Review

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GENOMIC SIGNATURE AND SUSCEPTIBILITY TO BREAST CANCER*

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ABSTRACT

Early parity is associated with a pronounced decrease in the risk of breast cancer, and additional live births reduce the risk even move. The protection afforded by early full-term pregnancy in women can be explained by the higher degree of differentiation of the mammary gland, which eliminates type 1 stem cells and creates a second type of stem cell (stem cell 2) that is able to metabolize carcinogens and repair DNA damage more efficiently than cells of the nulliparous breast. All though differentiation significantly reduces cell proliferation in the mammary gland, the epithelium remains capable of responding to a given stimulus, such as a new pregnancy. Under these circumstances, the cells that are stimulated to proliferate are derived from structures that have already been primed by the first cycle of differentiation. However, if the shift from stem cell 1 to stem cell 2 has not been completed, a sufficiently powerful carcinogenic stimulus may overburden the system, and successfully initiate a neoplastic process. Incomplete differentiation of this type may explain the development of breast cancer after a late first full-term pregnancy. The finding that differentiation is a powerful inhibitor of cancer initiation provides a strong rationale for pursuing the identification of the genes that control this process.

Key words: Breast Cancer, pregnancy, prevention, stem cell

INTRODUCTION

The incidence of breast cancer has gradually increased in the United States and in most Western societies over the last few decades [14]. Although the reasons for this increase are not certain, epidemiological, clinical, and experimental data indicate that the risk of developing breast cancer is strongly dependent on the ovary and on endocrine conditions modulated by ovarian function, such as early menarche, late menopause, and parity [14,19,30,78,79]. Women who give birth to a child when they are younger than 24 years of age exhibit a decrease in their lifetime risk of developing breast cancer, and additional pregnancies increase this protection [25]. The protective effect of full term pregnancy is a well established concept in humans and in rats and mice [18,22,29,30,38,50-52,62,65,66,84,85]. A plausible explanation for the lifetime protective effect of an event occurring so early in life is provided by the biological behavior of breast cancer and by comparative studies with experimental animal models [64].

Epidemiological observations indicate that women who have been irradiated have a higher incidence of breast cancer, but only in those in whom exposure occurred at a young age, particularly before 19 years of age, and not in those who were irradiated at older ages or after pregnancy [16]. In rodents, the maximal incidence of 7,12-dimethylbenz (a) anthracene (DMBA) - induced mammary cancer occurs when the carcinogen is administered to young, virgin, cycling rats, whereas the same carcinogen fails to induce tumors when given to rats after a full term pregnancy [38,65]. The high susceptibility of the young virgin rat mammary gland to develop malignancies is the result of the interaction of the carcinogen with rapidly dividing cells present in terminal end buds (TEBs), undifferentiated structures that represent the most active growth foci of the mammary parenchyma. Cancer initiation in this model is the result of a combination of factors, including a high rate of carcinogen binding to epithelial DNA, fixation of transformation, formation of polar metabolites, and deficient DNA repair [38,39,50,53,65,75,76].

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*Dedicated to Professor Benedicto de Campos Vidal on the occasion of his 75th birthday.

Although no specific etiologic agent for breast cancer has been identified, there are close similarities between the pathogenesis of this disease in women and that induced in rodents by chemical carcinogens. Ductal carcinoma, the most common breast malignancy, originates in type 1 lobules (Lob1), also called the terminal ductal lobular unit (TDLU), an undifferentiated structure that is considered to be equivalent to the TEB, the site of origin of ductal carcinomas in rodents [41,42,56,58,60,82]. Furthermore, *in vitro*, the same chemical carcinogens that induce mammary cancer in experimental animals [41,47,56,58,60] can transform human breast epithelial cells. These observations suggest that if the human breast is exposed to a carcinogenic insult, the Lob1 or TDLU would be the structure affected and the site of initiation of a malignancy [42,82]. The genomic damage caused by radiation, environmental carcinogens, hormonal imbalances, and/or other still unidentified factors, either alone or in combination with a genetic predisposition, may cause breast cancer in women. For cancer to develop, however, this multifactorial combination must occur during the window of high susceptibility that is encompassed between menarche and the first full-term pregnancy (FFTP), even though the damaged cells would be clinically detectable as a neoplasm only after several years of progressing along the various stages of transformation [41,47,49]. Hence, an initial mutagenic event occurring early in life, such as before or during puberty, in the primitive ductal structures of the breast can multiply during the process of branching and ductal elongation during puberty and sexual maturation.

The breast as a developmental organ

The breast tissue of normally cycling non pregnant adult women contains three types of lobules, namely, type 1 lobules (Lob 1) and the more developed type 2 (Lob 2) and type 3 (Lob 3) lobules (Fig. 1A) [47-49,53,61]. The lobular composition of the breast of sexually mature women is determined by numerous endogenous and exogenous factors, principally age, and hence, the number and regularity of menstrual cycles, as well as endocrine imbalances, the use of exogenous hormones, environmental exposures that could act as endocrine disruptors, and the physiological status of pregnancy.

In nulliparous women, the breast contains a large number of undifferentiated structures such as terminal ducts and Lob 1 (Fig. 1B). The percentage of Lob 1 remains almost constant throughout the lifespan of nulliparous women. Lob 2 are present in moderate numbers during the early reproductive years while the number of Lob 1 remains significantly higher, and Lob 3 are almost totally absent, suggesting that a certain percentage of Lob 1 may have progressed to Lob 2, but that very few Lob 2 progressed to Lob 3 (Figure 1B) [48,61].

