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Estrogen induced breast cancer is the result in the disruption of the asymmetric cell division of the stem cell

Jose Russo, MD, Kara Snider, BS, Julia S. Pereira, PhD, and Irma H. Russo, MD
Breast Cancer Research laboratory, Fox Chase Cancer Center, Philadelphia, PA, USA

Abstract

There is evidence that in the human breast there is a stem cell population that can give rise to many different cell types and have the unique potential to divide asymmetrically. In this way stem cells maintain the stem cell pool and simultaneously generate committed cells that reconstitute the organ for example for preparing the breast for a new pregnancy after the involution from a previous pregnancy and lactation process. In addition to the *in vivo* models of mammary morphogenesis there are *in vitro* systems that are more amenable to study in critically determined conditions the ductulogenic pattern of growth of the breast epithelia. Primary mammary epithelial cells grown in collagen matrix are able to form tree-like structures resembling *in vivo* ductulogenesis. The human breast epithelial cells MCF-10F formed tubules when grown in type I collagen and we demonstrated that treatment of these cells with 17 β -estradiol (E₂) induces phenotypical changes indicative of neoplastic transformation. The transformation of MCF-10F by E₂ is associated with impaired ductal morphogenesis by altering the stem cells unique potential to divide asymmetrically inducing formation of solid masses mimicking intraductal carcinoma that progress to invasive and tumorigenic phenotype. In the present work we present evidence for the mechanism of cell asymmetry leading to normal ductulogenesis and how the normal stem cell is transformed to cancer stem cell by altering this process. Furthermore, we demonstrate that the carcinogenic agent, in this case E₂, induces a defect in the asymmetric cell division program of the normal mammary stem cell.

Keywords

Asymmetric cell division; Breast cancer; Carcinogenesis; Estrogen; Stem cell

Introduction

One unresolved challenging question in breast cancer is that if the cancer stem cell originates from normal stem/progenitor cells or from mature cells that acquired stem cell phenotype because of a transformation event or subsequent mutations. There is evidence that in the human breast there is a stem cell population that can give rise to many different cell types and are important for breast development, pregnancy induced differentiation, lactation and involution (1). Stem cells have the unique potential to divide asymmetrically to generate daughters of distinct fate, one of which remains a stem cell and the other, which turns into a cell, committed to differentiation. By dividing asymmetrically, stem cells maintain the stem cell pool and simultaneously generate committed cells that reconstitute the organ for example for preparing the breast for a new pregnancy after the involution from a previous pregnancy and lactation process (2,3). However, in this cumulative knowledge we still do

¹ Corresponding author Address: Jose Russo, MD, Professor, Breast Cancer Research Laboratory, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, Phone: 215-728-4782, Fax: 215-728-2180, Jose.Russo@fccc.edu.

not know if the cancer stem cell originates from normal stem/progenitor cells or from mature cells that acquired stem cell phenotype because of a transformation event or subsequent mutations. Our starting hypothesis is that the normal stem cell divides asymmetrically and that the carcinogenic event disrupts this process leading to a neoplastic breast cancer stem cell. Furthermore, the main challenge has been to demonstrate that the asymmetric cell division takes place in the normal process of ductulogenesis and that the carcinogenic agent (17 β -estradiol) modifies this process (4-6). This unique model offers a stem cell with the characteristic of the basal cell type that differentiates normally in a tri-dimensional *in vitro* system forming ductular structures mimicking the lobules type 1 of the human breast (4-6). This in turn offers a structural model to understand how the normal cellular architecture is modified during the asymmetric cell division as the main driver of this normal morphogenetic process. In addition the basal stem cell when challenged by a carcinogenic agent loss the ability to form ductal structures and became neoplastic mimicking the histological appearance of the early neoplastic process like carcinoma *in situ* forming an epithelial multilayer with loss of polarity and increase cell proliferation [5,6]. Therefore, in this unique system we have been able to demonstrate that the normal stem cell is the one that under a carcinogenic insult switch the process of asymmetric cell division. Altogether, this model is offering a way to understand the mechanism of asymmetric cell division that is still elusive in the breast cells and to demonstrate how the disruption of this phenomenon by estrogen can trigger the initial step of cell transformation.

