[Frontiers in Bioscience 11, 151-172, January 1, 2006]

The concept of stem cell in the mammary gland and its implication in morphogenesis, cancer and prevention

Jose Russo, Gabriela A. Balogh, Jinqiang Chen, Sandra V. Fernandez, Rachael Fernbaugh, Rebecca Heulings, Daniel A. Mailo, Raquel Moral, Patricia A. Russo, Fathima Sheriff, Johana E. Vanegas, Richard Wang and Irma H. Russo

Breast Cancer Research Laboratory, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111

TABLE OF CONTENTS

1. ABSTRACT

The breast attains its maximum development during pregnancy and lactation. After menopause the breast regresses in both nulliparous and parous women containing lobular structures that have been designated lobules type 1. Despite the similarity in the lobular composition of the breast at menopause, the fact that nulliparous women are at higher risk of developing breast cancer than parous women, indicates that lobules type 1 in these two groups of women might be biologically different, or exhibit different susceptibility to carcinogenesis. Based on these observations it was postulated that the lobule type 1 found in the breast of nulliparous women and of parous women with breast cancer never went through the process of differentiation, retaining a high concentration of epithelial cells that are targets for carcinogens and therefore susceptible to undergo neoplastic transformation, these cell are called Stem cells 1, whereas lobules type 1 structures found in the breast of early parous postmenopausal women free of mammary pathology, on the other hand, are composed of an epithelial cell population that is refractory to transformation called Stem cells 2. It was further postulated that the degree of differentiation acquired through early pregnancy has changed the "*genomic signature*" that differentiates the lobule type 1 from the early parous women from that of the nulliparous women by shifting the Stem cell 1 to a Stem cell 2 that is refractory to carcinogenesis, making this the postulated mechanism of protection conferred by early full term pregnancy. The identification of a putative breast stem cell (Stem cell 1) has reached in the last decade a significant impulse and several markers also reported for other tissues have been found in the mammary epithelial cells of both rodents and humans. Although still more work needs to be done in order to better understand the role of the Stem cell 2 and its interaction with the genes that confer it a specific signature, collectively, the data presently available provides evidence that pregnancy, through the process of cell differentiation, shifts the Stem cell 1 to Stem cell 2, cells that exhibit a specific genomic signature that could be responsible for the refractoriness of the mammary gland to carcinogenesis.

2. BREAST DEVELOPMENT AND DIFFERENTIATION

The breast tissue of normally cycling women contains three identifiable types of lobules, the previously described lobules type 1 (Lob 1) and the more developed lobules type 2 (Lob 2) and type 3 (Lob 3) (1-3). The breast attains its maximum development during pregnancy. The rapid increase in number of newly formed ductules during pregnancy results in the progression of Lob 2 to Lob 3. This growth phase is followed by the functional or secretory phase, which is indicative of the progression from ductules to secretory acini that characterize the fully differentiated Lob 4. After post-lactational involution, Lob 4 regress to Lob 3, which remain present as the predominant structure in the breast until a woman reaches the fourth decade of life, decreasing thereafter due to their involution to Lob 2 and Lob 1 (1, 2). At difference of the breast of parous women, the nulliparous breast contains a great number of Lob 1, whose percentage remains almost constant throughout their lifespan. Lob 2 are present in moderate numbers and Lob 3 are almost totally absent during the early reproductive years, whereas the number of Lob 1 remains significantly higher, suggesting that a certain percentage of Lob 1 might have progressed to Lob 2, but very few of them had progressed to Lob 3 (2). After menopause the breast regresses in both nulliparous and parous women. This phenomenon is manifested as an increase in the number of Lob 1, and a concomitant decline in the number of Lob 2 and Lob 3. At the end of the fifth decade of life, the breast of both nulliparous and parous women contains predominantly Lob 1 (2). Despite the similarity in the lobular composition of the breast, the fact that nulliparous women are at higher risk of developing breast cancer than parous women, indicates that Lob 1 in these two groups of women might be biologically different, or exhibit different susceptibility to carcinogenesis (2, 3).

3. PATHOGENESIS OF BREAST CANCER

The Lob 1 has been identified as the site of origin of the most common breast malignancy, the ductal carcinoma, as demonstrated in comparative studies of normal and cancer-bearing breasts obtained at autopsy and from cancer-bearing lumpectomy or mastectomy specimens (3-5). The findings that the non tumoral parenchyma in cancer associated breasts contained a significantly higher number of hyperplastic terminal ducts, atypical Lob 1 and ductal carcinomas *in situ* originated in Lob 1 than those breasts of women free of breast cancer indicated that the Lob 1 develop preneoplastic as well as neoplastic lesions (4). More differentiated lobular structures have been found to originate tumors whose malignancy is inversely related to the degree of differentiation of the parent structure, i.e., Lob 2 originate lobular carcinomas *in situ*, and Lob 3 give rise to benign breast lesions, such as hyperplastic lobules, cysts, fibroadenomas and adenomas, and Lob 4 to lactating adenomas (4). The fact that each specific compartment of the breast gives origin to a specific type of lesion lends support to a new biological concept, that the differentiation of the breast determines its susceptibility to neoplastic transformation (4, 6). The architecture of the non-tumoral breast tissues from nulliparous women's cancer-bearing lumpectomy or mastectomy specimens do not differ from that of nulliparous females free of mammary pathology being both composed predominantly by Lob 1 (3, 7). Although in premenopausal parous women from the general population the breast contains predominantly Lob 3 and a very low percentage of Lob 1, in those parous women with or without family history that had developed breast cancer their breast tissues contain the Lob 1 as the predominant structure, appearing similar to that of nulliparous women (7). The similarities found between the architecture of the breast of nulliparous women and that of parous women with cancer support the hypothesis that the degree of breast development is of importance in the susceptibility to carcinogenesis, and, furthermore, that parous women who develop breast cancer might exhibit a defective response to the differentiating influence of the hormones of pregnancy (2) and therefore they are hosting cells that are susceptible to carcinogenesis. Based on these observations it was postulated by Russo and his coworkers in 1997 (8) (figure 1), that the Lob 1 found in the breast of nulliparous women and of parous women with breast cancer never went through the process of differentiation, retaining a high concentration of epithelial cells that are targets for carcinogens and therefore susceptible to undergo neoplastic transformation, these cells were called Stem cells 1 (8). Lob 1 structures found in the breast of early parous postmenopausal women free of mammary pathology, on the other hand, are composed of an epithelial cell population that is refractory to transformation and these cells are called Stem cell 2, making this the postulated mechanism of protection conferred by early full term pregnancy (2, 3, 6, 8). It was further postulated that the degree of differentiation acquired through early pregnancy has changed the "*genomic signature*" that differentiates the Lob 1 from the early parous women from that of the nulliparous women by shifting the Stem cell 1 to a Stem cell 2 that is refractory to carcinogenesis (figure 1).

4. EVIDENCE FOR A STEM CELL 1 IN THE MAMMARY GLAND.

Stem cells in adult structures have been defined by their ability for self-renewal and for generating a differentiated progeny. In the mammary gland, DeOme *et al.* (9) demonstrated that fragments of different parenchymal portions were able to generate fully functional mammary outgrowths in mice, forming ductal and lobuloalveolar structures composed by epithelial and myoepithelial cells. This concept was further developed by Kordon and Smith (10) who demonstrated that the progeny from a single cell may comprise the epithelial population of a fully developed lactating mammary outgrowth in mice. Thus, the development of the complete mammary tree from a small portion of a duct or from single cells attests of their multifaceted potential. However, it was not known whether these progenitor/stem cells would be capable of initiating cancer when exposed to a carcinogenic agent. This issue was addressed by Russo and coworkers (11-13), who demonstrated that cancer started in terminal end buds (TEBs) present in the mammary gland of young virgin rats. The analysis of these structures by electron microscopy

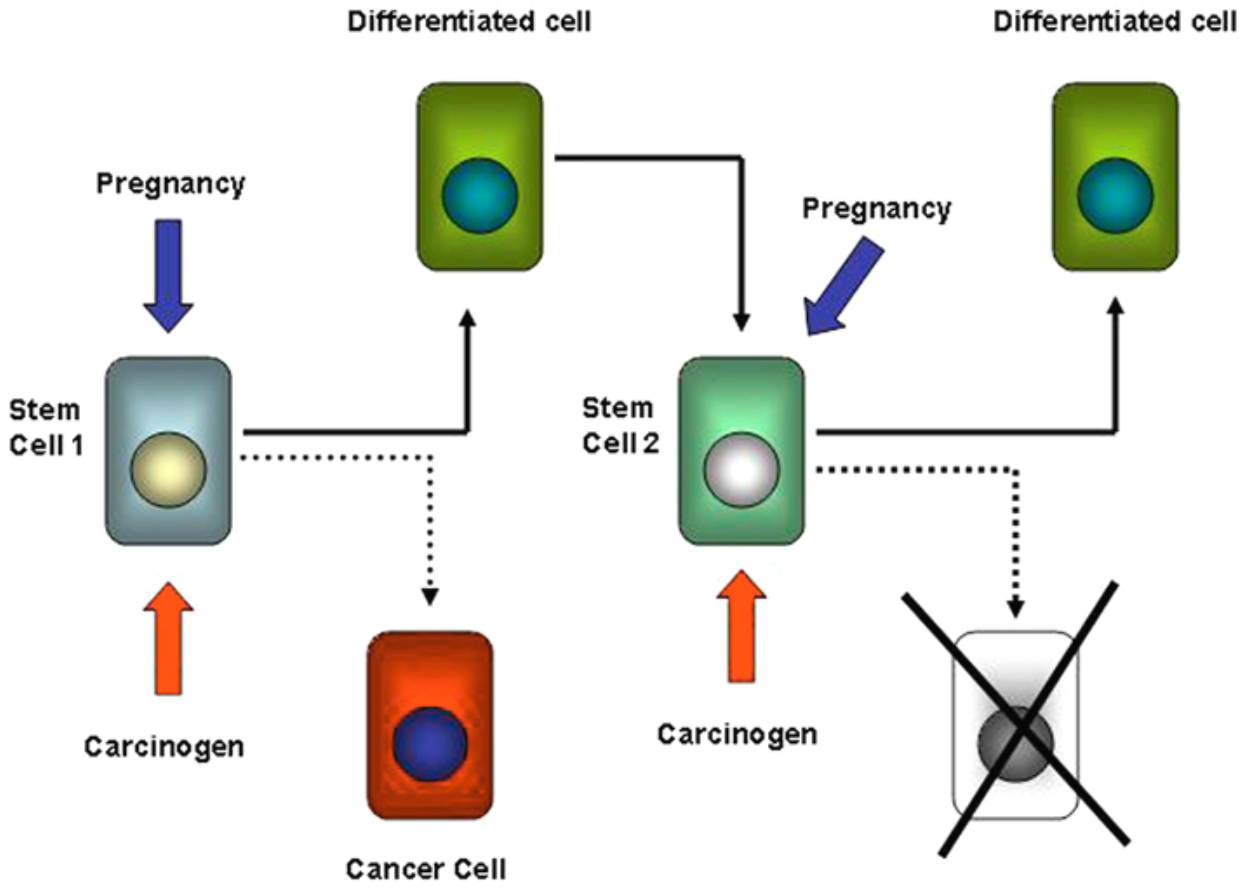


Figure 1. Breast cancer originates in undifferentiated terminal structures of the mammary gland, the lobule type 1, that contain the Stem cell 1 that is the target of the neoplastic event. Early parity induces differentiation of the mammary gland creating the Stem cell 2. Even though differentiation significantly reduces cell proliferation in the mammary gland, the mammary epithelium remains capable of responding with proliferation to given stimuli, such as a new pregnancy. Under these circumstances, however, the cells that are stimulated to proliferate are from structures that have already been primed by the first cycle of differentiation, that are able to metabolize the carcinogen and repair the induced DNA damage more efficiently than the cells of the nulliparous gland, and are less susceptible to carcinogenesis. However, if the shifting of Stem cell 1 to Stem cell 2 has not been completed a carcinogenic stimulus powerful enough may overburden the system, thereby initiating successfully a neoplastic process. (Reprinted with Permission from: Russo J., *et al Breast Cancer Res*, 2005 (129)).

allowed one to characterize their cellular composition based upon cell and nuclear size, nuclear-cytoplasmic ratio, amount of chromatin condensation, electron density of the cytoplasm, number and distribution of organelles, and presence or absence of Mg^{++} and $Na^{+}K^{+}$-dependent ATPases. Based upon these criteria, in addition to myoepithelial cells, three types of epithelial cells were identified: Light, intermediate and dark cells (12, 13). Dark cells were found to be the predominant type in TEBs, intermediate and myoepithelial cells were present in significantly lower percentages and light cells were only occasionally seen, therefore their percentage was combined with that of intermediate cells. The analysis of the DNA labeling index revealed that all the cell types proliferated, although at different rates, depending upon the type of cells and of their location within the mammary gland tree. Cell proliferation was maximal in intermediate cells located in TEBs, being significantly lower in dark and myoepithelial cells found in the same location. High cell proliferation was associated with greater incorporation of H^3-DMBA, and a progressive dominance of intermediate cells in DMBA-induced intraductal proliferations (IDPs) and in ductal carcinomas (13, 14). These results indicated that intermediate cells were not only the targets of the carcinogen but also the stem cells of mammary carcinomas. Further work by Bennett *et al.* (15) demonstrated that intermediate cells isolated from DMBA-induced mammary tumors originated two cell types in culture, the dark cell, representing a terminally differentiated cell or a class in transition to differentiation, and intermediate cells, which could represent an undifferentiated, or stem cell, a progenitor of dark and myoepithelial cells. Rudland and coworkers (16) isolated and characterized from the normal rat mammary gland and from DMBA-induced mammary adenocarcinomas epithelial cells that were cuboidal and gave rise to a mixture of cuboidal and spindle-shaped cells resembling fibroblasts. In confluent cultures, cuboidal cells acquired the morphology of a third type of cells, which

were dark, polygonal and with many small vacuoles, resembling the dark cells ultrastructurally described by Russo *et al.* (13). Chepko and Smith (17) differentiated three division-competent cell populations in the murine mammary epithelium, a subset of "large light cells" structurally and functionally compatible with early stages of secretory differentiation, "small light cells" that were the least differentiated, suggesting that the large light cells were a direct precursor to terminally differentiated cells, both secretory and myoepithelial.

4.1. Postulated cell markers for identifying the stem cell in the mammary gland

A shift from the pioneering work done for characterizing by morphology and by *in vitro* behavior the progenitor/stem cells started with the search for immunocytochemical and genomic markers. Smith *et al* (18) utilized the expression of keratins 6 and 14 in mouse mammary epithelium for defining subsets of morphologically distinct luminal mammary epithelial cells with kinetic properties expected for latent mammogenic stem cells. Keratin 6 was confined to a small number of mammary epithelial cells found in the growing end buds and among the luminal epithelium, whereas keratin 14 was expressed in basally located fusiform cells as the myoepithelial cells. These authors emphasized the usefulness of these markers for identifying mammary epithelium-specific primordial cells. Stingl *et al.* (19, 20) utilized new molecular markers for selecting subpopulations of cells with distinct differentiation potential. They described bipotent human mammary epithelial progenitor cells based on the expression of epithelial specific antigen (ESA), sialomucin 1 (MUC1), common acute lymphoblast antigen (CALLA/CD10,) and alpha-integrin, in combination with exclusion of rhodamine dye. Hebbard *et al* (21) observed that CD44, a member of the family of cell surface proteins that is expressed in breast carcinomas, is also expressed in the normal mammary gland. In rodents CD44 expression is first detected at puberty, and thereafter it is regulated by the estrous cycle; it disappears during lactation, reappearing during involution, suggesting that the expression of this protein is a marker of a stem cell. Novel studies in mice mammary gland (22) have identified stem cells in TEBs and ducts by pulse labeling HC-11 primary mammary epithelial cells with fluorescent TRITC-cell linker membrane label and BrdU, the cells were then transplanted into cleared juvenile syngenic mammary fat pads, in which they were identified as long-lived, label-retaining mammary epithelial cells (LRCs) in mammary ducts that were actively growing or static. This study demonstrated that LRCs are stem cells and their progeny (transitional cells) are arranged as transitional units (TUs) and that both express Zonula Occludens-1 and alpha-catenin proteins, data that suggest that transitional units retain stem cells.

