they are epigenetically regulated (unpublished data).

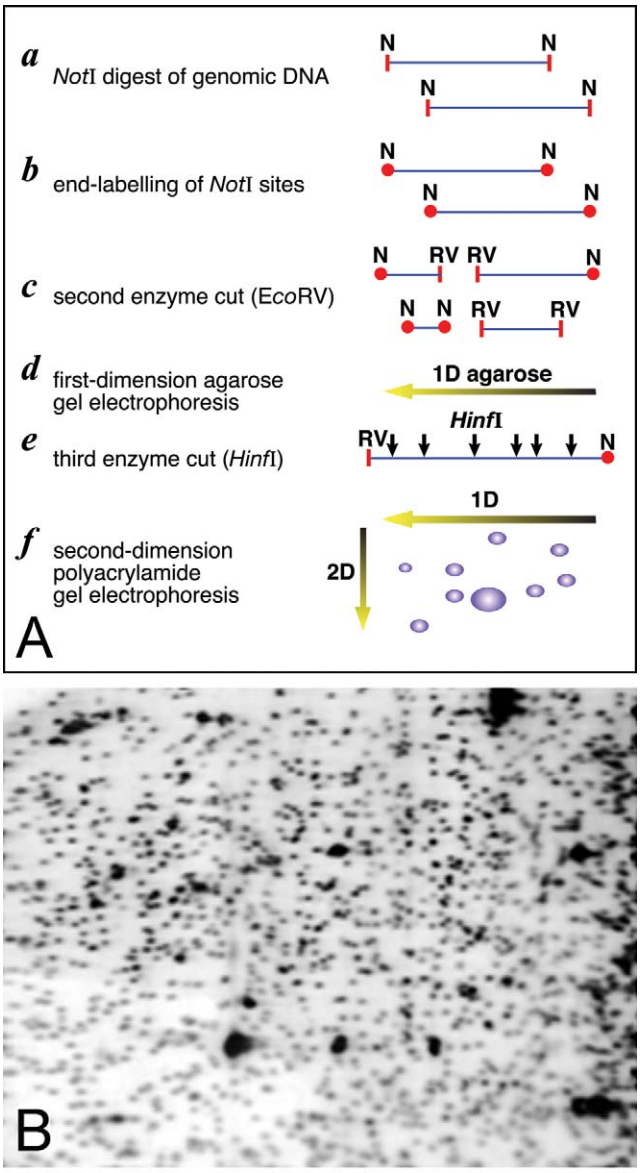

FIGURE 6.—Restriction landmark genomic scanning (RLGS). (A) The DNA is cut with the methylation-sensitive *Not*I. The recognition sequence for *Not*I, GC′GGCCGC, has two CpG sites, and *Not*I will not cut if the site is methylated. The overhangs of cut sites are filled with radiolabeled dCTP and dGTP. Afterward, the fragments were digested with EcoRV to get fragments into a resolvable size, and the labeled fragments were separated in first-dimension 0.8% agarose tube gel. The gel was treated with HinfI to cut the DNA into smaller fragments, and the tube gel was placed perpendicularly on top of 5% polyacrylamide gel and separated in second dimension. (B) Autoradiogram of the RLGS polyacrylamide gel. Sites of differential methylation are identified by comparison with a control profile; when comparing control and treated, hypomethylation would appear as greatly increased intensity of RLGS spots; RLGS spot loss is a result of hypermethylation of the *Not*I site. Each virtual spot is predefined by a specific sequence in a database.

The NRG1, or heregulin alpha (HRG), was down regulated very early in the transformation process (trMCF), and its expression remained low in the invasive and tumorigenic cells (bsMCF and caMCF), indicating that the epigenetic effect can be maintained in multiple cell generations (Figure 8). NRG1 has been shown to induce the growth and differentiation of epithelial cells and promotes branching morphogenesis (Y. Yang et al. 1995). NRG1 is a ligand for ErbB3 and ErbB4 and regulates both cell proliferation and terminal differentiation in the mammary gland (Long et al. 2003; Sartor et al. 2001; Tidcombe et al. 2003). We studied NRG1 expression in nine human breast tissue samples, and we found that its expression was low in six of six samples of invasive ductal carcinoma, whereas it was high in three of three samples of normal breast tissue (Table 4). Recently it was shown that NRG1 was hypermethylated in tumor samples, whereas it was unmethylated in normal breast tissue, and the authors proposed that NRG1 was a tumor suppressor gene (Chua et al. 2008). NRG1 was found to be heavily methylated in 76.5% of breast cancer cell lines that had no NRG1 expression, and it was relatively unmethylated in normal breast cell lines and cancer cell lines expressing this gene (Chua and Paw 2006).