There are four main supporting evidence for this hypothesis: i, *The model of human breast epithelial cell transformation in vitro in the context of breast cancer.* ii, *The basal cell is the normal stem cell that generate the ductal structures.* iii, *Demonstration that the basal cell type is a Stem cell of breast cancer.* iv, *Genomic alterations in the transformed cells (trMCF) indicating that the asymmetric cell division is the target mechanism of neoplastic transformation.*

i-The model of human breast epithelial cell transformation in vitro in the context of breast cancer

Invasive breast cancer that leads to metastasis is a heterogeneous disease that encompasses a variety of pathological features that are associated with specific clinical behavior (7). The discovery that the morphological heterogeneity of breast cancer is reflected at the transcriptome level has allowed the classification of breast cancer into five main groups: luminal A and B, normal breast-like, ERBB2 (HER2) and basal-like breast carcinomas (8,9). The luminal-like subtypes display moderate to high expression of ER α and luminal cytokeratins. The basal-like carcinomas, which have been reported to have a more aggressive clinical behavior, is the one that we will concentrate our attention as the basis for demonstrating our hypothesis that the normal stem cell divides asymmetrically and that the carcinogenic event disrupts this process leading to a neoplastic cancer stem cell. The basal-like carcinomas are composed of cells that consistently express genes usually found in normal basal/myoepithelial cells of the breast, including basal cytokeratins such as 5/6, 14 and 17, p-cadherin, and caveolin 1 and p53 (10,11). Molecular analyses of basal-like carcinomas have confirmed the frequent lack of expression of estrogen (ER) (12,13) and progesterone (PR) receptors and HER2 (14,15), high levels of expression of proliferation-related genes, and frequent mutation of the TP53 gene. Morphologically, basal-like breast carcinomas present with high histological grade, high mitotic indices, central necrotic zones, pushing borders and a conspicuous lymphocytic infiltrate. In addition, metaplastic elements and medullary/atypical medullary features have been reported. Similarities have been found between basal-like tumors and breast carcinomas occurring in BRCA1 mutation carriers in a premenopausal population of African American (AA) women, and in general, in the younger breast cancer patient population (16,17).

Our observations that ductal carcinomas originate in lobules type 1 (Lob.1) of the immature breast (Figure 1) (18), which are the structures with the highest proliferative activity provides a mechanistic explanation for the higher susceptibility of these structures to undergo neoplastic transformation when exposed to chemical carcinogens, as demonstrated in *in vitro* experiments (19). However, the role of ER α positive and ER α negative cells in the initiation of breast cancer is not clear. The fact that the cells that do proliferate in culture are ER α negative suggests that the stem cells that originate normal ductal structures and cancer are the ER α negative proliferating cells. This idea is supported by our observations that MCF-10F, a spontaneously immortalized ER α negative human breast epithelial cell line derived from breast tissues containing Lob.1 and Lob.2 (20), is able to form normal ductal structures in a tridimensional collagen matrix system (Figure 2a and 3). The ductal structures are lined by a monolayer of well polarized epithelial cells that becomes malignant after exposure to the chemical carcinogens benz(a)pyrene (19) or 17 β -estradiol (E₂) (4,21-23). In this model (Figure 4) (4), MCF-10F cells that are E₂-transformed (trMCF cells) progressively express phenotypes of *in vitro* cell transformation, including colony formation in agar methocel, and loss of the ductulogenic capacity (Figures 2b, 2c and 3). The cell lines derived from those tumors (caMCF) were poorly differentiated ER α , PR and ERBB2 negative adenocarcinomas (5,6). These characteristics are similar to the human basal cell-like carcinomas previously described (9). To better understand the molecular events associated with the progressive phenotypic changes that were observed during estrogen-mediated malignant cell transformation, we analyzed chromosomal copy number (CN), loss of heterozygosity (LOH), and gene expression changes that occurred at different stages of cell transformation. By integrating these data we were able to identify associations between CN changes, LOH, transcript expression and phenotypes of invasion and tumorigenicity, including a strong gene signature of epithelial to mesenchymal transition (EMT) that was confirmed by immunohistochemistry (6). The bcMCF (invasive) and caMCF (tumor-derived) cells showed dramatic changes in morphology, losing epithelial characteristics of polarity and acquiring mesenchymal characteristics of a fibroblast-like spindle shape and increased migratory behavior and invasiveness (Figure 3). Changes in gene and protein expression were characteristic of epithelial-mesenchymal transition (EMT), namely loss of intercellular adhesion (E-cadherin and occludins), down-regulation of epithelial makers (cytokeratins), and up-regulation of mesenchymal markers (vimentin and smooth muscle actin (5,6) (Figure 5).