The study of markers for other stem cells has been useful in the identification of mammary stem/progenitor cells. Sca1 (stem cell antigen 1) was first described in mice as a hematopoietic stem cell antigen (23). Welm *et al* (24) detected in the luminal epithelium of mice a Sca1+ cell population that is enriched for functional stem/progenitor cells. These cells are BrdU label retaining, lack expression of differentiation markers, and are progesterone receptor negative. The Sca1+ population also shows "side population" (SP) properties, a characteristic first defined in bone marrow cells (23), as cells with Hoeschst dye-effluxing properties that have phenotypic markers of multipotential hematopoietic stem cells. It has been proposed that the protein responsible for that phenotype is breast cancer resistance protein 1 (BCRP1), suggesting that the expression of this protein could serve as a marker for stem cells from various sources (25). Mammary epithelial cells with SP properties were also identified in human mammary gland. Alvi *et al.* (26) showed that 0.2-0.45% of both human and mouse epithelia were formed by distinct SP cells. These cells generated ductal and lobuloalveolar structures when transplanted into murine cleared mammary fat pads. The SP cells had a high expression of BCRP1, sca1, telomerase catalytic subunit, and low levels of differentiated markers for luminal (epithelial membrane antigen and cytokeratin 19) and myoepithelial cell types (cytokeratin 14). These cells were detected in all human breast samples studied, but their presence was not correlated with age, parity, contraceptive use or day of menstrual cycle. Further investigations identified new markers which may be specific for the human stem/progenitor cells. Gudjonsson *et al* (27) isolated a cell line derived from human mammary cells expressing epithelial specific antigen (ESA) and lacking sialomucin (MUC) expression that could give rise to both luminal epithelial and myoepithelial cells in culture. One single ESA+/MUC- cell had the ability of generating a terminal ductal-lobular unit-like structure in basement membrane gel, similar to that formed when the cell line was implanted in mice. In contrast, an ESA+/MUC+ subpopulation was differentiated, luminal epithelial-restricted without stem cell properties. Wicha and coworkers (28) developed a system to enrich the population of human mammary progenitor/stem cells by culturing them in suspension where they formed "nonadherent mammospheres". These structures were able to differentiate along all three mammary epithelial lineages and to clonally generate complex functional structures in 3D culture systems. Cytological and immunocytochemical analysis of secondary mammospheres revealed that these structures contained cells positive for alpha-6 integrin, cytokeratin 5, which was widely expressed, and CD10; ESA-positive and cytokeratin 14-positive cells were less frequently found. Muc 1, alpha-smooth muscle antigen (ASMA), and cytokeratin 18 were not detected. In addition to cells, mammospheres contained extracellular material (ECM). However, immunostains for fibronectin and collagen IV, the classical components of adult gland ECM material were negative, although ~20% of the mammospheres stained positive for laminin. In contrast, abundant expression of the embryonic ECM components tenascin and decorin, was detected in mammospheres (See Ref.28). Moreover, the comparison of the genomic profile of undifferentiated cells from mammospheres to that of differentiated cells cultured on collagen identified genes candidates for stem/progenitor cell markers. Some of these genes were already described as involved in stem/progenitor cell-specific functions or regulation of self-

renewal and abnormal expression of some of them has been correlated with breast cancer development such as proliferation, cell survival and invasion. Recently, new studies showed that the null mutation of the peroxisome proliferator-activated receptor-binding protein (PBP) resulted in defective mammary gland development and in the inability of the mammary epithelial cells to form mammospheres, suggesting a role of PBP in the survival of mammary stem cells or in mammosphere formation process (29)

4.2. Role of the Estrogen Receptor alpha as a marker of stem cells in the mammary gland

The identification of the stem cell and of its role in the development and differentiation of the mammary gland from birth to senesce requires an understanding of the effect of estrogen and its cognate ligand receptor alpha (ERalpha) in these processes. The importance of the role played by the ERalpha in mammary gland development has been highlighted by the development of the ERalphaKO mouse (30). At birth, the mammary gland of intact animals consists of a rudimentary ductal tree that develops and fills the stroma of the gland in response to increased ovarian estrogen at puberty. The mammary gland of ERalphaKO females does not grow beyond the rudimentary ducts, illustrating the role of estrogens in ductal elongation. The importance of active ductal growth driven by estrogen has been further emphasized by the higher susceptibility of the breast to be transformed during a "high risk" window in the lifespan of a female encompassed between menarche and a first full term pregnancy (14). This period is characterized by rapid ductal growth and active proliferative activity of the mammary epithelium of Lob 1. These structures are composed of a rapidly proliferating epithelium that has a high content of ERalpha and progesterone receptor (PR) positive cells. With the progressive maturation of Lob 1 to Lob 2, Lob 3, and Lob 4 there is a progressive decrease in the percentage of proliferating cells, a reduction in the percentage of cells positive for steroid hormone receptors, and a reduction in the susceptibility of the cells to be transformed by chemical carcinogens (31). These data indicate that the stem cells that originate the mammary tree as well as cancerous lesions are located in a specific compartment of the mammary parenchyma, namely the Lob 1 or TDLU; these are the cells that have been called Stem cell 1 by Russo and Russo (8). Supporting studies by Petersen *et al* (32) have shown that a subset of suprabasal breast luminal epithelial cells that are able to generate themselves as well as differentiated luminal epithelial and myoepithelial cells, and to form terminal ductal lobular unit (TDLU)-like structures are distinguished by cytokeratin 19. The suprabasal population of breast stem cells consists of undifferentiated "intermediate" cells with Hoechst dye-effluxing "side population" (SP) properties. These cells lack expression of myoepithelial and luminal apical membrane markers such as CALLA and MUC1. They are rich for ERalpha-positive cells and express several fold higher levels of the ERalpha, p21 (CIP1) and Msi1 genes than non-SP cells. They also form branching structures in matrigel which included cells of both luminal and myoepithelial lineages. These data suggest a model where scattered steroid receptor-positive cells are stem cells that self-renew through asymmetric cell division and generate patches of transit amplifying and differentiated cells (33, 34). ERalpha/PR+ breast cancers exhibit loss of the two key regulators of asymmetric cell division, Musashi-1 and Notch-1 and thus they may arise from symmetric division of the ERalpha/PR+ stem cell (33). These data are supported by the observations of Russo *et al* (31) that epithelial cells of the Lob 1 co-express ERalpha, PR and the proliferation marker Ki67, suggesting that these cells could originate ERalpha positive tumors. However, these cells represent less than 1% of the total cell population, whereas the majority of ERalpha/PR+ cells do not express the proliferation marker, an indication that the cells that contain the receptors are not capable of proliferating. The findings that proliferating cells are different from those that are ERalpha- and PR-positive support data that indicate that estrogen controls cell proliferation by an indirect mechanism. Further support is the finding that when Lob 1 of normal breast tissue are placed in culture, they lose the ERalpha-positive cells, indicating that only proliferating cells that are also ERalpha-negative can survive, representing a type of stem cell that may originate ER negative tumors (31). The fact that the majority of proliferating breast epithelial cells do not express ERalpha and PR could explain Clayton and coworkers (35) data that cells characterized as human mammary stem cells, present ESA expression, Hoechst dye exclusion, low levels of MUC-1 and CALLA, and lack detectable expression of ER alpha and beta. Cells expressing that phenotype had high cloning efficiency in culture from a single cell, generating mixed colonies containing luminal and myoepithelial cells.

4.3. Role of the Estrogen Receptor beta as a marker of stem cells

Estrogens and ERs are required for the proliferation of stem cells. For example, it was observed that the human breast carcinoma cells, PMC42, with stem cell characteristics, did not proliferate in serum-free medium unless 17-beta-estradiol (E_2) or progesterone was added, though their ER content is lower than that described for MCF-7 and T47D. The addition of both hormones induced a more than additive increase in proliferation whereas the addition of tamoxifen significantly decreased cell numbers and inhibited the stimulatory effects of E_2 (36). Estrogens also have profound effects on function and plasticity of rat neural stem cells. Both embryonic and adult rat neural stem cells express ERalpha and ERbeta. E_2 treatment increased the proliferation of embryonic neural stem cells and these effects were inhibited by the ER antagonist, ICI-182780, showing an involvement of ERs (37). It has also been shown that estrogens have significant effects on spermatogonial stem cells of the Japanese eel (*Anguilla japonica*) and these effects are mediated via ERs (38). Together, these observations indicate that estrogens and ERalpha/ERbeta are required for the proliferation of stem cells. However the data regarding the role of the second ER isoforms, ERbeta in regulation of cell proliferation are controversial (30-41). ERbeta has been assigned two different and opposed actions as an inhibitor or a stimulator of E_2-induced proliferation in human breast cells. For examples, there are several reports indicating that over-expression of ERbeta and its variants in breast cancer

cell lines and breast cancer cells inhibited ERalpha-mediated cell proliferation (42-44). In ERbeta-knock out mice, the presence of ERalpha but not ERbeta was necessary for the development of mouse mammary gland (45, 46). Thus, it was hypothesized that ERbeta is acting as antagonist for the actions of ERalpha, as an inhibitor of cell proliferation (42-44, 47-49). However, this hypothesis is challenged by the following data: a) There are proliferating cells in breast which express ERbeta (50), but the ERalpha-expressing cells in breast cancer do not express proliferation markers (51, 52), b) The expression of ERbeta in breast cancer cells correlated with the enhanced expression of cell proliferating markers (e.g. Ki67 and cyclin A) (50-53); c) ERbeta is by far the more abundant of the two ERs in human breast cells (54) and rat mammary gland (55, 56) and its expression is higher in normal breast cells than in breast cancer cells (57-61); d) both wild-type and ERbeta variants are expressed in higher grade human breast tumors (52-64) and the expression of ERbeta correlated with accepted prognostic indicators including lymph node status and tumor grades (65); e) high percentage ERalpha-negative tumors were positive for ERbeta; The presence of ERbeta was associated with an increase in survival rates in women treated with tamoxifen (66); f) In addition, it has been found an inverse relationship between the methylation rate of the ERbeta gene and tamoxifen resistance. The tamoxifen-resistant tumors showed denser methylation of the ERbeta gene than control tumors (67).

There are several lines of evidence suggesting that ERbeta is involved in the control of stem cell proliferation. Proliferation of pluripotent, bone marrow stem cells, which develop to lymphoid and myeloid progenitors, is negatively regulated by estrogens. In estrogen deficiency and in ERbetaKO mice, there is significant alteration in bone marrow hematopoiesis. Shim *et al.* (68) observed that 1.5 years old ERbetaKO mice developed pronounced splenomegaly that is much more severe in females than in males. Further characterization of these mice revealed myelogenous hyperplasia in bone marrow, an increase in the number of granulocytes and B lymphocytes in blood, lymphadenopathy, and infiltration of leukocytes in the liver and lung. Analysis by flow cytometry of the bone marrow cells revealed that the percentage and total number of Gr-1hi/Mac-1hi-positive granulocytes were increased. The numbers of B cells in the bone marrow and spleen were significantly higher in ERbeta$^{-/-}$ mice than in WT littermates. Some of the ERbetaKO mice also had a severe lymphoproliferative phenotype. Thus the absence of ERbeta results in a myeloproliferative disease resembling human chronic myeloid leukemia with lymphoid blast crisis. These results indicate a role for ERbeta in regulating the differentiation of pluripotent hematopoietic progenitor cells (68). In the prostate there are high levels of ERbeta but no ERalpha, suggesting a direct estrogenic influence on prostatic epithelium mediated by ERbeta. Imamov *et al* (69) reported that loss of ERbeta results in epithelial hypercellularity in the ventral prostate. In ERbeta$^{-/-}$ mouse prostates, there is over-expression of the androgen receptor and of the antiapoptotic factor Bcl-2 in the prostate. It is normally expressed only in the basal cells in the prostate. This apparent expansion of the 'stem cell-like population' in the ERbeta$^{-/-}$ mouse prostate has been further examined. They found a higher expression of cytokeratin 5 in ERbeta$^{-/-}$ mouse prostates so that the ratio of cytokeratin 5 to that of 19 is much higher in ERbeta$^{-/-}$ than in wild-type littermates. In addition, labeling of DNA with BrdU showed a 3.5-fold higher proliferation rate in ERbeta$^{-/-}$ mouse prostate. Despite these clear differences, the piling up of epithelial cells never progressed to high-grade prostatic intraepithelial neoplasia (PIN)-like lesions. Hyperplastic foci in ERbeta$^{-/-}$ mice show accumulation of cells without signs of atypia, resembling low-grade PIN in humans. The reason for this appeared to be a high rate of cellular detachment and subsequent fall off into the lumen in ERbeta$^{-/-}$ mice. The fall off phenomenon is possibly related to the finding that the expression of the cell adhesion molecule E-cadherin was reduced. It is concluded that in ERbeta$^{-/-}$ mouse prostates, the epithelial cell population contains more epithelial cells in the intermediate stage of differentiation, possessing both the ability to proliferate as the basal cells and the ability to secrete as the highly differentiated luminal epithelium.

Estrogen exposure has been linked to a risk for the development of testicular germ cell cancers. Pais *et al.* (70) compared the localization and expression levels of these ER subtypes in testicular germ cell cancers with normal testis. They observed that ERalpha was not expressed in the human normal testis and was also absent in all of the testicular germ cell cancers studied. In contrast, ERbeta was strongly expressed in various germ cells of the normal testis, suggesting that only ERbeta mediates the action of estrogen in the human male gonad (70). Palmieri *et al.* (71) evaluated the expression of ERalpha and ERbeta by immunochemistry in normal breast tissue samples, and in purified human breast fibroblasts by Western blotting, RT-PCR analysis and ligand-binding sucrose gradient assay. They observed that ERbeta variants, including ERbeta1, ERbeta2, ERbeta5, and ERbeta delta but not ERalpha, are expressed in human adult mammary fibroblasts. These results are supported by the findings that an ERbeta-selective ligand, BAG, but not the ERalpha high-affinity ligand E_2, can induce fibroblast growth factor-7 release and activate transcription from an estrogen-responsive element promoter in these adult human mammary fibroblasts. Together, these observations revealed that, in the adult breast and in breast cancer, the proliferative signals derived from the stroma of adult mammary glands in response to estrogen are not mediated by ERalpha and provide new insights into the nature of stromal-epithelial interactions in the adult mammary gland. In addition, the expression of these ERbeta variants in cells where there is no ERalpha suggested that these ERbeta splice forms may have functions other than that of modulating ERalpha activity (71).