Bone morphogenetic protein 6 (BMP6) showed reduced expression in the transformed (trMCF), invasive, and tumor stages (bsMCF and caMCF) (Figure 8). Bone morphogenetic protein 6 regulate growth, differentiation, and apoptosis of various cells, including epithelial cells, and play critical roles during embryogenesis and the morphogenesis of various organs. Expression of BMP6 was reduced in eighteen of forty-four breast carcinoma samples compared with tumor-free resection margins lines (Clement et al. 1999). Amisyn (STXBP6) showed reduced expression in the transformed and invasive cells (trMCF and bsMCF); this protein binds components of the SNARE complex that has a central role in vesicle targeting and fusion in eukaryotic cells; it has been shown that epimorphin, one of the SNARE proteins, directs epithelial morphogenesis (Hirai et al. 1998). Chondroitin sulfate synthase 3, or CHSY2 (CSS3), showed increased expression in the transformed cells (trMCF), and it was expressed in neither the invasive nor in the tumorigenic stages (bsMCF and caMCF), suggesting its hypermethylation at these stages (Figure 8); CSS3 is a glycosyltransferase that transfers glucuronic acid (GlcUA) to the nonreducing end of the elongating chondroitin polymer. Some chondroitin sulfate proteoglycans (CSPG) provide high osmotic pressure and water retention, and others modulate not only cell adhesion to extracellular matrix, cell migration, cell proliferation, and morphogenesis but also cytokine signals (Schwartz and Domowicz 2002). SNIP was down-regulated in the invasive and tumor cells (bsMCF and caMCF) (Figure 8). SNIP is a signaling molecule involved in the linkage of actin cytoskeleton to the extracellular matrix during cell migration, invasion, and transformation (Bouton et al. 2001). Sprouty homolog 1 (SPRY1) expression was low in the invasive and tumor cells (bsMCF and caMCF) (Figure 8). Sprouty homolog 1 proteins were found to be endogenous inhibitors of the Ras/mitogen-activated protein kinase pathway that play an important role in the remodeling of branching tissues. It has been shown that SPRY1 and SPRY2 were down regulated consistently in breast cancers (Lo et al. 2004). SPRY1 and SPRY2

TABLE 2.—Hypermethylated and hypomethylated genes in the in vitro–in vivo model of cell transformation induced by 17β-estradiol.

	Chromosome	CpG location	Gene description
Hypermethylated genes			
MAPK11	22q13.33	body	mitogen-activated protein kinase 11
HOXA9	7p15.2	5′	homeo box A9
MAF	16q23.1	5′ + body	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)
KCNC1	11p15.1	5′	potassium voltage-gated channel,
EPB49	8p21.3	Body	erythrocyte membrane protein band 4.9
SYNE1	6q25.2	5′	spectrin repeat containing, nuclear envelope 1
KCNK9	8q24.3	5′	potassium channel, subfamily K, member 9
FOXD1	5q12-q13	5′	forkhead box D1
AB020689	4q31.21	5′	Hypothetical protein KIAA0882
ITGA11	15q23	5′	integrin, alpha 11
GRM6	5q35	5′	glutamate receptor, metabotropic 6
STXBP6	14q11.2	5′	syntaxin binding protein 6 (amisyn)
KCNIP2	10q24.32	5′	Kv channel interacting protein 2
HR	8p21.3	5′	hairless homolog (mouse)
NRG1	8p21.1	5′	neuregulin 1
SULT4A1	22q13.2	5′	sulfotransferase family 4A, member 1
TM4SF9	4q23	5′	transmembrane 4 superfamily, member 9
CSS3	5q23.3	5′	chondroitin sulfate synthase 3
HOXB5	17q21.32	3′	homeo box B5
TLL1	4q32.3	5′	tolloid-like 1
OTP	5q14.1	5′	orthopedia homolog (Drosophila)
PDE6D	2q37.1	5′	phosphodiesterase 6D, cGMP-specific,
FGF14	13q33.1	5′	fibroblast growth factor 14
ASB18	2q37.3	Body	ankyrin repeat and SOCS box-containing 18
TBR1	2q24.2	3′	T-box, brain, 1
ABR	17p13.3	5′	active BCR-related gene
GNAL	18p11.21	5′	guanine nucleotide binding protein,
C9orf58	9q34.13	5′ + body	chromosome 9 open reading frame 58
SNIP	17q21.2	Body	Hypothetical protein KIAA1684
AK096941	Chr5	5′	hypothetical gene supported by AK096941
AB018254	8p23.3	5′	Hypothetical protein KIAA0711
KCNA2	1p13.3	5′	potassium voltage-gated channel,
SGPP2	2q36.1	Body	sphingosine-1-phosphate phosphotase 2
ITPK1	14q32.12	5′ + body	inositol 1,3,4-triphosphate 5/6 kinase
SPRY1	4q28.1	5′	sprouty homolog 1,
AB014526	4q33	5′	Hypothetical protein KIAA0626
LOC375323	3p25.3	5′	lipoma HMGIC fusion partner-like protein 4
BMP6	6p24.3	5′	bone morphogenetic protein 6
Hypomethylated genes			
CAST	5q15-q21	5′	calpastatin
ALX3	1p13.3	5′	aristaless-like homeobox 3
BACH2	6q15	5′	BTB and CNC homology 1
POGZ	1q21.3	5′	pogo transposable element with ZNF domain