ii-The basal cell as the normal stem cell that generates the ductal structures

MCF-10F cells grown in collagen matrix (3D-cultures) (Figures 2a and 3). One of the advantages of using type I collagen matrix is that the human breast epithelial cells like MCF-10F forms tubules mimicking the normal ductules of the human breast. After the cells are treated with E₂ the ductulogenesis is quantitatively evaluated by estimating the ability of the cell to form tubules. When MCF-10F cells grow in regular media or in media with DMSO and plated in collagen, the cells formed tubules (Figure 3). When the cells are treated with E₂ alone and plated in collagen, cells form semi solid masses of $178 \pm 33.16 \mu\text{m}$ in diameter (Figure 3). The treatment with E₂ increased cell proliferation evaluated using an antibody against Ki-67 antigen. The Ki67 antigen is a cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase) and it is absent in resting (G0) cells. The cross sections of the tubular structures showed a monolayer of epithelial cells around the lumen (Figure 3) whereas in the E₂ treated cells, the cross section of the tubules showed an increase of both number of epithelial layers (Figure 3) and Ki-67 positive cells (Proliferative index= 91.16). The epithelial cells layering the ducts showed to be estrogen receptor alpha (ER α) negative and they have a significantly low expression of cadherins, laminin, and fibronectin. The filling of the lumen is the result of decreased in central apoptosis, enhanced cellular proliferation or their combination (24).

Luminal filling is the earliest morphologic alteration and is commonly reported in neoplastic processes (24). Luminal filling is commonly seen in atypical ductal hyperplasia and ductal carcinoma in situ (DCIS). In summary this phenotypical changes are representative of the phenomena observed in the breast providing an adequate model for addressing the double question of the role of asymmetry in stem cell division and the role of a carcinogenic insult on the stem cell.

iii-Demonstration that the basal cell type is a Stem Cell of breast cancer

The MCF-10F cells are non-transformed, non-tumorigenic and ER negative. Because malignant cell transformation of these cells (4,5,21-23) produced poorly differentiated tumors characteristic of basal-like carcinomas, we chose to classify these cells relative to the breast cell lines described by Charafe-Jauffret et al. (25). MCF-10F and derived cell lines clustered in the branch containing the basal breast cell lines. In our molecular characterization of malignant cell transformation (6) we identified the “intermediate filament” component enriched in Gene Ontology (GO) analysis, separating the non-tumorigenic MCF-10F and trMCF cells from the tumorigenic bcMCF and caMCF cells. Numerous cytokeratins were suppressed or absent, whereas vimentin was strongly induced in bcMCF (7.0-fold) and caMCF (8.1-fold). Because of these findings, we generated a gene list from published literature for EMT markers and their regulators (6). The 52 genes in this list were filtered by low stringency criteria of combined coefficient of variation > 0.3 and ‘Present calls’ in more than 30% of the samples. The 27 genes passing these criteria were used for sample and gene clustering (Figure 5A). Two sample groups and two gene groups were identified. The nontumorigenic MCF-10F and trMCF cells were grouped into sample cluster κ , while the tumorigenic bcMCF and caMCF cells were grouped into cluster λ . On the other side, the genes were grouped into cluster α and β based on their expression pattern. The epithelial markers *E-cadherin*, *occludin*, *desmoplakin* and *cytokeratins* were decreased, while the mesenchymal markers *fibronectin*, *vimentin* and *N-cadherin* were increased in bcMCF and caMCF cells (Figure 5A). By real time RT-PCR, it was confirmed that the expression of FN1, S100A4, SNAIL2, HRAS and TGF β 1 was increased, while CDH1 (*E-cadherin*) was decreased in bcMCF and caMCF cells (6). Immunocytochemical analysis using antibodies against epithelial membrane antigen EMA (also called MUC1) and *E-cadherin* displayed significant loss of these epithelial markers and increased expression of the mesenchymal marker vimentin in tumorigenic cells (Figure 5B). These findings confirmed the EMT phenotype revealed by gene expression profile in Figure 5A.

iv-Genomic alterations in the trMCF cells indicating that the asymmetric cell division is the target mechanism of neoplastic transformation

Loss of the ductulogenic capacity is the earliest phenotype observed during the neoplastic transformation of MCF-10F by 17 β -estradiol. Asymmetric cell division takes place in the normal process of ductulogenesis and this process is modified by the carcinogenic agent. Specifically we have demonstrated that 17 β -estradiol acting in the basal cell type induce architectural changes in the stemness, the cell polarity, the partitioning of the cell component and the mitotic apparatus and the interaction with the niche or extracellular matrix.