MCF-10F cells are immortalized human breast epithelial cells (HBEC) negative for ERalpha but positive for ERbeta. The lower passages of these cells have low levels of ERbeta mRNA expression but when they were transformed either with chemical carcinogen, or estrogens

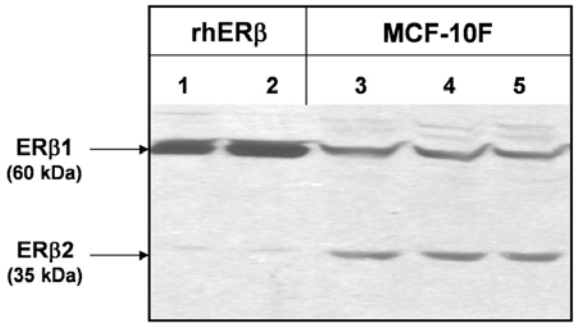


Figure 2. Detection of ERbeta Protein in MCF-10F Cells. RhER beta (60 ng/lane) and total cellular extracts (100 microgram/lane) prepared from MCF-10F, were separated via SDS-Page, transferred to Nitrocellulose membrane and probed with ERbeta antibody, followed by secondary goat anti-rabbit IgG-HRP. The nitrocellulose membrane was developed with ECL Western blot Analysis System (Amersham Biosciences) and exposed to X-ray film.

or its metabolites (72-74), the levels of ERbeta expression were enhanced. Figure 2 shows the Western blot for ERbeta in MCF-10F cells. Recombinant human ERbeta (rhERbeta) (60 ng/lane) was included as a positive control and was detected by this ERbeta antibody (figure 2, lanes 1 and 2). Two ERbeta antibody-reactive proteins (ERbeta 1, 60 kDa and ERbeta 2, 35 kDa) indicated by the arrow heads were detected in cellular extracts (100 microgram/lane) prepared from MCF-10F cells (lanes 3-5). These ERbeta-reactive protein bands appear to be ERbeta isoforms as they have the mobility similar to those of rhERbeta in same SDS-PAGE. These results confirm the presence of ERbeta proteins in MCF-10F. E_2, 2-OH E_2 and 4-OH E_2 are capable of enhancing cell proliferation, and of transforming HBEC and that these E2-induced phenotypes were not abrogated by antiestrogen, ICI182780 suggesting that ERbeta may play a role in E2-mediated cell proliferation and transformation (72, 75, 76) and that these cells have the property of the Stem cell 1 (figure 1). However, the precise role of ERbeta in these E_2-mediated processes in HBEC requires further investigations.

4.4. Influence of the stroma in the genomic profile of the Stem cell 1 *in vitro*

Considering that the HBEC MCF-10F could behave like a Stem cell 1 we aimed to determine the role of the stroma in the growth pattern of these cells *in vitro*. We have found that MCF-10F cells growing in collagen matrix form ductular structures mimicking the breast epithelia *in vivo* (figure 3). We performed cDNA microarray in order to determine the differential gene expression profile between the cells growing in monolayer and those growing in a tri-dimensional matrix and determining the role of the stroma in the ductulogenic process (figure 3). We found 161 genes differentially expressed and up-modulated (log mean of difference >2.0, with $p<0.05$) in the ductular structures in comparison to the MCF-10F cells in monolayer (table 1). Those genes are related to several biological functions such as gene transcription or regulation of transcription (e.i. Myeloid cell nuclear differentiation antigen) (77-83), protein biosynthesis (such as Stromal cell-derived factor 2) (84), amino acid transport and membrane trafficking (collagen, type IV, alpha 5 and RAB4, member RAS oncogene family), DNA repair system (such as ADP-ribosyltransferase (NAD+, poly (ADP ribose) polymerase)-like 2) (85-95) and genes related to regulation of cell transformation such as transforming growth factor, beta receptor III (table 1). Two of the genes that are highly relevant are the myeloid nuclear differentiation antigen of MNDA and the ADP-ribosyltransferase (NAD+, poly (ADP ribose) polymerase)-like 2 or PARP. The myeloid nuclear differentiation antigen (MNDA) is expressed in a lineage-specific manner in myeloid cells (80-82). MNDA may have an important role in myelomonocytic cell differentiation by exerting an antiproliferative effect on myeloid cell growth. In our specific model the MCF-10F cells express it under the stroma like effect of the collagen matrix and if could be involved in the expression of the organization of the ductal structures. MNDA may be related to the stem cell differentiation process in the human breast epithelial cells, to generate the Stem Cell type 2 (figure 1).

ADP-ribosyltransferase (NAD+, poly (ADP ribose) polymerase)-like 2 is also upregulated in the ductular structures and is thought to participate in chromatin condensation, maintenance of genomic stability, and the repair of oxidative DNA damage (85). PARP binds to double and single DNA strand breaks, generated by reactive oxygen species and the DNA-bound repair enzymes during the repair process. Upon binding to strand breaks sites via two zinc fingers in its N-terminal region, PARP's catalytic activity increases 500-fold (86, 87). PARP catalyzes the transfer of the ADP-ribose moiety of NAD+ onto a host of acceptor proteins such as histones, DNA topoisomerases, p53, DNA-dependent protein kinase, and other DNA binding proteins, including itself, thus forming long branched polymers of ADP-ribose (88-91). The high negative charge associated with poly ADP-ribosylation electrostatically repels the modified proteins from DNA and this is thought to clear the damaged site of chromatin and other extraneous proteins and facilitate repair (92, 93). Although PARP is necessary for the repair of damaged DNA that allows continued cell survival, it is widely recognized that in the face of extensive DNA strand breaks, PARP activation can lead to depletion of NAD+, decreases in intracellular ATP levels, and cell death (94, 95). All properties that are part of the genomic make up of a differentiated cell like the Stem Cell 2 (figure 1).

We also found 269 genes differentially expressed in MCF-10F monolayer cells respect to the ductular structures ($p<0.05$). These genes are related to the biological functions indicated in table 2. Among these genes are those related to the positive gene transcription DNA-dependent such as TRAF and INF receptor associated protein (96) and transcriptional co-activator with PDZ-binding motif (TAZ) (97-99). Genes related to process protein ubiquitination, proteolysis and peptidolysis were found over-expressed in the monolayer respect to the ductular structures (such as putative dimethyladenosine transferase, ESTs, Highly similar to beta-1,3-N-

Table 1 . Genes up-regulated in ductulus

Gene ID	Gene Name	Ductulus	MCF-10F	p Value	Function
N29376	myeloid cell nuclear differentiation antigen	1.41 ± 0.48	0.48 ± 0.41	0.022	Gene Transcription and Regulation of Transcription/ Chromatin modification
R45235	Stromal cell-derived factor 2	1.13 ± 0.56	0.06 ± 0.62	0.010	Protein biosynthesis/ Amino acid metabolism/Protein folding/Protein degradation
AA953254	Collagen, type IV, alpha 5 (Alport syndrome)	2.16 ± 0.61	-2.68 ± 0.02	0.040	Amino acid transport/membrane trafficking/Intracellular protein transport.
AA478440	RAB4, member RAS oncogene family	1.38 ± 0.04	-1.63 ± 0.04	0.040	Amino acid transport/membrane trafficking/Intracellular protein transport.
H23985	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase)-like 2	1.35 ± 0.25	0.63 ± 0.16	0.044	DNA repair Pathways
N26658	Transforming growth factor, beta receptor III (betaglycan, 300kD)	1.41 ± 0.13	0.45 ± 0.09	0.006	Cell Transformation/tumor suppressor gene

Table 2. Genes up-regulated in MCF-10F growing in a monolayer

Gene ID	Gene Name	Ductulus	MCF-10F	P Value	Function
TTRAP	TRAF and TNF receptor-associated protein	0.32 ± 0.28	4.97 ± 0.18	0.0091	Regulation of gene transcription DNA-dependent
WWTR1	Transcriptional co-activator with PDZ-binding motif (TAZ)	-2.26 ± 0.01	0.92 ± 0.16	0.0224	Regulation of gene transcription DNA-dependent
HSA9761	Putative dimethyladenosine transferase	-2.33 ± 0.18	2.03 ± 0.00	0.0190	Process Protein ubiquitination/Proteolysis and peptidolysis
B3GALT6	ESTs, Highly similar to beta-1,3-N-acetylglucosaminyltransferase [H.sapiens]	0.30 ± 0.30	3.30 ± 0.02	0.0434	Process Protein ubiquitination/Proteolysis and peptidolysis
HSPH1	Heat shock 105kD	0.27 ± 0.16	1.15 ± 0.06	0.0470	Process Protein ubiquitination/Proteolysis and peptidolysis
AA629897	Laminin receptor 1 (67kD, ribosomal protein SA)	-0.13 ± 0.27	3.65 ± 0.08	0.0041	Process Protein ubiquitination/Proteolysis and peptidolysis
W86466	Kinesin family member 13B	0.75 ± 0.01	2.10 ± 0.11	0.0321	Signal transduction/ Protein dimerization activity/ Protein targeting
AA126623	RAN binding protein 3	0.59 ± 0.29	1.54 ± 0.38	0.0459	Signal transduction/ Protein dimerization activity/ Protein targeting
W86466	Kinesin family member 13B	0.75 ± 0.01	2.10 ± 0.11	0.0321	Gene related to T cell activation**.**
AA994796	LIM and SH3 protein 1	-1.93 ± 0.06	1.89 ± 0.14	0.0236	Biological Process of ion protein lipid transport
AA448191	Vesicle-associated membrane protein 4	-2.48 ± 0.09	1.95 ± 0.15	0.0245	Biological Process of ion protein lipid transport
AA598595	Karyopherin (importin) beta 3	0.40 ± 0.1	1.40 ± 0.04	0.0337	Biological Process of ion protein lipid transport
H13623	Epidermal growth factor receptor pathway substrate 8	0.81 ± 0.28	1.17 ± 0.30	0.0315	Growth factors on cell proliferation**.**
AA018591	Spectrin, beta, non-erythrocytic 1	0.82 ± 0.10	3.01 ± 0.01	0.0160	Actin cytoskeleton organization and biogenesis/ Actin binding
AA626349	A kinase (PRKA) anchor protein 3	0.33 ± 0.30	2.86 ± 0.35	0.0084	Actin cytoskeleton organization and biogenesis/ Actin binding
AA936779	keratin 6A	0.23 ± 0.23	4.51 ± 0.04	0.0215	Actin cytoskeleton organization and biogenesis/ Actin binding
AA449336	Protein regulator of cytokinesis 1	-0.09 ± 0.29	1.68 ± 0.00	0.0099	Regulatory Microtubule associated protein/cell cycle protein

cetylglucosaminyltransferase (*H. sapiens*), Heat shock 105kD and Laminin receptor 1 (67kD, ribosomal protein SA) (table 2). This later gene is of importance because several laminin receptors are included in the integrin family of extracellular matrix receptors. Ligand binding by integrin heterodimers results in signal transduction events controlling cell motility (100). Altogether the data summarized here emphasizes the importance of the use of MCF-10F cells as a model of human breast epithelial cells proven valuable for the isolation and characterization of mammary stem cells and early progenitors, and for the study of the pathways involved in normal growth, differentiation, and lineage-specific commitment of human mammary epithelial cells. This system may also be amenable to the addition of stromal elements in order to study stromal-stem cell interactions involved in mammary cell commitment and differentiation. Furthermore, because mammary stem cells may be a target for cellular

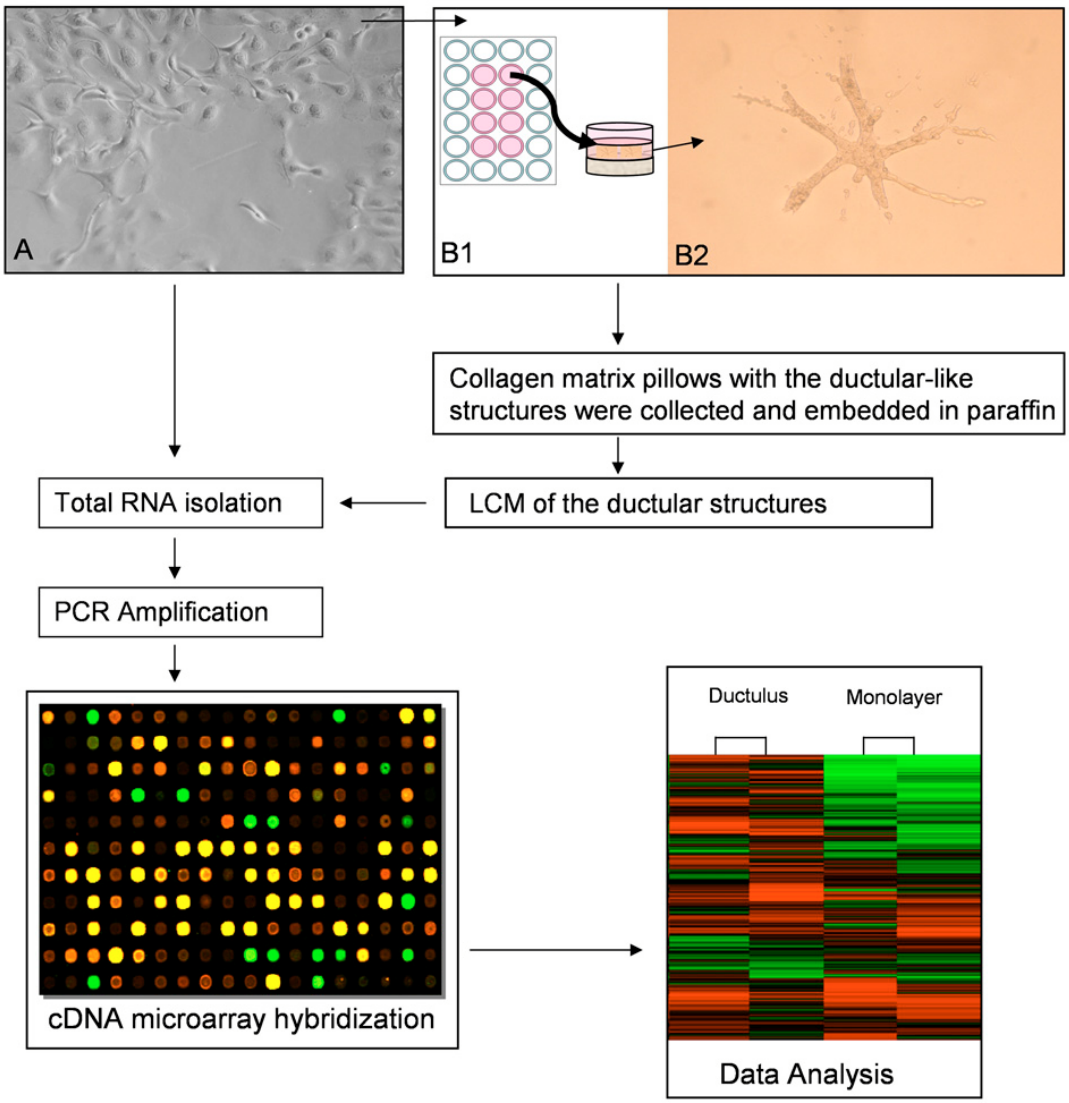


Figure 3. Schematic representation of the steps to determine the differential gene expression profile between MCF-10F cells growing in a monolayer (A) and those forming ductular-like structures in collagen matrix after 21 days (B1 and B2).

transformation, this system has proved to be useful in studying mammary carcinogenesis as is described in the next section.