TABLE 3.—Expression studies in the in vitro–in vivo model of cell transformation. The expressions of different genes were studied by real-time RT-PCR. The values express fold induction (± *SD*) compared to MCF-10F. The real-time RT-PCR was performed in triplicate for each sample/primer, and the standard deviations are indicated.

	SPRY1	NRG1	STXBP6	BMP6	CSS3	SNIP
MCF-10F	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
trMCF	1.17 ± 0.03	0.37 ± 0.03	0.11 ± 0.02	0.62 ± 0.08	4.79 ± 1.34	1.89 ± 0.14
bsMCF	0.35 ± 0.04	0.004 ± 0.01	0.39 ± 0.07	0.75 ± 0.007	0 ± 0	0.22 ± 0.03
Tumor cell lines:						
caMCF1	0.41 ± 0.06	0 ± 0	4.27 ± 0.15	0.33 ± 0.03	0 ± 0	0.18 ± 0.05
caMCF2	0.40 ± 0.04	0 ± 0	2.96 ± 0.26	0.36 ± 0.02	0 ± 0	0.10 ± 0.01
caMCF3	0.71 ± 0.19	0 ± 0	4.52 ± 0.26	0.20 ± 0.02	0 ± 0	0.19 ± 0.02
caMCF4	0.36 ± 0.18	0 ± 0	6.7 ± 0.07	0.21 ± 0.01	0 ± 0	0.34 ± 0.02

Abbreviation: RT-PCR, reverse transcriptase polymerase chain reaction; SD, standard deviation.

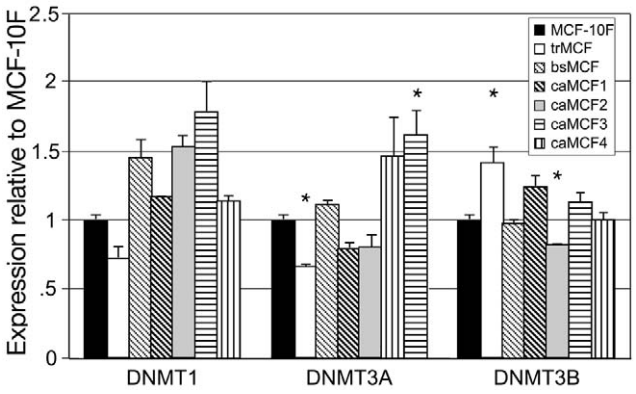

FIGURE 7.—DNMT1, DNMT3A, and DNMT3B expression in the in vitro–in vivo model of cell transformation. The expression of the different DNA methyltransferases was studied by real-time reverse transcriptase polymerase chain reaction (RT-PCR) in trMCF, bsMCF, and caMCF cells and compared to the expression in MCF-10F. The real-time RT-PCR was done in triplicate for each sample/primer/probe. Student *t*-test was used for comparison between treatments and control, and $p \leq .01$ was accepted as statistically significant. The mean ($\pm SD$) and the statistically significant differences from the control MCF-10F (*) are indicated.

were expressed specifically in the luminal epithelial cells of breast ducts, with higher expression during stages of tissue remodeling, when the epithelial ducts are forming and branching (Lo et al. 2004). These findings suggest that SPRYs might be involved as a modeling counterbalance and surveillance against inappropriate epithelial expansion. The Sprouty proteins are increasingly being recognized as deregulated in various types of cancer; therefore, the finding that this gene can be epigenetically regulated in our HBEC model of cell transformation is especially relevant.