iv-a-Stemness in asymmetric cell division—*CD44⁺/CD24^{-/low}* is the cell surface marker of tumorigenic breast cancer cells in which the tumorigenic capacity is further increased by additional expression of *ESA*. These cells are characterized by a 186-gene “invasiveness” gene signature that is associated with risk of death and metastasis in breast cancer (26). *CD24* encodes a small, heavily glycosylated cell-surface adhesion protein. *CD44* undergoes extensive alternative splicing within its central region spanning exon 6a to 14, also termed as variable exon v1 to v10. The two variants expressed in our model are mRNA precursor variant 3 with exon 6a to 11 spliced out and variant 4 with exon 6a to 14

spliced out, corresponding to *CD44E* and *CD44H*, respectively (27).. The *CD44H* is mainly expressed on cells of lymphohematopoietic origin; it plays an important role in cell adhesion and its expression promotes tumor cell migration (28).. *CD44E* is preferentially expressed on epithelial cells and it is involved in the recognition of a common determinant in *CD44H* and *CD44E* promoting homotypic cellular aggregation (29). Microarray and RT-PCR analysis of *CD44* and *CD24* revealed an expression pattern of *CD44H^{high}/CD44E⁻/CD24⁻* in bcMCF and caMCF. The increased ratio of *CD44H/CD44E* in trMCF cells might represent an early marker for E₂-transformed HBECs (Figures 6A, B, C and D). The significant increase of *CD44H* and complete loss of *CD44E* might be a novel phenotype associated with the tumorigenic capacity. In addition, the loss of *ESA* in bcMCF and caMCF indicated that *ESA* expression is not required for the tumorigenic capacity in our model.

iv-b-Cell partitioning in asymmetric cell division—In table 1 and Figures 7 and 8 are listed some of the genes that may contribute in altering the cell partitioning during the asymmetric cell division. Among them is *NOTCH3*, which is significantly downregulated in the estrogen-transformed cells that have lost the ability to form ductal components in the collagen matrix. As it is depicted in Figure 9, *NOTCH3* is polarized in their localization in mCF10F cells and when the cell divides one of the daughter cells carry the expression of *NOTCH3* and the other one no. Instead in the E₂ treated cells or trMCF most of the cells have very low or no expression of *NOTCH3*. Therefore we postulate that *NOTCH3* could be asymmetrically partitioned in the breast epithelial cells and that daughter cells that are transformed by estradiol will have a loss of the expression of this gene. There are supporting evidence in the literature indicating that Notch-1 and Notch-3 have been shown to affect stem cell fates, suggesting that Notch function in specifying cell fate might also be evolutionarily conserved (30-33). Interestingly, the Notch pathway plays multiple roles in hematopoiesis, including differentiation, proliferation and survival, and may be critical for HSC activity (34,35). Notch1 was found in the CD34⁺CD38⁻Lin⁻ subpopulation of human bone marrow cells (SAGE) and in two enriched stem cell populations derived from the fetal liver (Sca-1⁺AA4.1⁺Lin^{lo/-}cKit⁺ cells) (SCDb) (36,37). In the *Drosophila* suggests that asymmetrical localization/segregation of Numb in neural precursors enables sibling cells to respond differently to extrinsic Notch signaling and, thus, to adopt distinct fates (A or B). During the asymmetric precursor division, Numb segregates exclusively into one daughter cell, the future B cell. The Notch ligand Delta signals both progenies to adopt the A-cell fate. In the A cell, Delta activation of Notch induces the cleavage of the Notch receptor and the subsequent translocation of the Notch intracellular domain to the nucleus, where it regulates transcription of specific target genes and allows the cell to adopt the A fate. In the B cell, Numb blocks reception and/or transduction of the Notch signal, possibly through its endocytosis. The absence of active Notch signaling in these cells is deterministic for B-cell fate (38). The silencing of *NOTCH 3* in the trMCF or the daughter cell generated in the transformation of MCF could be due to degradation. Protein ubiquitination and deubiquitination are dynamic processes implicated in the regulation of numerous cellular pathways. In table 1 is listed genes that are modified during the transformation induced by 17β-estradiol. Among them are; *TRIM22* and *TRIM5* (39-44), *SLPI* (45-47), *USP1* (48), *UBE2Q2* (49,50), and *UBE2S* components of the ubiquitin pathway leading to proteasome degradation that are significantly dysregulated in the trMCF cells supporting the interpretation that the localization of this complex could be asymmetrically located in the trMCF cells under the treatment with estradiol disrupting the asymmetrical process.