4.5. The MCF-10F as the Stem cell 1 in estrogen induced carcinogenesis

There is substantial amount of epidemiological, clinical and experimental evidence pointing to estrogens, e.g. 17beta-estradiol (E_2), as being one of the most important etiological factors for the development and progression of breast cancer (See reference 72). To test the transforming ability of estrogens on MCF-10F cells that is ERalpha negative, ERbeta positive and progesterone receptor negative, and for this purpose considered as a Stem cell 1, were treated twice a week during two weeks with 70 nM E_2 (72). These cell expressed transformation phenotypes such as the formation of colonies in agar methocel, and the loss of the ductulogenic capacity when they grew in a collagen matrix. In order to identify the more aggressive transformed cells capable of forming tumors after E_2 treatment, we have selected the highly invasive populations of MCF-10F cells treated with E_2 in their 9th passage by seeding them in Boyden chambers (figure 4). Those cells crossing the membrane were collected, expanded, and designated B2, B3, B4, B5, C2, C3, C4 and C5. Four of them, B2, C3, C4 and C5 cells were injected to severe combined immune depressed (SCID) mice. Only C3 and C5 cells were tumorigenic in 2/12 and 9/10 animals injected, respectively (figure 4). The tumors were poorly differentiated adenocarcinomas, ERalpha and PR negative, and expressed basic keratin of high molecular weight, E-Cadherin, CAM5.2, and vimentin. The genomic profile of C3 and C5 cell analyzed by cDNA microarray, revealed that C5 cells overexpressed

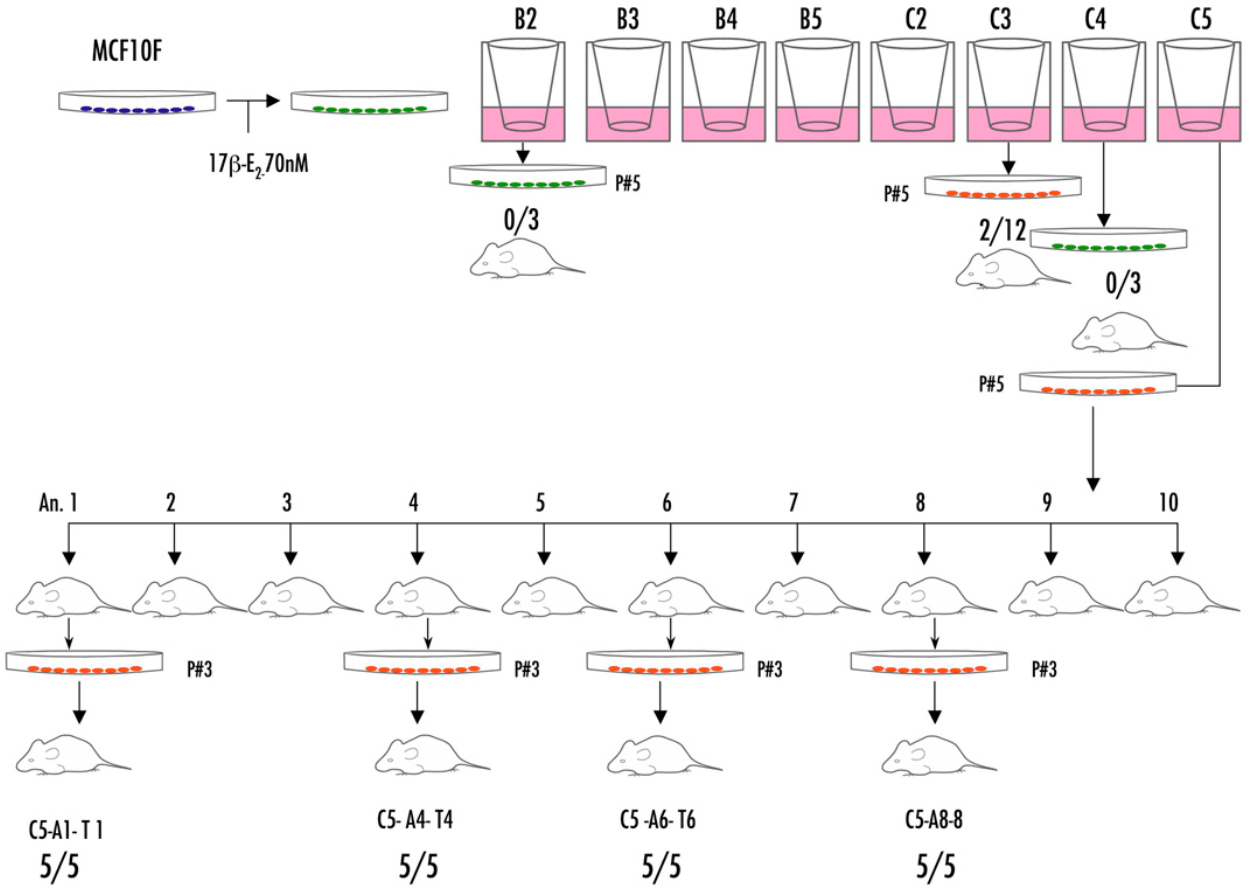


Figure 4. Schematic representation of the evolution of MCF-10F during transformation and tumorigenesis induced by 17beta-estradiol.

Table 3. Study of the genomic changes in the Stem Cell 1 during the process of cell transformation by estrogen by Comparative Genomic Hybridization (CGH).

	CGH	
Cell type	**Gains**	**Losses**
MCF-10F	8q24.1	
70nM E_2	8q24.1	9p11-13
C5	8q24.1	9p11-13
Tumors (An1, An 4, An 6, An 8)	High level gain of 1p HLG	3p12.3-13
	5q15-qter	4
	8q24.1	8p11.1-21
		9p21-pter
		18q
Cell lines derivate from tumors (C5 tumor1, C5 tumor 4 C5 tumor 6, C5 tumor 8)	1p	3p12.3-13
	5q15-qter	4
	8q24.1-qter	8p11.1-21
		9p21-pter
		18q

In the CGH results, only gains and losses that reach the 1.2 or 0.8 threshold values are shown; alterations present in less than 50% of the populations are not shown in this table.
HLG: high level gains.

more than 5-fold tankyrase (chr 8p23.1), claudin 1 (chr 3q28), homeobox C10 (HOX-C10; chr 12q13.3), and Notch homolog 3 (chr 19p13.12). It also exhibited downregulation of telomeric repeat binding factor 1 (chr 8q21.11) and tumor metastasis suppressor LASS2 (chr 1q21.3) genes. Four tumoral cell lines were obtained from four of the nine tumors derived from C5: C5-A1-T1, C5-A4-T4, C5-A6-T6 and C5-A8-T8 and all of them produced tumors when they were injected to SCID mice (figure 4). Comparative genomic hybridization (CGH) analysis was performed to identify gains and losses of genetic material in the different cell lines during the tumorigenic process (table 3). CGH

analyses shown that the cells treated with E_2 have not differences when compared with untreated MCF-10F cells, except a lost in 9p11-13. The four tumors (An1, An 4, An6 and An 8) showed identical pattern of genomic imbalances. CGH analysis showed similar genomic patterns between the four tumors (A1, A4, A6 and A8) and the four cell lines derived from them (C5-A1-T1, C5-A4-T4, C5-A6-T6 and C5-A8-T8); and there was no additional chromosomal alterations after *in vitro* cell culture. All the tumors and derived cell lines showed gains of 1p, 5q15-qter and 8q24.1-qter and losses of chromosome 4, 3p12.3-13, 8p11.1-21, 9p21-pter and 18q24.1 (table 3). The gain of 8q 24.1 shown in the tumors have also been shown by MCF-10F, the cells treated with E_2 and C5 (table 3). The parental cell line C5 has shown a tendency for gain of 1p and 5q15-qter and loss of chromosome 4 and it is likely that in the tumors derived from C5, a sub-clone with these changes had a selective advantage *in vivo* and became more distinct. Losses of 3p12.3-13, 8p11.1-21, 9p21-pter and 18q appear to be new changes in the tumor (table 3). Interestingly, C5 and the cells treated with E_2 had loss of 9p11-13 while in the tumors the 9p21-pter was lost (table 3). The chromosomal alterations that we found *in vitro* are similar to those reported in primary breast cancer. Altogether the data shows both that 17beta-estradiol is able to transform a HBEC and that the MCF-10F has all the properties of a Stem cell 1 that is able to generate a tumor when challenged with the carcinogen agent.

5. THE EVIDENCE FOR THE STEM CELL 2 FOUND POST PREGNANCY IN THE MAMMARY GLAND

Epidemiological studies in humans and experimental carcinogenesis models have provided wide evidence of the protective effect of pregnancy from breast cancer development (101-110). Russo and coworkers (108, 111, 112) have postulated that the mechanism of pregnancy-induced protection is mediated by the induction of mammary gland differentiation driven by the hormonal milieu of pregnancy, which creates a specific genomic signature in the mammary gland that makes this organ permanently refractory to carcinogenesis (figure 1). Alternative explanations attributed the protective effect of pregnancy to changes in the environmental milieu (113) and/or alterations in the immunological profile of the host (105). A further refinement of the hypothesis of how pregnancy could be affecting cancer susceptibility through induction of differentiation of the mammary gland was first proposed by Russo and Russo (8), who postulated that the Lob 1 and the TEB found in the breast of nulliparous women or of young virgin rats, respectively, had not completed their differentiation into Lob 2, Lob 3 and Lob 4, retaining a high concentration of stem cells called Stem cells 1, which are susceptible to undergo neoplastic transformation when exposed to a carcinogenic agent (See previous section) (8) (figure 1). After the postmenopausal involution of the mammary gland, the architecture of the parous breast is similar that of the nulliparous breast, containing predominantly Lob 1 composed of Stem cell 2, an epithelial cell population that is refractory to transformation. It was further postulated that the degree of differentiation acquired through early pregnancy permanently changes the "*genomic signature*" that differentiate the Lob 1 from early parous women from that of nulliparous women, shifting the Stem cell 1 to a Stem cell 2 that is refractory to carcinogenesis. These cells were called Stem cell 2 because after post-lactational involution, the mammary epithelium remains capable of responding with proliferation and differentiation to the stimulus of a new pregnancy; however, these cells are refractory to carcinogenesis, even though they are stimulated to proliferate and to regenerate the whole mammary gland (figure 1). The Stem cell 2 is characterized by having a genomic signature that has been induced by the first cycle of differentiation. During the last eight years supporting evidence to this hypothesis has been generated by Russo and coworkers as well as by other researchers. Recent studies by Smith and coworkers (114-116) using transgenic WAP-driven Cre and Rosa 26-fl-stop-fl-LacZ mice provided evidence of a new mammary epithelial cell population that originates from differentiated cells during pregnancy; 5-10% of this parity-induced epithelium survives postlactational involution after the first pregnancy. With successive pregnancies their percentage increases, reaching 60% of the total epithelium in multiparous females. The parity-induced mammary epithelial cells (PI-MEC) is equivalent to the Stem cell 2 postulated by Russo *et al*, (8) since these cells show capacity for self-renewal and contribute to mammary outgrowth in transplantation studies. PI-MEC can function as alveolar progenitors in subsequent pregnancies, and it is thought that they would be related to differences in response to hormonal stimulation and carcinogenic agents observed between nulliparous and parous females (114-116).

The crucial role of the number of mammary stem cells in breast cancer risk has also been recently postulated by Trichopoulos (117), number that would be reduced through the process of terminal differentiation after the first full-term pregnancy. Several authors have focused in finding molecular changes as a mechanism of the pregnancy-induced protection (118-125). Russo and coworkers have found that the post-pregnancy involuted mammary gland exhibits a genomic signature characterized by elevated expression of genes involved in the apoptotic pathways, such as testosterone repressed prostate message 2 (TRPM2), interleukin 1beta-converting enzyme (ICE), bcl-XL, bcl-XS, p53, p21, and c-myc, which can be from 3 to 5 fold upregulated (118, 119, 126). The activation of programmed cell death genes occurs through a p53-dependent process, modulated by c-myc and with partial dependence on the bcl2-family related genes. In addition, inhibin A and B, heterodimeric non-steroidal secreted glycoproteins with tumor suppressor activity are also upregulated (118, 119, 126, 127). Genes whose level of expression progressively increases with time of pregnancy reaching their highest levels between 21 and 42 days post-partum are those coding for a fragment of glycogen phosphorylase, AMP activated kinase, bone morphogenetic protein 4 and vesicle-associated protein 1. G/T mismatch-specific thymine DNA glycosylase gene is also increased by five-fold in this model. These data indicate that the activation of genes involved in the DNA repair process is part of the signature induced in the mammary gland by

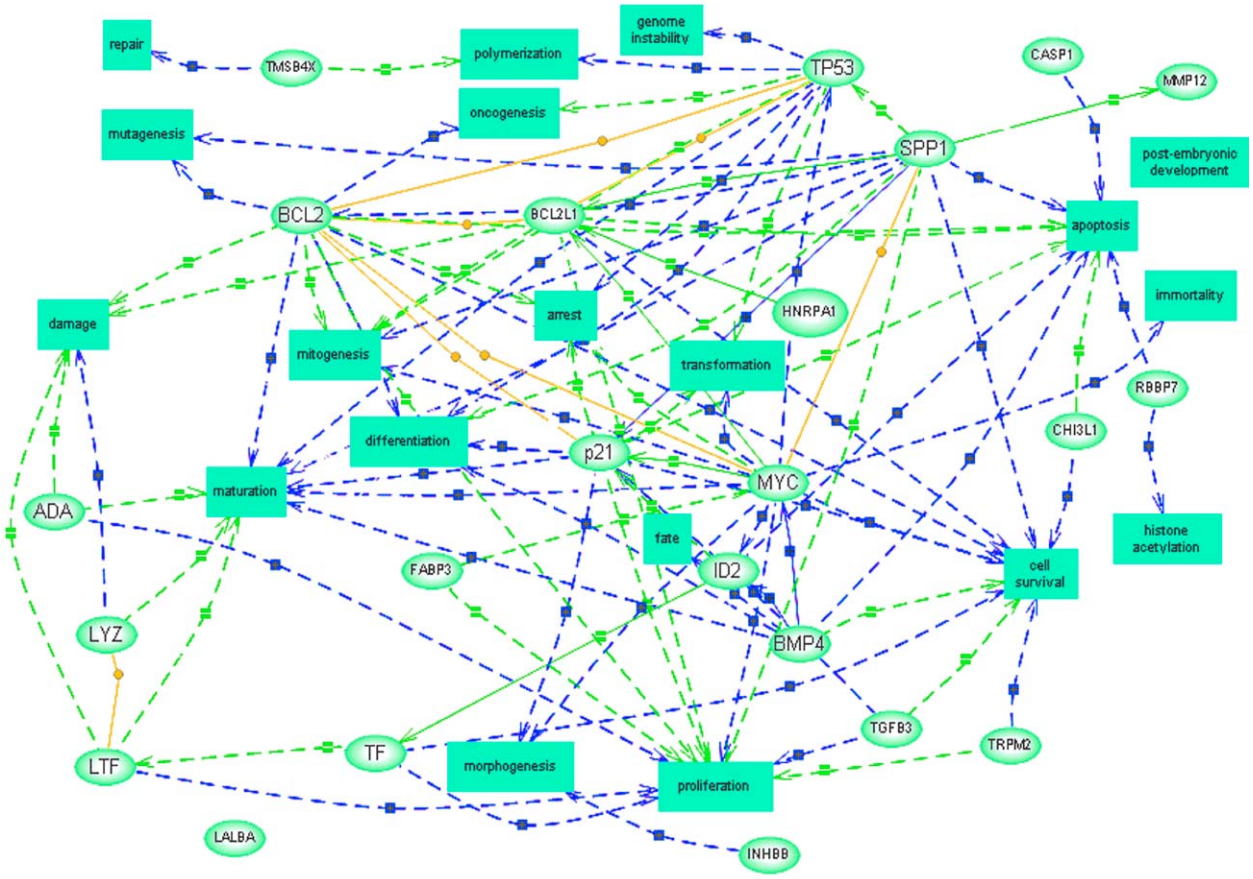


Figure 5. Interactions among the proteins codified by the genes related to the putative Stem cell 2. Ovals represent the proteins, boxes represent cellular processes, blue lines represent positive regulations, green lines represent negative regulations, yellow lines represent bindings.