Therefore, the genomic and epigenetic changes triggered by estrogen exposure that lead normal cells to tumorigenesis confirm the role of this steroid hormone in cancer initiation (Figure 8).

Relevance of Neoplastic Transformation of Human Breast Epithelial Cells In Vitro by Estrogens and Xenoestrogens

Altogether, our data indicate that BPA as well as E2 are able to induce neoplastic transformation of human breast epithelial cells. Importantly, we have also demonstrated that MCF-10F cells treated with E2 formed tumors when they were injected into SCID mice. The model not only mimics the primary breast tumors, but it also provides a unique system for understanding genomic changes that lead to specific neoplastic phenotypes (Figure 8). More importantly, this model may be used to test the functional role of specific genomic and epigenetic changes that take place during cell transformation, invasion, and tumorigenesis. Our data also support the concept that E2 can act as a carcinogenic agent without ERα, although we cannot rule out the possibility that ERβ, or other receptors not yet identified, could play a role in the early stages of cell transformation, invasion, and tumorigenesis. Although this model is extremely valuable in furthering our understanding of estrogen-induced carcinogenesis in MCF-10F cells, an immortalized cell line, we also presented evidence that primary breast epithelial cells are also transformed by natural estrogen and xenoestrogens as well. More importantly, we present for the first time evidence that epigenetic changes are involved in the early stages of cancer initiation by altering ductulogenesis (Figure 8). Epigenetic changes of different genes arise early at the initiation stage. Data supporting this hypothesis came from the analysis of morphologically normal tissue surrounded by invasive carcinomas where hypermethylation of NRG1 and RAR β was detected (unpublished data). DNA mutations and other epigenetic modifications appear in the transformation stage (carcinoma in situ). We postulate that the initiation and transformation (carcinoma in situ) stages could be reverted to a normal stage by drugs that are capable of modifying the methylation pattern of affected genes. In the invasion and tumor stages (invasive carcinomas), important chromosomal gains and losses appear (Figure 8). Furthermore, we found that BPA was able to induce transformation of human breast MCF-10F epithelial cells. After treatment with BPA, the cells produced fewer tubules in collagen and an increased number of solid masses, and this compound also increased the invasion capacity of the cells. Treatment with BBP did not increase solid mass formation, although it increased the invasion capacity of the cells.

Acknowledgments

Grant R21 ES015894 and U01 ES/CA 12771 from the NIH, USA, supported this work. The authors thank Kara Snider and Patricia A. Russo for the technical work. The RLGS was done in collaboration with Dr. Christopher Plass from Ohio State University, and the analysis of metaphase-CGH was performed by Dr. Binaifer Balsara from the Research Cytogenetic and Genomic Facility of the Fox Chase Cancer Center.