Of great interest is *TRIM22*, a functional E3 ubiquitin ligase, that is also ubiquitinated itself and is downregulated in the trMCF cells indicating a possible role in the partitioned position in the stem cells MCF (Table 1). There is 41 fold down regulation in trMCF when compared to the stem cell MCF. Its potential role in the cell asymmetry is suggested to us by the work of Herr et al (40), showing that human and rhesus *TRIM22* localize to different sub cellular

compartments and that this difference can be assigned to the positively selected B30.2 domain. It is possible that TRIM22 is asymmetrically located in the MCF cells and that during transformation the daughter cells trMCF lose or alternatively silence the expression of this gene. Further support is the fact that TRIM22 underwent self-ubiquitylation in vitro in combination with the E2 enzyme UbcH5B and the ubiquitylation was dependent on its RING finger domain. Further evidences showed that TRIM22 could also be self-ubiquitylated in vivo. Importantly, TRIM22 was conjugated with poly-ubiquitin chains and stabilized by the proteasome inhibitor in 293T cells, suggesting that TRIM22 targeted itself for proteasomal degradation through the poly-ubiquitylation (41). Another evidence that it could be involved in the asymmetry of cell division is the finding that interferon induces TRIM22 as a p53 target gene, with possible involvement in proliferation and differentiation of leukemia cells. Expression of TRIM22 correlates inversely to differentiation, as TRIM22 is highly expressed in CD34(+) human bone marrow progenitor cells, but declines in mature populations. The erythroid lineage appears as a special case, as TRIM22 expression shows an extreme decrease during late erythroid maturation and is completely undetectable in nucleated erythroid populations in contrast to other lineages (42,43). A similar phenomenon has been reported in T cells (44). Therefore it is possible that TRIM22 is compartmentalized in one of the poles of the stem cell MCF and that during the estrogen induced transformation process the daughter or trMCF cells lost it completely.

Besides NOTCH3 there are other candidate proteins coded by the genes MLF1 (51-54), NEBL (55-58), PACS1 (59-61), SEC14L2 (62), TRIOBP (63-65), TSPAN1 and PSIP1 (Table 1) that may have a regulatory effect on the partition distribution of proteins and organelles affecting the asymmetric cell division. For example the Myeloid leukemia factor 1 (MLF1) expresses normally in a variety of tissues including hematopoietic stem cells and also in the MCF cells but it is unregulated several folds in the trMCF cells (Table 1). MFL1 is a negative regulator of cell cycle progression functioning upstream of the tumor suppressor p53 (51,53,54) stabilizing the activity of the tumor suppressor p53 by suppressing E3 ubiquitin ligase and the shuttling of MFL1 is critical for the regulation of cell proliferation and a disturbance in the shuttling balance increases the cell's susceptibility to oncogenic transformation (52). Therefore this gene product is a target for evaluation in the loss of asymmetric division induced by E2 in the MCF cells. There are also three genes PACS1 (59-61), SEC14L2 (62) and TRIOBP (63-65) that are significantly down regulated in the trMCF cells and because their role in cell sorting and interaction with actin filaments may play an unsuspected role in asymmetric cell division.

iv-c-Mitotic apparatus—A significant amplification in the expression of Aurora Kinase B (AURKB), polo-like kinase 1 (PLK1), centromere protein A (CENPA), and kinesin family member C (KIF2C) are observed in the E2 cells with respect to the MCF10 cells (Table 1 and Figure 8). This could indicate that the dysregulation of these genes by the carcinogenic agent 17 β -estradiol are contributing in altering the asymmetric pattern of cell division of the stem cell MCF. Supporting evidence for this is the knowledge that the binding of kinetochores to spindle microtubules during mitosis regulates the trafficking of macropinosomes to the cytoskeleton during antigen presentation. The centromere protein A or CENPA, is a histone H3-like protein that is thought to be involved in the nucleosomal packaging of centromeric DNA. It is a marker of embryonic stem cells (66,67) and highly expressed in breast cancer cells. CENPA interacts with poly(ADP-ribose) polymerase 2 (PARP-2) in a cell-cycle-dependent manner, accumulating at centromeres during prometaphase and metaphase, disassociating during anaphase, and disappearing from the centromeres by telophase (68,69). The KIF2C /mitotic centromere-associated kinesin (MCAK) is associated to the growth of breast cancer cells. It is expressed at undetectable levels in normal human tissues and upregulated in the trMC cells as well as in breast cancer tissue. Treatment of breast cancer cells with small interfering RNA against KIF2C/MCAK