pregnancy. These observations confirm previous findings that *in vivo* the ability of the cells to repair carcinogen-induced damage by unscheduled DNA synthesis and adduct removal is more efficient in the parous and animal mammary gland (111). In concordance with the studies of Srivastava *et al* (118), Siveraman *et al* (122) observed that p53 can be implicated in the protective effect of parity, which can be mimicked by treatment of virgin rats with estrogen and progesterone. Studies by Medina *et al.* (120, 121) in the same hormonal model reported that the function of p53 is required for the hormone-mediated protection of DMBA-induced mammary tumorigenesis in mice. Genomic analysis of the mammary gland of virgin rats treated with estrogen and progesterone at doses that have been reported to mimic pregnancy, showed downregulation of certain growth-promoting molecules, whereas markers involved in cell cycle control or the modulation of transforming growth factor beta (TGF-beta) signaling pathway were upregulated in the post-treatment involuted mammary gland (123). In this study, an unknown noncoding RNA (designated G.B7) and RbAp46, which has been implicated in a number of complexes involving chromatin remodeling, were found to be persistently up-regulated in the lobules of the regressed glands. Using gene profile analysis, D'Cruz and coworkers (125) also observed down regulation of growth factors potentially involved in epithelial proliferation as well as persistent upregulation of TGF-beta3 and several of its transcripts targets in the involuted gland of parous rats and mice. The proposed model of parity-induced specific changes (8) has been further confirmed by Ginger and Rosen (124), who reported that pregnancy induces multiple changes in the mammary epithelial cells, including nuclear accumulation of p53 and induction of whey acidic protein (WAP). During involution, a large component of the epithelium is eliminated through apoptosis, and a specific subpopulation of epithelial cells survives this process. The involuted mammary gland has persistent changes in gene expression, nuclear localization of p53, and an altered proliferative capacity in response to a carcinogen. Pregnancy would induce epigenetic changes, such as chromatin remodeling, DNA methylation/demethylation, and histone modifications, affecting cell fate in the parous mammary gland. As it is depicted in figure 5 all the genes that have been related to the Stem cell 2 seem to work in a different functional pathways than those described for the Stem cell 1.

Collectively, the data described above present evidence that pregnancy, through the process of cell

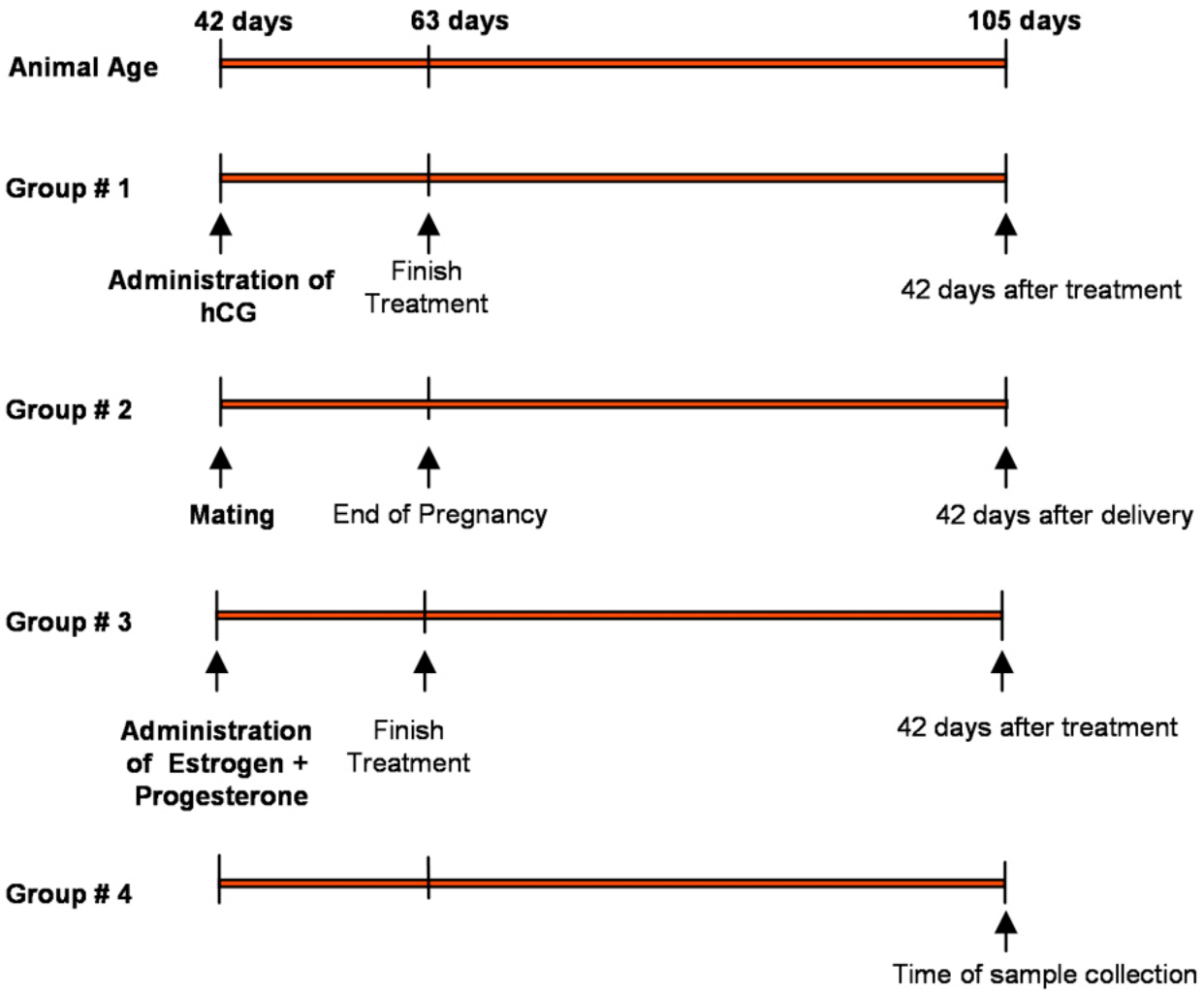


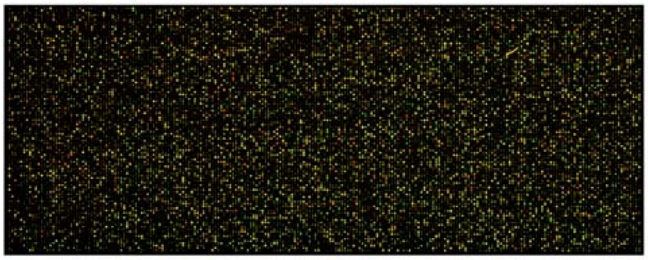
Figure 6. The experimental model. These studies have been carried out utilizing four groups of virgin Sprague-Dawley rats that have been maintained in an environmentally controlled clean air room with a 12hour light/12 hour darkness cycle. Starting when the rats are 42 days old, the first group, identified as (hCG) has received a daily intraperitoneal (ip) injection of 100 IU/hCG; for the second group, identified as (P) the rats have been mated; the third group, identified as (E+P) have been implanted subcutaneously (sc) a pellet of estrogen and progesterone; and the fourth group consisted of age-matched virgin control rats.

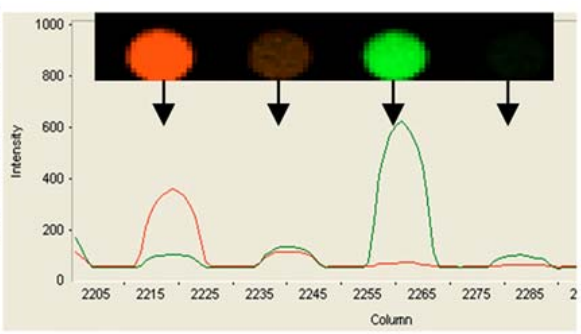
differentiation, shifts the Stem cell 1 to Stem cell 2 (figure 1), cells that exhibit a specific genomic signature that could be responsible for the refractoriness of the mammary gland to carcinogenesis.

6. NEW EVIDENCE FOR A STEM CELL 2 INDUCED BY PREGNANCY

6.1. Evidence in the rat mammary gland

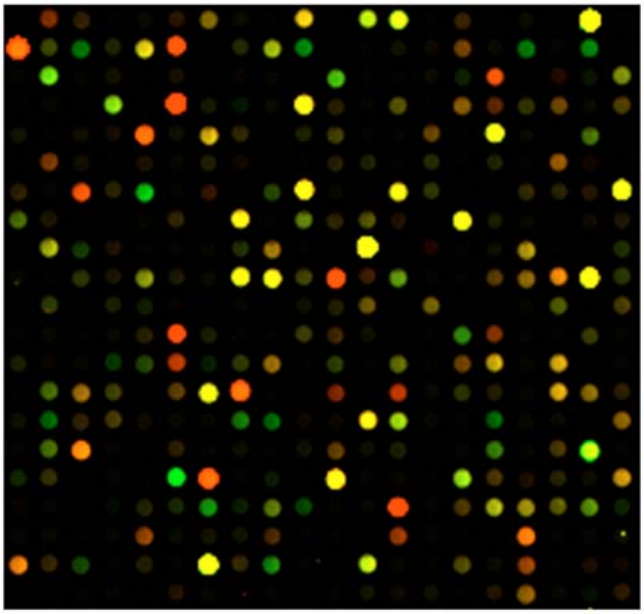
The epidemiological data suggest that early parous women exhibit a lifetime decrease in breast cancer risk indicating hormonal and reproductive factors in the prevention of breast cancer development (128). Studies in the rat mammary model of DMBA induced carcinogenesis full term pregnancy or the administration of the placental hormone chorionic gonadotropin (hCG) inhibited mammary cancer initiation and inhibit tumor progression (8). The protective effect of pregnancy and or hCG have been explained by a mechanism by which the adult stem cell pre-existing in mammary gland in nulliparous gland described as Stem cell 1 are induced to differentiate to a new progenitor or Stem cell 2 which is refractory to carcinogenesis (8). To further this hypothesis we have used 42 days old Sprague Dawley female rats divide in four groups (figure 6). One group was treated with recombinant hCG 100 IU by intraperitoneally injection during 21 days, a second group of pregnant rats and a third group (E+P group) (figure 6) that was treated with inter-scapular pellets containing Estrogen (20 mg/ml) + Progesterone (20 mg/ml) during 21 days. The last group was an untreated or control group of virgin animals. After 42 days post-pregnancy or post-treatment the gene expression profile was studied employing the oligonucleotide rat microarray technology (Agilent Technologies, CA), containing 22,500 features.





A

C



	Red Channel	Green Channel
Saturated Features	1 out 22,500	1 out 22,500
Non-uniformity outliers Features	64	220
Features Population Outliers	0	0
Background non-uniformity Outliers	24	18
Background Population Outliers	167 (0.74%)	161 (0.71%)

B

D

Figure 7. Rat Oligonucleotide Microarray from mammary gland after 42-treatment. A shows the image of the oligonucleotide microarray of 22,500 features from rat mammary gland of hCG after 42 days of treatment. The glass microarray was hybridized in red channel (Cy5) with the sample and in the green Channel (Cy3) with the rat universal RNA reference (Stratagene). B shows the amplification of the spots in the microarray using the Feature Extraction software (Agilent). C and D show the fluorescence intensity distribution of the spots. Red lines means the intensity distribution in the red channel and green lines means the intensity distribution of the green channel. Table D indicates the number of saturated features in the whole microarray in channels (red and green), the number of non-uniformly distributed outliers' features, the number of features outliers in the population, the background non-uniformly distributed, and the population of the background outlier.

The microarrays were hybridized using 1.5 microgram of cDNA labeled for both channels; the green channel or the Cy3 fluorescent dye was used for the rat universal reference and the Cy5 or the red channel corresponded with the samples. Figure 7 shows the quality of the oligonucleotide microarray and the image analysis software that was used (Feature Extraction from Agilent technologies, CA). After normalization of the data with Lowess methodology we combined the replicates of the 5 animals in each group in order to obtain the mean (log scale) of the genes expressed with the standard deviation. We selected the up- and down-modulated genes by design a cut off of 2.5 log scale respect to the control group or untreated, with $p<0.01$. We found 194 genes significantly ($p<0.01$) up-modulated (>2.5 $\log_2$ folds) in the hCG-treated group that were commonly expressed with the pregnancy group and were not expressed in the E+P group. Those genes corresponded to different biological functions involving: activators or repressors of transcription (CREB1, Inhibitor of DNA binding 1, p21, GADD153, etc), hormonal receptors (such as GABA, 5-hydroxytryptamine receptor 2A receptor, corticotropin-releasing factor receptor 2, androgen receptor between others), apoptosis (caspase 1, activator of apoptosis Harakiri, etc), molecular transporters (such as ATPase, hydrogen-potassium, alpha 2a subunit, calcium-transporting sarcoplasmic reticulum type ATPase class 2 isoform, sodium-dependent serotonin transporter; 5-hydroxytryptamine transporter, etc), growth factors (such as beta-nerve growth factor, leukemia inhibitory/cholinergic neuronal differentiation factor,

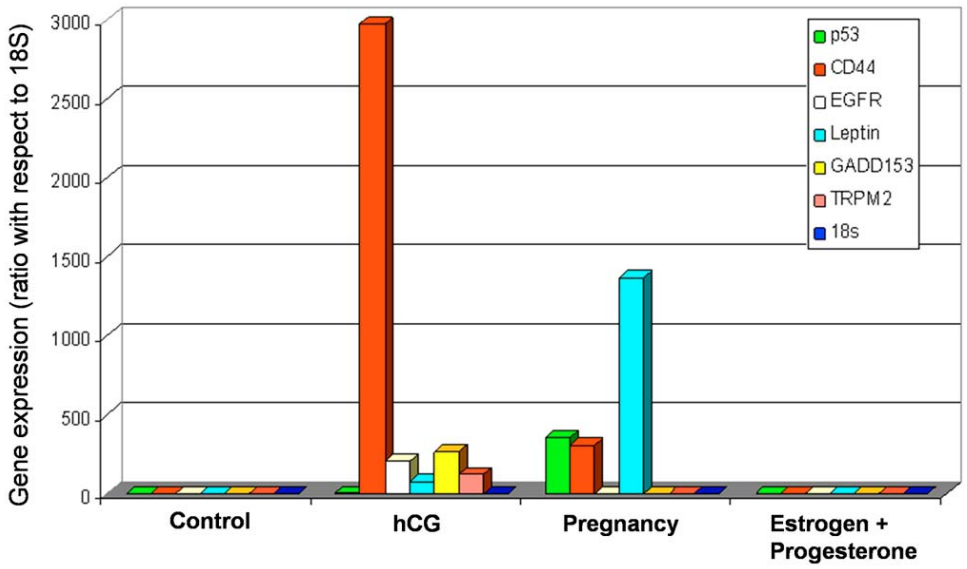


Figure 8. Gene expression validation using Real Time PCR of rat mammary gland at the 42 days after-treatment.