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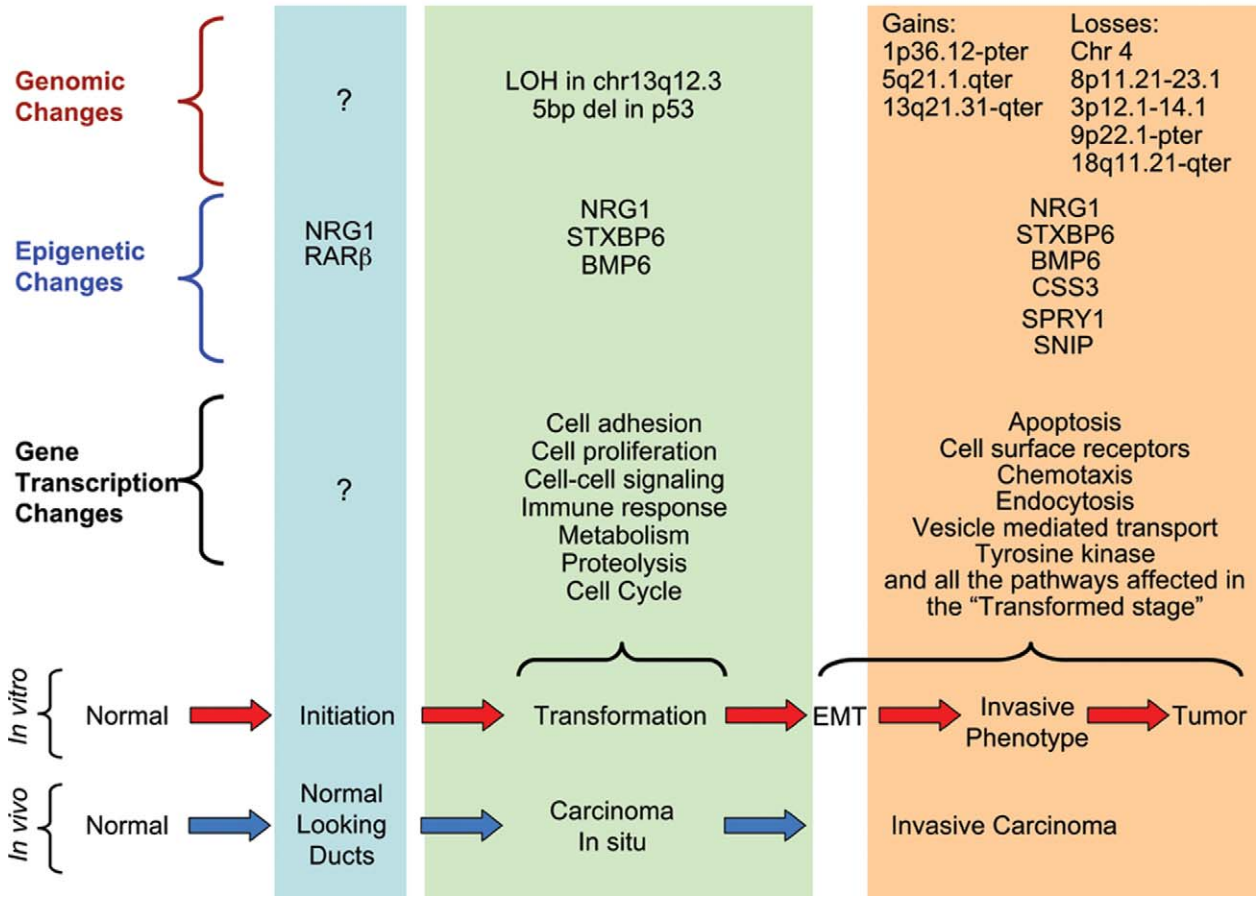

FIGURE 8.—Molecular pathway of neoplastic transformation of breast epithelial cells. In the lower portion of the figure, the phenotypes of breast cancer progression in vivo are compared with those in the in vitro model. The genomic, epigenetic, and gene transcription changes are listed and compared among the different stages of cancer progression. In the epigenetic changes, the hypermethylated genes at the different stages are indicated. In the transformation stage, loss of heterozygosity (LOH) in chromosome 13q12.3 and a five-base-pair deletion in p53 exon 4 were detected. In the invasive and tumor stages, several gains and losses have been described. EMT, epithelial–mesenchymal transition.

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TABLE 4.—NRG1 expression in normal and tumor breast samples. The NRG1 expression was studied using real-time RT-PCR in normal and tumor breast samples. Assays were done in triplicate. The values express NRG1 fold induction (± *SD*) compared to NRG1 expression in sample #1.

Breast tissue identification sample #		Relative NRG1 expression
1	Normal tissue	1 ± 0.13
4	Normal tissue	2.44 ± 0.25
7	Normal tissue	1.53 ± 0.11
4582 T	Invasive carcinoma	0.027 ± 0.023
99-101 T	Invasive carcinoma	0.011 ± 0.00016
99-223 T	Invasive carcinoma	0.0045 ± 0.001471
00- 126 T	Invasive carcinoma	0.01 ± 0.001
99- 190 T	Invasive carcinoma	0.003 ± 0.0009
99-218 T	Invasive carcinoma	0.015 ± 0.0011

Abbreviation: RT-PCR, reverse transcriptase polymerase chain reaction; SD, standard deviation.

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