effectively suppressed KIF2C/MCAK expression and inhibited the growth of the breast cancer cell lines T47D and HBC5 (70). The importance of these kinetochore related proteins is that few-fold reductions in kinetochore-microtubule turnover, particularly in early mitosis, induce severe chromosome segregation defects. And interestingly enough stimulation of microtubule dynamics at kinetochores restores stability to chromosomally unstable tumor cell lines, establishing a causal relationship between deregulation of kinetochore-microtubule dynamics and chromosomal instability. Thus, temporal control of microtubule attachment to chromosomes during mitosis is central to genome stability in human cells (71). In the *Drosophila* sensory organ precursor cells, Aurora A regulates the asymmetrical localization of Numb (72). Mammalian Auroras (member of the serine/threonine kinase family) regulate centrosome separation, chromosome segregation and cytokinesis. Auroras are overexpressed in many cancers and high protein levels correlated with chromosomal instability (73-75). A key regulator in the process of cell division is the polo-like kinase 1 (PLK1). It controls cytokinesis, the final stage of cell division and is a novel player in maintaining genomic stability during DNA replication and is an important modulator of the DNA damage checkpoint. Importantly it has been shown to be a link between developmental processes and the cell cycle machinery during asymmetric cell division in flies and worms (76,77). Therefore, the role of CENPA, KIF2A, KIF2B, and MCAK (KIF2C) in the asymmetric cell division of breast epithelial cells are a focus of our research in specific aims 1 and 2.

Two genes YPEL5 and EPLIN identified by us, may have an unsuspected role in the control of asymmetric cell division in the human breast epithelial cells. YPEL5 gene is down regulated in the 17 β -estradiol transformed cells and the coded protein is localized to the centrosome and nucleus during interphase and at the mitotic spindle during mitosis. This sub cellular localization in association with centrosome or mitotic spindle suggests a novel function involved in the cell division (78). The second gene EPLIN (epithelial protein lost in neoplasm; also known as Lima-1) (Table 1) (79) is also significantly down regulated in the trMCF cells and play an important role in the first phase of the cytokinesis that is the membrane ingression. The ingression phase generates a cleavage furrow and this requires co-operative function of the actin-myosin II contractile ring and septin filaments (80). Therefore, it is possible to postulate that EPLIN play an important role in the asymmetric cell division of the stem cell MCF and that is silenced in the trMCF cells. This concept is supported by data showing low level of EPLIN in tumor tissues (81). Of interest is that EPLIN couples with alpha-catenin and, in turn, links the cadherin-catenin complex to F-actin (82). EPLIN is a substrate for ERK. ERK phosphorylates Ser360, Ser602, and Ser692 on EPLIN in vitro and in intact cells. Phosphorylation of the C-terminal region of EPLIN reduces its affinity for actin filaments (83). EPLIN increases the number and size of actin stress fibers and inhibits membrane ruffling induced by Rac and the reduced expression may contribute to the motility of invasive tumor cells (84,85). All of these data points toward a need of a detail analysis in the architectural localization of these gene products in the MCF cells during the normal process of ductulogenesis and during the loss of the asymmetric properties of these cells induced by 17- β -estradiol.

IV-e-Cell polarity and asymmetric cell division—As it is depicted in Table 1 there are several genes that are significantly dysregulated in the trMCF such as CDC42, EPSL8L2, FLNB, FXYD3, and NRP2. Whereas the role of EPSL8L2, FLNB, FXYD3, and NRP2 is less clear, the CDC42 is known to regulates the generation of cell polarity from yeast to man and in a wide range of biological contexts and the downstream target Par6 (86,87). Epithelial morphogenesis involves the establishment of an apical surface in an individual cell and the formation of cadherin-based adherents junctions and claudin-based tight junctions between adjacent cells. Accompanying reorganization of the actin and microtubule cytoskeletons and polarized vesicle trafficking reinforces these interactions,

leading to a stable tissue of polarized cells. Expression of dominant-negative or constitutively active versions of Cdc42 in the dog kidney epithelial cell line MDCK grown on a two-dimensional surface leads to defective tight junction formation (typically a delay) as well as mislocalized delivery of basolateral proteins (87-90). Similar experiments performed with MDCK grown in three dimensions to provide a more physiological growth context have concluded that Cdc42 is required to form an apical surface through regulated trafficking of a vacuolar apical compartment (91,92). In Caco-2, a human intestinal epithelial cell line, which, when grown in a three-dimensional matrix, generates polarized cysts with a single central lumen. Cdc42 is not required for the formation of an apical surface, but instead is required to position the apical surface with respect to the growing three-dimensional structure. Cdc42 regulates apical surface positioning by controlling spindle orientation during cell division.