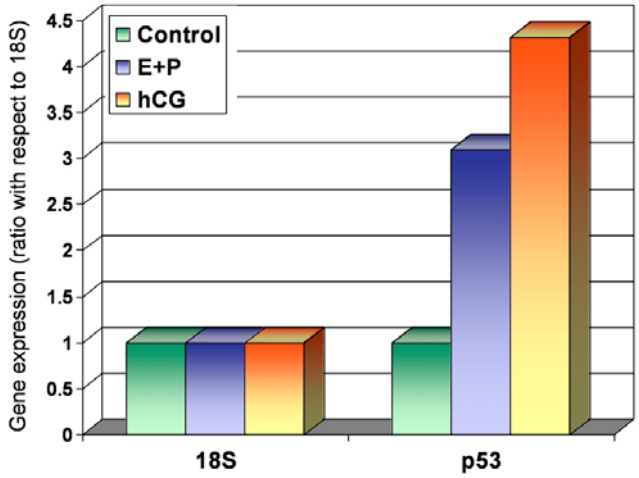


Figure 9. Tumor suppressor p53 gene expression validation using Real Time PCR of rat mammary gland in hCG and E+P groups at 42 days after treatment.

transforming growth factor beta 3 (TGF-beta3); antiproliferative growth factor, etc), cell division control (such as p21 Cdk 1 inhibitor, cell division control protein 2 homolog, arrestin, etc), DNA repair genes (such as GADD153, casein kinase delta, etc), lipid metabolism (such as leptin, obese factor, etc), enzymes of metabolic pathways (such as hydroxysteroid 20-alpha dehydrogenase, pancreatic cholesterol esterase, cytosolic acyl-CoA thioester hydrolase 1, etc), tumoral suppressors (such as p53, retinoblastoma protein Rb, etc), cell-surface antigens (such as B7.1, CD-28, BIG-1 protein; neural cell adhesion protein, CD44 antigen; phagocytic glycoprotein I, etc) and functionally uncharacterized proteins (such as opposite gonadotropin-releasing hormone, protein tyrosine phosphatase 4A1 (PTP4A1) and PRL1). We validated the gene expression of p53, p21, GADD153, leptin, CD28 and casein kinase delta 1, by real time PCR, RT-PCR (figures 8 and 9). The gene expression profile of p53, casein kinase 1 delta, TRPM2, TGFbII, p21, activator of apoptosis Harakiri, TNFbeta,GADD153, HMG1 and opposite GnRH are different in the E+P group compared with hCG-treatment and pregnancy groups. These data indicate that hCG, as pregnancy, induce early genomic changes that control de progression of the differentiation pathway, and these changes are permanently imprinted in the mammary gland. In addition we observed that the genomic signature induced by hCG is different from the one induced by the combination of the hormones E+P, this may explain that the protection induced by these hormones does not have a long lasting effect in the rat mammary gland by shifting the Stem cell 1 to Stem cell 2 (figure 1).

6.2. Evidence for a Stem cell 2 in the human breast

The advances of the human genome project and the availability of new tools for genomic analysis, such as cDNA array, tissue array, laser capture microdissection

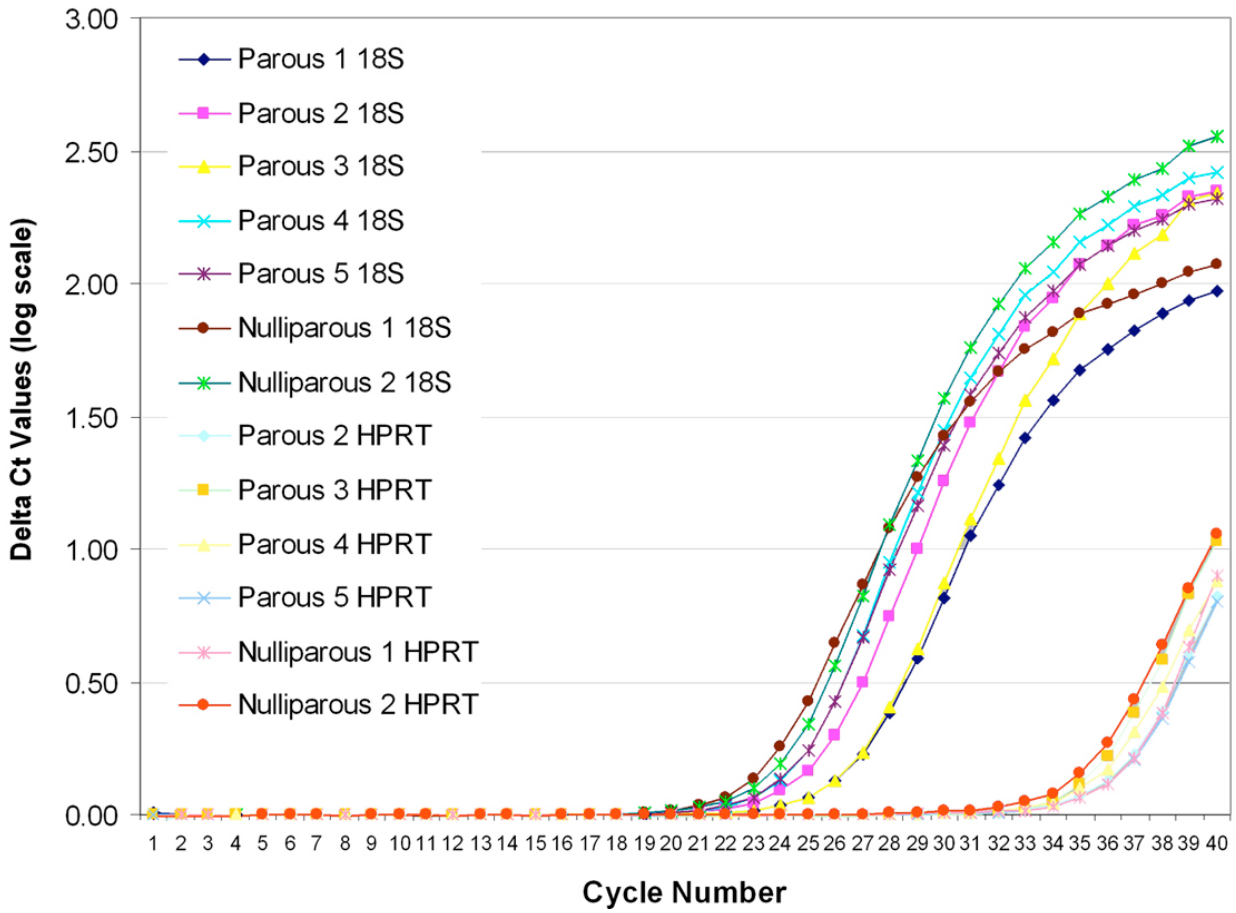


Figure 10. Amplified RNA quality controls. In order to check the quality and the integrity of the amplified RNA, we used Real Time PCR assay to amplify the housekeeping gene 18S and HPRT. The figure shows the amplification curves of the 18S and HPRT for the epithelium of lobule 1 in nulliparous and parous women.

(LCM), and bioinformatics techniques have allowed us to determine whether there are clusters of genes that are differentially expressed in populations that differ in their breast cancer risk. Furthermore, those clusters of genes whose expression may be affected by early pregnancy and that can be proven to be functionally relevant in protecting the breast from cancer development could serve as markers for evaluating cancer risk in large populations. We have demonstrated this by comparing the genomic profile of the epithelium and the stroma of normal breast tissues obtained from reduction mammoplasty specimens from three postmenopausal nulliparous and five postmenopausal parous women.

The epithelium and the stroma were separately dissected using laser capture microdissection (LCM) and the RNA of each compartment and each sample was isolated, amplified using PCR methodology oligo-dT7-based methodology. The quality of the amplified RNA after two rounds of amplification, using the bioanalyzer (Agilent Technologies, CA), and the integrity of this RNA was checked by the expression of two housekeeping genes by real time PCR (figure 10). Fluorescent probe was synthesized by triplicate in order to hybridize to cDNA glass-microarrays containing 40,000 genes, placing in the green channel (Cy3) the human reference RNA and in the red channel (Cy5) the breast tissue samples. The cDNA microarray hybridization was checked using the control negative spots printed in the microarrays, also we checked the hybridization in both the red and green channels, measuring the total number of genes expressed in all the microarrays. After wash, the cDNA microarrays were scanned using the Affymetrix 428 fluorescent scanner (MWG Biotechnology, INC). The image of the cDNA microarrays was analyzed using the ImaGene version 5.5 (Biodiscovery, CA). Data were analyzed employing the GeneSight software version 4.2 (Biodiscovery, CA) after Lowess normalization eliminating all the outlier spots with Flags 1 to 5. Using the Confidence analysis with 99% level we select only the genes differentially expressed between same tissue compartments in each group, selecting a cut off of ± 2.0 log up or down respect to the reference ($p<0.05$). Our analysis revealed that the breast of nulliparous and parous women contained 4,828 epithelial and 4,055 stromal genes that were commonly expressed. In the nulliparous breast there were 37 epithelial and 50 stromal genes that were overexpressed and significantly different ($p<0.05$) from 164 epithelial and 66 stromal genes that were

Table 4. Genes differentially expressed in the Stem Cell 2 of the lobules type 1 of the parous women in comparison to those expressed in the Stem Cell 1 of the nulliparous women

Gene ID	Gene name	Symbol	parous	nulliparous	P value	Function
AA926649	Excision repair cross-complementing rodent repair deficiency, complementation group 2 (xeroderma pigmentosum D)	ERCC2	1.62 ± 0.13	0.94 ± 0.32	0.027	DNA repair
AI076461	RAD51-like 1 (S. cerevisiae)	RAD51 L1	2.01 ± 0.06	0.79 ± 0.38	0.1917	DNA repair
AA907277	Excision repair cross-complementing rodent repair deficiency, complementation group 6	PGBD3	1.67 ± 0.41	0.6 ± 0.49	0.044	DNA repair
AA708793	TP53 activated protein 1		.-0.87 ± 0.24	1.77 ± 0.1	0.0001	DNA repair
AI027434	Homo sapiens, Similar to NBR2, clone MGC:5031, mRNA, complete cds	LOC513 26	1.60 ± 0.43	0.83 ± 0.18	0.046	DNA repair
AA046713	ribosomal protein S3	RPS3	1.74 ± 0.11	0.77 ± 0.31	0.1458	DNA repair
AI168124	likely ortholog of mouse putative IKK regulator SIMPL		1.67 ± 0.15	0.98 ± 0.31	0.027	Programmed cell death
AI565203	BCL2-associated X protein	BAX	1.91 ± 0.15	0.95 ± 0.13	0.0011	Programmed cell death
AI049803	LIM domain binding 2	LDB2	1.82 ± 0.11	0.92 ± 0.27	0.0059	Transcription
N68754	heart and neural crest derivatives expressed 2		0.87 ± 0.36	1.52 ± 0.02	0.0003	Transcription
AI122680	Bromodomain adjacent to zinc finger domain, 2A	BAZ2A	1.69 ± 0.38	0.90 ± 0.16	0.03	Transcription
AI066778	Zinc finger CCCH type domain containing 5	ZC3HD C5	1.56 ± 0.08	0.83 ± 0.16	0.0023	Transcription
AI636025	zinc finger protein and BTB domain containing 11	ZBTB11	1.64 ± 0.22	0.66 ± 0.22	0.0052	Transcription
AI292221	BarH-like homeobox 1	BARX1	1.57 ± 0.26	0.62 ± 0.3	0.015	Transcription
AI056507	SOX2 overlapping transcript (non-coding RNA)	SOX2OT	1.54 ± 0.07	0.6 ± 0.26	0.0036	Transcription
AA703557	BTB and CNC homology 1, basic leucine zipper transcription factor 2		0.93 ± 0.19	1.83 ± 0.28	0.01	Transcription
AI015522	Ring finger protein 146	RNF146	1.67 ± 0.12	0.73 ± 0.09	0.1309	Transcription

overexpressed in the parous breast. We identified sets of genes that were differentially expressed in the epithelial cells of the parous breast that could be considered their genomic signature. Among the known genes that are differentially expressed between the nulliparous and parous epithelial breast tissue we have separated them according to some putative functional groups (table 4). We also found genes transcripts related to: signal transduction pathway, transport and cell trafficking, cell adhesion functions, to protein synthesis and degradation functions, and to general or undetermined cell functions. There are 107 EST sequences and 8 sequences of gene transcripts differentially expressed among the two groups under study.

In the interlobular stroma there is less number of known genes differentially expressed. The parous breast stroma contains 17 upregulated genes; among them are: neuropilin 2; chaperonin containing TCP1, subunit 6B (zeta 2); integrin, alpha M (complement component receptor 3, alpha; also known as CD11b (p170); macrophage antigen alpha polypeptide), Thy-1 cell surface antigen; and jun B proto-oncogene. There are 49 and 44 ESTs differentially expressed in the parous and nulliparous stroma of the lobules type 1 respectively. We have selected 12 genes for the validation of the cDNA microarray employing real time RT-PCR. The genes selected were: fatty acid binding protein 6, BCL2-associated X protein, leukemia inhibitory factor receptor, RAD51-like 1, neuroligin 3, BarH-like homeobox 1, somatostatin receptor 1, excision repair cross-complementing rodent repair deficiency, p300/CBP-associated factor, SRY (sex determining region Y)-box 30, ubiquitin-conjugating enzyme E2D3 and cryptic gene. Our data led us to conclude that the breast epithelium of parous postmenopausal women contains a genomic signature significantly different from that of the nulliparous breast as a reflection of the shifting from a progenitor or Stem cell 1 to a progenitor or Stem cell 2 (figure 1).

7. UNIFYING CONCEPTS

Breast cancer originates in undifferentiated terminal structures of the mammary gland. The terminal ducts of the Lob 1 of the human female breast, which are the sites of origin of ductal carcinomas, are at their peak of cell replication during early adulthood, a period during which the breast is more susceptible to carcinogenesis. The susceptibility of Lob 1 to undergo neoplastic transformation has been confirmed by *in vitro* studies, which have shown that this structure has the highest proliferative activity and rate of carcinogen binding to the DNA (14). More importantly, when treated with carcinogens *in vitro* its epithelial cells express phenotypes indicative of cell transformation (1). These studies indicate that in the human breast the target cell of carcinogens is found in a specific compartment whose characteristics are the determinant factors in the initiation event. These target cells will become the stem cells (Stem cell 1) of the neoplastic event, depending upon: (a) topographic location within the mammary gland tree, (b) age at exposure to a known or putative genotoxic agent, and (c) reproductive history of the host. The higher incidence of breast cancer observed in nulliparous women supports this concept, because it parallels the higher cancer incidence elicited by carcinogens in rodents when exposure occurs at a young age. In addition, it has been shown that early parity is associated with a pronounced decrease in the risk of breast

cancer an additional live births confer greater risk reduction (102). Thus, the protection afforded by early full-term pregnancy in women could be explained by the higher degree of differentiation of the mammary gland at the time at which an etiologic agent or agents act. Even though differentiation significantly reduces cell proliferation in the mammary gland, the mammary epithelium remains capable of responding with proliferation to given stimuli, such as a new pregnancy. Under these circumstances, however, the cells that are stimulated to proliferate are from structures that have already been primed by the first cycle of differentiation, becoming the Stem cell 2 that are able to metabolize carcinogen(s) and repair DNA damage more efficiently than the cells of the virginal gland, thus becoming less susceptible to carcinogenesis, as it has been demonstrated in the rodent experimental system. However, if the shift of Stem cell 1 to Stem cell 2 has not been completed, a carcinogenic stimulus powerful enough may overburden the system, thereby initiating successfully a neoplastic process. Such conditions may explain the small fraction of women developing breast cancer after an early first full-term pregnancy, i.e. because they have not had a full completion of the first cycle of differentiation. The findings that differentiation is a powerful inhibitor of cancer initiation provide a strong rationale for pursuing the identification of the stem cells susceptible to carcinogenesis and of the genes that control this process. The knowledge gained will provide novel tools for developing rational strategies for breast cancer prevention.