IV-f-Cell positioning an asymmetric cell division—The interrelation between extrinsic determinants (niche), stem cells and the regulation of cell fate is better characterized in germline cells of lower organisms (93,94). In the mammalian cells like the breast epithelia could be determined by the extracellular matrix. Among the proteins that in the breast epithelial cells that are involved are the E cadherin and the bone morphogenetic protein. Both of them are expressed in the normal cells and lost in the trMCF cells. In the *Drosophila* germinal stem cell of the female gonads, DE-cadherin-mediated cell adhesion was shown to be essential for anchoring ovarian gonadal stem cells to their niches and stimulating their proliferation (95). Decapentaplegic (Dpp) (the *Drosophila* homolog of human bone morphogenetic protein 2/4) is expressed in anterior somatic cells of the gonad and is essential for PGC proliferation. PGC mutants for *thick veins*, an essential Dpp receptor, are impaired in their ability to clonally populate a niche, further suggesting that Dpp is one of the extrinsic mitotic signals that promote the clonal expansion of gonadal stem cells in the niche (96). Recent studies identify the osteoblasts and the BMP signaling pathway as potential *in vivo* regulators of the HSC niches. In mice BMP signaling promotes the generation of astrocytes and mature, myelinating oligodendrocytes *in vivo* but does not affect oligodendrocyte precursor development, thus suggesting tight regulation of BMP signaling to ensure proper gliogenesis.

The concept of asymmetric cell division also needs to be studied in the context of the activation of cell proliferation, apoptosis and cell junction gene products that are significantly modified during the transformation of MCF cells. Some of these genes are listed in Table 1 and among them are TOP2A (97,98), MK62, RFC3, BIRC5 (99-101) and GSN. Of interest is the up regulation of BIRC5 or Survivin (Table 1 and Figure 10), a member of inhibitor of apoptosis family protein. A vector-based small interfering RNAs (siRNAs) silenced Survivin expression in prostate cancer cells, resulting in significantly reduced cell proliferation and enhanced apoptosis, and increased the sensitivity of prostate cancer cells to the apoptosis-inducing agent, platinol (102). Signal transducer and activator of transcription 3 (Stat3) and survivin have been shown to exert oncogenic effects in various human neoplasms. Global gene expression analysis shows that BIRC5 or Survivin, an anti-apoptotic oncofetal gene, is highly expressed in human embryonic stem (hES) cells and teratomas but not in embryoid bodies. Genetic and pharmacological ablation of Survivin induces apoptosis in hES cells and in teratomas both *in vitro* and *in vivo*.

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Figure 1.

Lobule type 1 or the terminal ductal lobular unit is the nsite of origin of ductal carcinoma in situ

Figure 2.

Loss of branching by treatment of MCF-10F cells with 17β -estradiol (E_2)-(A,B and C, f) Cells in collagen matrix. Phase contrast (10 $\times$).A) MCF-10F cells treated with DMSO (control) forming ductules lined by a double layer of low cuboidal epithelial cells; B) MCF-10F cells treated with 0.007nM estradiol tend to form spheres lined by a multilayered epithelium with secondary lumina; C) MCF-10F cells treated with 70nM estradiol form large spherical masses lined by a hyperplastic epithelium.

Figure 3.
The MCF basal cell type from normal ductal structures that loss the ductulogenic capacity forming the trMCF cells that evolves to bcMCF and caMCF cells.

Figure 4.

In vitro-in vivo model of cell transformation of Human breast epithelial cells with 17 b estradiol (Modified from: Huang, Y., Fernandez, S., Goodwin, S., Russo, P.A., Russo, I. H., Sutter, T., and Russo, J. Epithelial to Mesenchymal Transition in Human Breast Epithelial Cells Transformed by 17- beta- Estradiol. *Cancer Res* 67 11147-11157, 2007)

Figure 5.

Expression profile of EMT markers and their regulators during malignant cell transformation. A. Hierarchical clustering of cell lines and genes was performed using dChip software. Two sample clusters (κ and λ) and two gene clusters (α and β) were identified. The red, white, and blue colors represent level above, at, and below mean expression, respectively. B. Detection of epithelial and mesenchymal markers by immunocytochemistry (100 $\times$). a: Histological sections of MCF-10F cells, reacted with pre-immune mouse serum, were used as the negative control; b, c, d: MCF-10F reacted for EMA, E-Cadherin, vimentin, respectively; e: trMCF cells reacted with pre-immune mouse serum used as negative control; f, g, h: trMCF cells reacted for EMA, E-cadherin and vimentin, respectively; i: bsMCF cells reacted with pre-immune mouse serum as a negative control; j, k, l: bsMCF cells reacted for EMA, E-cadherin and vimentin, respectively; m: caMCF tumor cell line cells reacted with pre-immune mouse serum used as negative control; n, o, p: caMCF tumor cell lines reacted for EMA, E-cadherin and vimentin, respectively; q and r: invasive ductal carcinoma of the breast as positive control and immunoreacted for EMA and E-cadherin, respectively; s: histological section of an invasive adenocarcinoma immunoreacted for vimentin. (Reprinted with permission from: Huang, Y., Fernandez, S., Goodwin, S., Russo, P.A., Russo, I. H., Sutter, T., and Russo, J. Epithelial to Mesenchymal Transition in Human Breast Epithelial Cells Transformed by 17- beta- Estradiol. *Cancer Res* 67 11147-11157, 2007).

Figure 6.

Characterization of the expression of CD44 and CD24. A and B, microarray expression values of CD44 and CD24, respectively. Significance: $P < 0.05$ in unpaired t test compared with MCF-10F. C, RT-PCR validation for CD44 (top) and CD24 (middle) expression levels. D, densitometry ratio of CD44H to CD44E detected in RTPCR. (Reprinted with permission from: Huang, Y., Fernandez, S., Goodwin, S., Russo, P.A., Russo, I. H., Sutter, T., and Russo, J. Epithelial to Mesenchymal Transition in Human Breast Epithelial Cells Transformed by 17- beta- Estradiol. *Cancer Res* 67 11147-11157, 2007)

Figure 7.

Heat maps showing clusters of genes up regulated (on the left) or down regulated (on the right) in the trMCF cells. Arrows indicate some of the genes that were validated through real time RT-PCR, red represent higher values of expression and green lower expression.

Figure 8.

Graphs showing the results from the real time RT-PCR of some of the listed in table 1. Gene expressions are relative to the MCF10F cells. Graph on the top shows the down regulated genes and the graph below shows the up regulated genes; “*” represents the genes that had p<0.001 compared to control and “+” p<0.03.

Figure 9.

Immunofluorescence studies of NOTCH 3. MCF10F and E2 cells were plated onto round cover slips and grown overnight at 37 C. Cells were fixed using 4% Formaldehyde and incubated with the primary antibody (Anti-NOTCH3, Abcam 2ug/ml) for 50 minutes at room temperature. Slides were viewed and images recorded using the Nikon Eclipse 2000-U C1 LSCM and a 40× - NA 1.3 oil objective lens. Nikon EZ-C1 software was used to view images.

Figure 10. Schematic representation showing the role of asymmetric cell division in ductulogenesis and the effect of estradiol in the loss of this property.

Dysregulated genes in the trMCF cells

List of genes showed to be dysregulated in the trMCF cells in the microarrays. Fold change is related to the MCF10F cells.

Table 1

Down regulated genes						
Symbol	Fold change	q-value	Symbol	Fold change	q-value	Symbol
BMP1	2.14	0.042	FXYD3	2.25	0.025	NTN4
BMPR2	2.01	0.019	GSN	3.77	0.014	PACS1
CDC42	1.74	0.047	ITGAV	1.81	0.034	SEC14L2
COL17A1	3.69	0.009	ITGB4	1.93	0.010	SLITRK6
COL1A1	6.12	0.030	ITGB6	2.64	0.010	SLPI
COL4A5	1.81	0.015	KIF13B	2.24	0.038	TNC
DSC2	1.85	0.014	LAMA3	3.48	0.021	TRIM22
EPLIN	1.71	0.029	LAMB3	2.70	0.048	TRIM5
EPS8L2	2.04	0.006	LAMC2	3.61	0.013	TRIOBP
EVA1	1.79	0.041	NEBL	2.25	0.032	TSPAN1
FLNB	2.12	0.036	NOTCH3	3.81	0.012	UBE2Q2
FN1	18.88	0.011	NRP2	2.40	0.034	YPEL5

Up regulated genes						
Symbol	Fold change	q-value	Symbol	Fold change	q-value	Symbol
AURKB	1.84	0.013	MKI67	1.78	0.045	RFC3
BIRC5	1.71	0.048	MLF1	3.16	0.009	TOP2A
CENPA	1.91	0.044	PLK1	1.70	0.056	UBE2S
COL12A1	1.99	0.073	POLE2	2.68	0.012	USP1
KIF2C	2.03	0.042	PSIP1	1.82	0.030	