8. ACKNOWLEDGEMENTS

This study was supported by Grant RO1-CA093599 awarded by the NIH, PHS, USA.

9. REFERENCES

1. J Russo & I H Russo: Development of human mammary gland. In: The Mammary Gland Development, Regulation and Function. Eds: Neville M C, Daniel C. Plenum Publishing Corp., New York. 67-93 (1987)
2. Russo J, R. Rivera & I. H. Russo: Influence of age and parity on the development of the human breast. *Breast Cancer Res Treat* 23, 211-218 (1992)
3. Russo J & I. H. Russo: Toward a physiological approach to breast cancer prevention. *Cancer Epidemiol Biomarkers Prev* 3, 353-364 (1994)
4. Russo J, B. A. Gusterson, A. E. Rogers, I. H. Russo, S. R. Wellings & M. J. van Zwieten: Comparative study of human and rat mammary tumorigenesis. *Lab Invest* 62, 244-278 (1990)
5. Wellings S. R, H. M. Jensen & R. G. Marcum: An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions. *J Natl Cancer Inst* 55, 231-273 (1975)
6. Russo J, A. L. Romero & I. H. Russo: Architectural pattern of the normal and cancerous breast under the influence of parity. *Cancer Epidemiol Biomarkers Prev* 3, 219-224 (1994)
7. Russo J, H. Lynch & I. H. Russo: Mammary gland architecture as a determining factor in the susceptibility of the human breast to cancer. *Breast J* 7, 278-291 (2001)
8. Russo J & I. H. Russo: Role of differentiation in the pathogenesis and prevention of breast cancer. *J of Endocrinology, Endocrine-Related Cancer* 4, 7-21 (1997)
9. Deome K. B, L. J. Faulkin, Jr., H. A. Bern & P. B. Blair: Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res* 19, 515-520 (1959)
10. Kordon E. C & G. H. Smith: An entire functional mammary gland may comprise the progeny from a single cell. *Development* 125, 1921-1930 (1998)
11. Russo J, J. Saby, W. Isenberg & I. H. Russo: Pathogenesis of mammary carcinomas induced in rats by 7,12-dimethylbenz(a)anthracene. *J Natl Cancer Inst* 59, 436-445 (1977)
12. Russo J, W. Isenberg, M. Ireland & I. H. Russo: Ultrastructural changes in the mammary epithelial cell population during neoplastic development induced by a chemical carcinogen. In: Electron Microscopy Society of America Proceedings. Vol. 34. Electron Microscopy Society of America. 250-251 (1976)
13. Russo J, L. Tait & I. H. Russo: Susceptibility of the mammary gland to carcinogenesis. III. The cell of origin of rat mammary carcinoma. *Am J Pathol* 113, 50-66 (1983)
14. Russo J, L. K. Tay & I. H. Russo: Differentiation of the mammary gland and susceptibility to carcinogenesis. *Breast Cancer Res Treat* 2, 5-73 (1982)
15. Bennett D. C, L. A. Peachey, H. Durbin & P. S. Rudland: A possible mammary stem cell line. *Cell* 15, 283-298 (1978)
16. Rudland P. S, D. C. Bennett & M. J. Warburton: Isolation and characterization of epithelial stem-cell cell lines from the rat mammary gland. *Br J Cancer* 41, 666-668 (1980)
17. Chepko G & G. H. Smith: Three division-competent, structurally-distinct cell populations contribute to murine mammary epithelial renewal. *Tissue Cell* 29, 239-253 (1997)
18. Smith G. H, T. Mehrel & D. R. Roop: Differential keratin gene expression in developing, differentiating, preneoplastic, and neoplastic mouse mammary epithelium. *Cell Growth Differ* 1, 161-170 (1990)
19. Stingl J, C. J. Eaves, U. Kuusk & J. T. Emerman: Phenotypic and functional characterization in vitro of a multipotent epithelial cell present in the normal adult human breast. *Differentiation* 63, 201-213 (1998)
20. Stingl J, C. J. Eaves, I. Zandieh & J. T. Emerman: Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. *Breast Cancer Res Treat* 67, 93-109 (2001)
21. Hebbard L, A. Steffen, V. Zawadzki, C. Fieber, N. Howells, J. Moll, H. Ponta, M. Hofmann & J. Sleeman: CD44 expression and regulation during mammary gland development and function. *J Cell Sci* 113 (Pt 14), 2619-2630 (2000)
22. Kenney N. J, G. H. Smith, E. Lawrence, J. C. Barrett & D. S. Salomon: Identification of stem cell units in the terminal end bud and duct of the mouse mammary Gland. *J Biomed Biotechnol* 1, 133-143 (2001)
23. Spangrude G. J, Y. Aihara, I. L. Weissman & J. Klein: The stem cell antigens Sca-1 and Sca-2 subdivide thymic and peripheral T lymphocytes into unique subsets. *J Immunol* 141, 3697-3707 (1988)

24. Welm B. E, S. B. Tepera, T. Venezia, T. A. Graubert, J. M. Rosen & M. A. Goodell: Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. *Dev Biol* 245, 42-56 (2002)
25. Zhou S, J. D. Schuetz, K. D. Bunting, A. M. Colapietro, J. Sampath, J. J. Morris, I. Lagutina, G. C. Grosveld, M. Osawa, H. Nakauchi & B. P. Sorrentino: The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 7, 1028-1034 (2001)
26. Alvi A. J, H. Clayton, C. Joshi, T. Enver, A. Ashworth, M. M. Vivanco, T. C. Dale & M. J. Smalley: Functional and molecular characterisation of mammary side population cells. *Breast Cancer Res* 5, R1-8 (2003)
27. Gudjonsson T, R. Villadsen, H. L. Nielsen, L. Ronnov-Jessen, M. J. Bissell & O. W. Petersen: Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. *Genes Dev* 16, 693-706 (2002)
28. Dontu G, W. M. Abdallah, J. M. Foley, K. W. Jackson, M. F. Clarke, M. J. Kawamura & M. S. Wicha: In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* 17, 1253-1270 (2003)
29. Jia Y, C. Qi, Z. Zhang, Y. T. Zhu, S. M. Rao & Y. J. Zhu: Peroxisome proliferator-activated receptor-binding protein null mutation results in defective mammary gland development. *J Biol Chem* 280, 10766-10773 (2005)
30. Couse J. F & K. S. Korach: Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* 20, 358-417 (1999)
31. Russo J, X. Ao, C. Grill & I. H. Russo: Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. *Breast Cancer Res Treat* 53, 217-227 (1999)
32. Petersen O. W, T. Gudjonsson, R. Villadsen, M. J. Bissell & L. Ronnov-Jessen: Epithelial progenitor cell lines as models of normal breast morphogenesis and neoplasia. *Cell Prolif* 36 Suppl 1, 33-44 (2003)
33. Clarke R. B, E. Anderson, A. Howell & C. S. Potten: Regulation of human breast epithelial stem cells. *Cell Prolif* 36 Suppl 1, 45-58 (2003)
34. Clarke R. B, K. Spence, E. Anderson, A. Howell, H. Okano & C. S. Potten: A putative human breast stem cell population is enriched for steroid receptor-positive cells. *Dev Biol* 277, 443-456 (2005)
35. Clayton H, I. Titley & M. Vivanco: Growth and differentiation of progenitor/stem cells derived from the human mammary gland. *Exp Cell Res* 297, 444-460 (2004)
36. Whitehead R. H, S. J. Quirk, A. A. Vitali, J. W. Funder, R. L. Sutherland & L. C. Murphy: A new human breast carcinoma cell line (PMC42) with stem cell characteristics. III. Hormone receptor status and responsiveness. *J Natl Cancer Inst* 73, 643-648 (1984)
37. Brannvall K, L. Korhonen & D. Lindholm: Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. *Mol Cell Neurosci* 21, 512-520 (2002)
38. Miura T, C. Miura, T. Ohta, M. R. Nader, T. Todo & K. Yamauchi: Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. *Biochem Biophys Res Commun* 264, 230-234 (1999)
39. Kuiper G. G, E. Enmark, M. Pelto-Huikko, S. Nilsson & J. A. Gustafsson: Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* 93, 5925-5930 (1996)
40. Ogawa S, S. Inoue, T. Watanabe, H. Hiroi, A. Orimo, T. Hosoi, Y. Ouchi & M. Muramatsu: The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. *Biochem Biophys Res Commun* 243, 122-126 (1998)
41. Balfe P. J, A. H. McCann, H. M. Welch & M. J. Kerin: Estrogen receptor beta and breast cancer. *Eur J Surg Oncol* 30, 1043-1050 (2004)
42. Skliris G. P, K. Munot, S. M. Bell, P. J. Carder, S. Lane, K. Horgan, M. R. Lansdown, A. T. Parkes, A. M. Hanby, A. F. Markham & V. Speirs: Reduced expression of oestrogen receptor beta in invasive breast cancer and its re-expression using DNA methyl transferase inhibitors in a cell line model. *J Pathol* 201, 213-220 (2003)
43. Paruthiyil S, H. Parmar, V. Kerekatte, G. R. Cunha, G. L. Firestone & D. C. Leitman: Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res* 64, 423-428 (2004)
44. Shaaban A. M, P. A. O'Neill, M. P. Davies, R. Sibson, C. R. West, P. H. Smith & C. S. Foster: Declining estrogen receptor-beta expression defines malignant progression of human breast neoplasia. *Am J Surg Pathol* 27, 1502-1512 (2003)
45. Krege J. H, J. B. Hodgin, J. F. Couse, E. Enmark, M. Warner, J. F. Mahler, M. Sar, K. S. Korach, J. A. Gustafsson & O. Smithies: Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci U S A* 95, 15677-15682 (1998)
46. Korach K. S, J. F. Couse, S. W. Curtis, T. F. Washburn, J. Lindzey, K. S. Kimbro, E. M. Eddy, S. Migliaccio, S. M. Snedeker, D. B. Lubahn, D. W. Schomberg & E. P. Smith: Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog Horm Res* 51, 159-186; discussion 186-158 (1996)
47. Weihua Z, S. Makela, L. C. Andersson, S. Salmi, S. Saji, J. I. Webster, E. V. Jensen, S. Nilsson, M. Warner & J. A. Gustafsson: A role for estrogen receptor beta in the regulation of growth of the ventral prostate. *Proc Natl Acad Sci U S A* 98, 6330-6335 (2001)
48. Weihua Z, S. Saji, S. Makinen, G. Cheng, E. V. Jensen, M. Warner & J. A. Gustafsson: Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. *Proc Natl Acad Sci U S A* 97, 5936-5941 (2000)
49. Weihua Z, M. Warner & J. A. Gustafsson: Estrogen receptor beta in the prostate. *Mol Cell Endocrinol* 193, 1-5 (2002)
50. Jensen E. V, G. Cheng, C. Palmieri, S. Saji, S. Makela, S. Van Noorden, T. Wahlstrom, M. Warner, R. C. Coombes & J. A. Gustafsson: Estrogen receptors and proliferation markers in primary and recurrent breast cancer. *Proc Natl Acad Sci U S A* 98, 15197-15202 (2001)
51. Clarke R. B, A. Howell & E. Anderson: 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, 121-133 (1997)
52. Clarke R. B, A. Howell, C. S. Potten & E. Anderson: Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Res* 57, 4987-4991 (1997)
53. Tonetti D. A, R. Rubenstein, M. DeLeon, H. Zhao, S. G. Pappas, D. J. Bentrem, B. Chen, A. Constantinou & V. Craig Jordan: Stable transfection of an estrogen receptor beta cDNA isoform into MDA-MB-231 breast cancer cells. *J Steroid Biochem Mol Biol* 87, 47-55 (2003)
54. Gustafsson J. A & M. Warner: Estrogen receptor beta in the breast: role in estrogen responsiveness and development of breast cancer. *J Steroid Biochem Mol Biol* 74, 245-248 (2000)
55. Saji S, E. V. Jensen, S. Nilsson, T. Rylander, M. Warner & J. A. Gustafsson: Estrogen receptors alpha and beta in the rodent mammary gland. *Proc Natl Acad Sci U S A* 97, 337-342 (2000)
56. Saji S, H. Sakaguchi, S. Andersson, M. Warner & J. Gustafsson: Quantitative analysis of estrogen receptor proteins in rat mammary gland. *Endocrinology* 142, 3177-3186 (2001)
57. Foley E. F, A. A. Jazaeri, M. A. Shupnik, O. Jazaeri & L. W. Rice: Selective loss of estrogen receptor beta in malignant human colon. *Cancer Res* 60, 245-248 (2000)
58. Iwao K, Y. Miyoshi, C. Egawa, N. Ikeda & S. Noguchi: Quantitative analysis of estrogen receptor-beta mRNA and its variants in human breast cancers. *Int J Cancer* 88, 733-736 (2000)
59. Roger P, M. E. Sahla, S. Makela, J. A. Gustafsson, P. Baldet & H. Rochefort: Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. *Cancer Res* 61, 2537-2541 (2001)
60. Rutherford T, W. D. Brown, E. Sapi, S. Aschkenazi, A. Munoz & G. Mor: Absence of estrogen receptor-beta expression in metastatic ovarian cancer. *Obstet Gynecol* 96, 417-421 (2000)
61. Leygue E, H. Dotzlaw, P. H. Watson & L. C. Murphy: Altered estrogen receptor alpha and beta messenger RNA expression during human breast tumorigenesis. *Cancer Res* 58, 3197-3201 (1998)
62. Speirs V, A. T. Parkes, M. J. Kerin, D. S. Walton, P. J. Carleton, J. N. Fox & S. L. Atkin: Coexpression of estrogen receptor alpha and beta: poor prognostic factors in human breast cancer? *Cancer Res* 59, 525-528 (1999)
63. Speirs V, C. Malone, D. S. Walton, M. J. Kerin & S. L. Atkin: Increased expression of estrogen receptor beta mRNA in tamoxifen-resistant breast cancer patients. *Cancer Res* 59, 5421-5424 (1999)
64. Speirs V, I. P. Adams, D. S. Walton & S. L. Atkin: Identification of wild-type and exon 5 deletion variants of estrogen receptor beta in normal human mammary gland. *J Clin Endocrinol Metab* 85, 1601-1605 (2000)
65. Speirs V & M. J. Kerin: Prognostic significance of oestrogen receptor beta in breast cancer. *Br J Surg* 87, 405-409 (2000)
66. Swain S. M: Tamoxifen for patients with estrogen receptor-negative breast cancer. *J Clin Oncol* 19, 93S-97S (2001)
67. Chang H. G, S. J. Kim, K. W. Chung, D. Y. Noh, Y. Kwon, E. S. Lee & H. S. Kang: Tamoxifen-resistant breast cancers show less frequent methylation of the estrogen receptor beta but not the estrogen receptor alpha gene. *J Mol Med* 83, 132-139 (2005)
68. Shim G. J, L. Wang, S. Andersson, N. Nagy, L. L. Kis, Q. Zhang, S. Makela, M. Warner & J. A. Gustafsson: Disruption of the estrogen receptor beta gene in mice causes myeloproliferative disease resembling chronic myeloid leukemia with lymphoid blast crisis. *Proc Natl Acad Sci U S A* 100, 6694-6699 (2003)
69. Imamov O, A. Morani, G. J. Shim, Y. Omoto, C. Thulin-Andersson, M. Warner & J. A. Gustafsson: Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate. *Proc Natl Acad Sci U S A* 101, 9375-9380 (2004)
70. Pais V, I. Leav, K. M. Lau, Z. Jiang & S. M. Ho: Estrogen receptor-beta expression in human testicular germ cell tumors. *Clin Cancer Res* 9, 4475-4482 (2003)
71. Palmieri C, S. Saji, H. Sakaguchi, G. Cheng, A. Sunters, M. J. O'Hare, M. Warner, J. A. Gustafsson, R. C. Coombes & E. W. Lam: The expression of oestrogen receptor (ER)-beta and its variants, but not ERalpha, in adult human mammary fibroblasts. *J Mol Endocrinol* 33, 35-50 (2004)
72. Russo J, M. Hasan Lareef, G. Balogh, S. Guo & I. H. Russo: Estrogen and its metabolites are carcinogenic agents in human breast epithelial cells. *J Steroid Biochem Mol Biol* 87, 1-25 (2003)
73. Hu Y. F, K. M. Lau, S. M. Ho & J. Russo: Increased expression of estrogen receptor beta in chemically transformed human breast epithelial cells. *Int J Oncol* 12, 1225-1228 (1998)
74. Russo J, M. H. Lareef, Q. Tahin, Y. F. Hu, C. Slater, X. Ao & I. H. Russo: 17Beta-estradiol is carcinogenic in human breast epithelial cells. *J Steroid Biochem Mol Biol* 80, 149-162 (2002)
75. Lareef M. H, J. Garber, P. A. Russo, I. H. Russo, R. Heulings & J. Russo: The estrogen antagonist ICI-182-780 does not inhibit the transformation phenotypes induced by 17-beta-estradiol and 4-OH estradiol in human breast epithelial cells. *Int J Oncol* 26, 423-429 (2005)
76. Fernandez S. V, I. H. Russo, M. Lareef, B. Balsara & J. Russo: Comparative genomic hybridization of human breast epithelial cells transformed by estrogen and its metabolites. *Int J Oncol* 26, 691-695 (2005)
77. Kehrl J. H: Hematopoietic lineage commitment: role of transcription factors. *Stem Cells* 13, 223-241 (1995)
78. Shivdasani R. A & S. H. Orkin: Erythropoiesis and globin gene expression in mice lacking the transcription factor NF-E2. *Proc Natl Acad Sci U S A* 92, 8690-8694 (1995)
79. Tsai S, S. Bartelmez, E. Sitnicka & S. Collins: Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development. *Genes Dev* 8, 2831-2841 (1994)
80. Briggs J. A, G. R. Burrus, B. D. Stickney & R. C. Briggs: Cloning and expression of the human myeloid cell nuclear differentiation antigen: regulation by interferon alpha. *J Cell Biochem* 49, 82-92 (1992)
81. Briggs R, L. Dworkin, J. Briggs, E. Dessypris, J. Stein, G. Stein & J. Lian: Interferon alpha selectively affects expression of the human myeloid cell nuclear

differentiation antigen in late stage cells in the monocytic but not the granulocytic lineage. *J Cell Biochem* 54, 198-206 (1994)
82. Briggs R. C, J. A. Briggs, J. Ozer, L. Sealy, L. L. Dworkin, S. F. Kingsmore, M. F. Seldin, G. P. Kaur, R. S. Athwal & E. N. Dessypris: The human myeloid cell nuclear differentiation antigen gene is one of at least two related interferon-inducible genes located on chromosome 1q that are expressed specifically in hematopoietic cells. *Blood* 83, 2153-2162 (1994)
83. Briggs R. C, W. Y. Kao, L. L. Dworkin, J. A. Briggs, E. N. Dessypris & J. Clark: Regulation and specificity of MNDA expression in monocytes, macrophages, and leukemia/B lymphoma cell lines. *J Cell Biochem* 56, 559-567 (1994)
84. Hamada T, K. Tashiro, H. Tada, J. Inazawa, M. Shirozu, K. Shibahara, T. Nakamura, N. Martina, T. Nakano & T. Honjo: Isolation and characterization of a novel secretory protein, stromal cell-derived factor-2 (SDF-2) using the signal sequence trap method. *Gene* 176, 211-214 (1996)
85. Lindahl T, M. S. Satoh, G. G. Poirier & A. Klungland: Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. *Trends Biochem Sci* 20, 405-411 (1995)
86. Menissier-de Murcia J, M. Molinete, G. Gradwohl, F. Simonin & G. de Murcia: Zinc-binding domain of poly(ADP-ribose)polymerase participates in the recognition of single strand breaks on DNA. *J Mol Biol* 210, 229-233 (1989)
87. Boulikas T: Relation between carcinogenesis, chromatin structure and poly(ADP-ribosylation) (review). *Anticancer Res* 11, 489-527 (1991)
88. Althaus F. R, L. Hofferer, H. E. Kleczkowska, M. Malanga, H. Naegeli, P. L. Panzeter & C. A. Realini: Histone shuttling by poly ADP-ribosylation. *Mol Cell Biochem* 138, 53-59 (1994)
89. Malanga M, J. M. Pleschke, H. E. Kleczkowska & F. R. Althaus: Poly(ADP-ribose) binds to specific domains of p53 and alters its DNA binding functions. *J Biol Chem* 273, 11839-11843 (1998)
90. Pleschke J. M, H. E. Kleczkowska, M. Strohm & F. R. Althaus: Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. *J Biol Chem* 275, 40974-40980 (2000)
91. Zahradka P & K. Ebisuzaki: A shuttle mechanism for DNA-protein interactions. The regulation of poly(ADP-ribose) polymerase. *Eur J Biochem* 127, 579-585 (1982)
92. Le Rhun Y, J. B. Kirkland & G. M. Shah: Cellular responses to DNA damage in the absence of Poly(ADP-ribose) polymerase. *Biochem Biophys Res Commun* 245, 1-10 (1998)
93. Cosi C & M. Marien: Implication of poly (ADP-ribose) polymerase (PARP) in neurodegeneration and brain energy metabolism. Decreases in mouse brain NAD+ and ATP caused by MPTP are prevented by the PARP inhibitor benzamide. *Ann N Y Acad Sci* 890, 227-239 (1999)
94. Ha H. C & S. H. Snyder: Poly(ADP-ribose) polymerase-1 in the nervous system. *Neurobiol Dis* 7, 225-239 (2000)
95. Pieper A. A, A. Verma, J. Zhang & S. H. Snyder: Poly (ADP-ribose) polymerase, nitric oxide and cell death. *Trends Pharmacol Sci* 20, 171-181 (1999)
96. Park Y. C, H. Ye, C. Hsia, D. Segal, R. L. Rich, H. C. Liou, D. G. Myszka & H. Wu: A novel mechanism of TRAF signaling revealed by structural and functional analyses of the TRADD-TRAF2 interaction. *Cell* 101, 777-787 (2000)
97. Kanai F, P. A. Marignani, D. Sarbassova, R. Yagi, R. A. Hall, M. Donowitz, A. Hisaminato, T. Fujiwara, Y. Ito, L. C. Cantley & M. B. Yaffe: TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. *Embo J* 19, 6778-6791 (2000)
98. Sudol M: Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product. *Oncogene* 9, 2145-2152 (1994)
99. Bedford M. T, D. Sarbassova, J. Xu, P. Leder & M. B. Yaffe: A novel pro-Arg motif recognized by WW domains. *J Biol Chem* 275, 10359-10369 (2000)
100. Cooper H. M, R. N. Tamura & V. Quaranta: The major laminin receptor of mouse embryonic stem cells is a novel isoform of the alpha 6 beta 1 integrin. *J Cell Biol* 115, 843-850 (1991)
101. MacMahon B, P. Cole, T. M. Lin, C. R. Lowe, A. P. Mirra, B. Ravnihar, E. J. Salber, V. G. Valaoras & S. Yuasa: Age at first birth and breast cancer risk. *Bull World Health Organ* 43, 209-221 (1970)
102. Lambe M, C. C. Hsieh, H. W. Chan, A. Ekbom, D. Trichopoulos & H. O. Adami: Parity, age at first and last birth, and risk of breast cancer: a population-based study in Sweden. *Breast Cancer Res Treat* 38, 305-311 (1996)
103. Kelsey J. L, M. D. Gammon & E. M. John: Reproductive factors and breast cancer. *Epidemiol Rev* 15, 36-47 (1993)
104. R C Moon: Influence of pregnancy and lactation on experimental mammary carcinogenesis. In: Banbury Report 8 Hormones and Breast Cancer. Eds: Pike M C, Siiteri P K, Welsch C W. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 353–364 (1981)
105. Sinha D. K, J. E. Pazik & T. L. Dao: Prevention of mammary carcinogenesis in rats by pregnancy: effect of full-term and interrupted pregnancy. *Br J Cancer* 57, 390-394 (1988)
106. Yang J, K. Yoshizawa, S. Nandi & A. Tsubura: Protective effects of pregnancy and lactation against N-methyl-N-nitrosourea-induced mammary carcinomas in female Lewis rats. *Carcinogenesis* 20, 623-628 (1999)
107. Welsch C. W: Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. *Cancer Res* 45, 3415-3443 (1985)
108. Russo J & I. H. Russo: Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. *Cancer Res* 40, 2677-2687 (1980)
109. Swanson S. M, L. M. Whitaker, C. R. Stockard, R. B. Myers, D. Oelschlager, W. E. Grizzle, M. M. Juliana & C. J. Grubbs: Hormone levels and mammary epithelial cell proliferation in rats treated with a regimen of estradiol and progesterone that mimics the preventive effect of pregnancy against mammary cancer. *Anticancer Res* 17, 4639-4645 (1997)
110. Rajkumar L, R. C. Guzman, J. Yang, G. Thordarson, F. Talamantes & S. Nandi: Short-term exposure to pregnancy levels of estrogen prevents mammary

carcinogenesis. *Proc Natl Acad Sci U S A* 98, 11755-11759 (2001)
111. Tay L. K & J. Russo: Formation and removal of 7,12-dimethylbenz[a]anthracene--nucleic acid adducts in rat mammary epithelial cells with different susceptibility to carcinogenesis. *Carcinogenesis* 2, 1327-1333 (1981)
112. Russo I. H, M. Koszalka & J. Russo: Comparative study of the influence of pregnancy and hormonal treatment on mammary carcinogenesis. *Br J Cancer* 64, 481-484 (1991)
113. Thordarson G, E. Jin, R. C. Guzman, S. M. Swanson, S. Nandi & F. Talamantes: Refractoriness to mammary tumorigenesis in parous rats: is it caused by persistent changes in the hormonal environment or permanent biochemical alterations in the mammary epithelia? *Carcinogenesis* 16, 2847-2853 (1995)
114. Wagner K. U, C. A. Boulanger, M. D. Henry, M. Sgagias, L. Hennighausen & G. H. Smith: An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. *Development* 129, 1377-1386 (2002)
115. Henry M. D, A. A. Triplett, K. B. Oh, G. H. Smith & K. U. Wagner: Parity-induced mammary epithelial cells facilitate tumorigenesis in MMTV-neu transgenic mice. *Oncogene* 23, 6980-6985 (2004)
116. Boulanger C. A, K. U. Wagner & G. H. Smith: Parity-induced mouse mammary epithelial cells are pluripotent, self-renewing and sensitive to TGF-beta1 expression. *Oncogene* 24, 552-560 (2005)
117. Trichopoulos D, P. Lagiou & H. O. Adami: Towards an integrated model for breast cancer etiology: the crucial role of the number of mammary tissue-specific stem cells. *Breast Cancer Res* 7, 13-17 (2005)
118. Srivastava P, J. Russo & I. H. Russo: Chorionic gonadotropin inhibits rat mammary carcinogenesis through activation of programmed cell death. *Carcinogenesis* 18, 1799-1808 (1997).
119. Srivastava P, J. Russo & I. H. Russo: Inhibition of rat mammary tumorigenesis by human chorionic gonadotropin associated with increased expression of inhibin. *Mol Carcinog* 26, 10-19 (1999)
120. Medina D & F. S. Kittrell: p53 function is required for hormone-mediated protection of mouse mammary tumorigenesis. *Cancer Res* 63, 6140-6143 (2003)
121. Medina D: Breast cancer: the protective effect of pregnancy. *Clin Cancer Res* 10, 380S-384S (2004)
122. Sivaraman L, O. M. Conneely, D. Medina & B. W. O'Malley: p53 is a potential mediator of pregnancy and hormone-induced resistance to mammary carcinogenesis. *Proc Natl Acad Sci U S A* 98, 12379-12384 (2001)
123. Ginger M. R, M. F. Gonzalez-Rimbau, J. P. Gay & J. M. Rosen: Persistent changes in gene expression induced by estrogen and progesterone in the rat mammary gland. *Mol Endocrinol* 15, 1993-2009 (2001)
124. Ginger M. R & J. M. Rosen: Pregnancy-induced changes in cell-fate in the mammary gland. *Breast Cancer Res* 5, 192-197 (2003)
125. D'Cruz C. M, S. E. Moody, S. R. Master, J. L. Hartman, E. A. Keiper, M. B. Imielinski, J. D. Cox, J. Y. Wang, S. I. Ha, B. A. Keister & L. A. Chodosh: Persistent parity-induced changes in growth factors, TGF-beta3, and differentiation in the rodent mammary gland. *Mol Endocrinol* 16, 2034-2051 (2002)
126. J Russo & I H Russo: Endocrine control of breast development. In: molecular basis of breast cancer: Prevention and treatment. Eds: Russo J, Russo I H Springer-Verlag, Berlin, Heidelberg, New York. 64-67 (2004)
127. Russo I. H & J. Russo: Role of hCG and inhibin in breast cancer. *Int J Cancer* 4, 297-306 (1997)
128. Greenlee R. T, T. Murray, S. Bolden & P. A. Wingo: Cancer statistics, 2000. *CA Cancer J Clin* 50, 7-33 (2000)
129. Russo J, R. Moral, G. A. Balogh, D. Mailo & I. H. Russo: The protective role of pregnancy in breast cancer. *Breast Cancer Res* 7, 131-142 (2005)

Key Words: Stem cell, mammary gland, pregnancy, differentiation, cancer, prevention, Review

Send correspondence to: Jose Russo, MD, Director, Breast Cancer Research Laboratory, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, Tel: 215-728-4782, Fax: 215-728-2180, E-mail: J_russo@fccc.edu

http://www.bioscience.org/current/vol11.htm