In parous women, on the other hand, a history of one or more full-term pregnancies between the ages of 14 to 20 years correlates with a significant increase in the number of Lob 3. These lobules persist as the predominant structure until a woman reaches the age of 40. Their percentage decreases after the fourth decade of life, through involution to Lob1 [36,37,54,61].

The breast attains its maximum development during pregnancy. This development occurs in two major phases: an early stage, characterized by ductal lengthening and profuse branching, that is sustained by active cell proliferation at the distal end of the ductal tree, with a rapid increase in the number of newly formed ductules that results in the progression of Lob 2 to Lob 3 [54,61], and a late stage in which the beginning of secretory activity is indicative of a progression from ductules to secretory acini, that are characteristic of the fully differentiated Lob 4 (Fig. 1A) [36,37,54,61].

Genetic influences are responsible of at least 5% of breast cancer cases; and also influence the pattern of breast development and differentiation, as shown by the study of breast tissue collected during prophylactic mastectomy from women with familial breast and breast/ovarian cancer, or proven to be carriers of the BRCA1 gene based on linkage analysis [45,61]. The morphological and architectural characteristics of these tissue samples were similar in breasts obtained from nulliparous and parous women. In both groups of women, the breast tissues consisted predominantely of Lob 1, with only a few specimens containing Lob 2 and Lob 3, in frank contrast to the predominance of Lob 3 found in parous women without a familial history of breast cancer [45,61]. The developmental pattern of the breast in parous women of the familial breast cancer group was similar to that of nulliparous women, and less developed than the breast of parous women without a history of familial breast cancer. The breasts of women belonging to the familial breast cancer group also differed in the branching pattern of

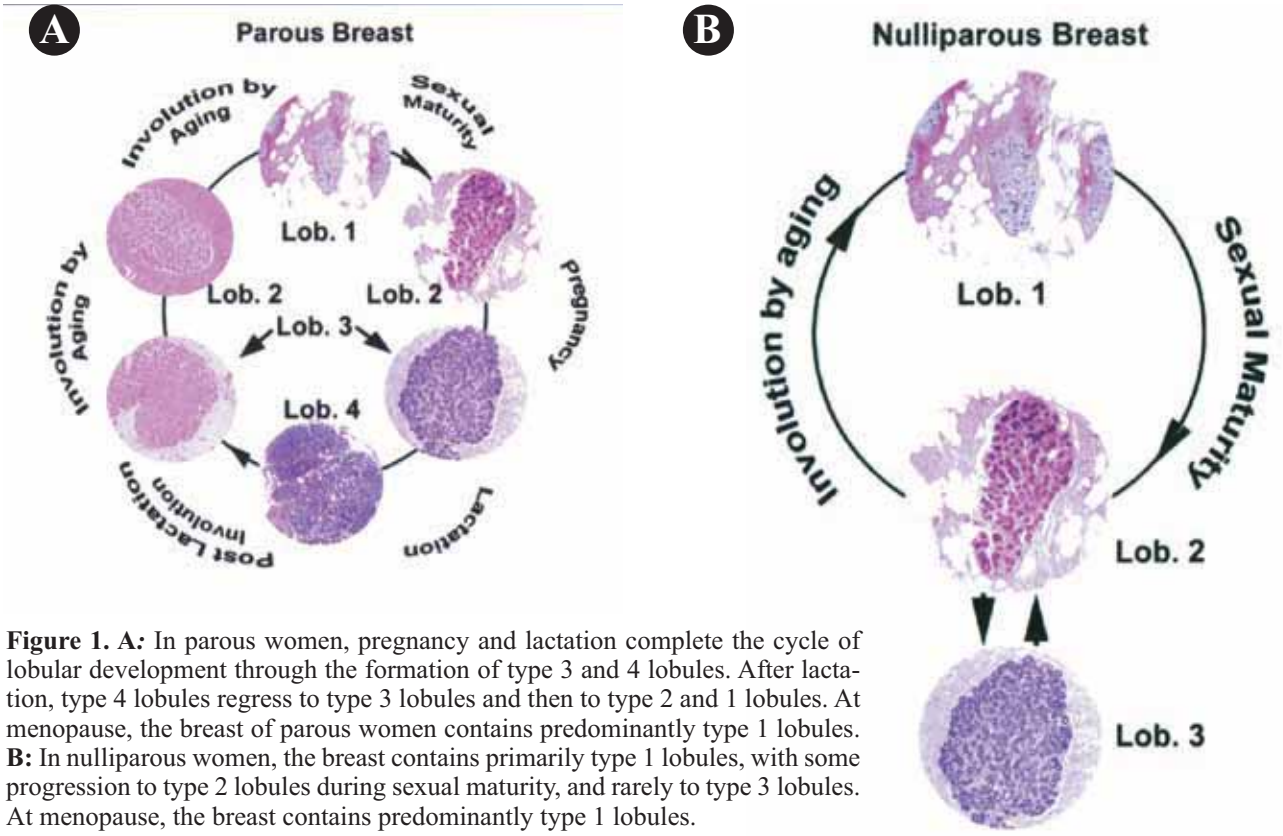

Figure 1. A*:* In parous women, pregnancy and lactation complete the cycle of lobular development through the formation of type 3 and 4 lobules. After lactation, type 4 lobules regress to type 3 lobules and then to type 2 and 1 lobules. At menopause, the breast of parous women contains predominantly type 1 lobules. **B:** In nulliparous women, the breast contains primarily type 1 lobules, with some progression to type 2 lobules during sexual maturity, and rarely to type 3 lobules. At menopause, the breast contains predominantly type 1 lobules.

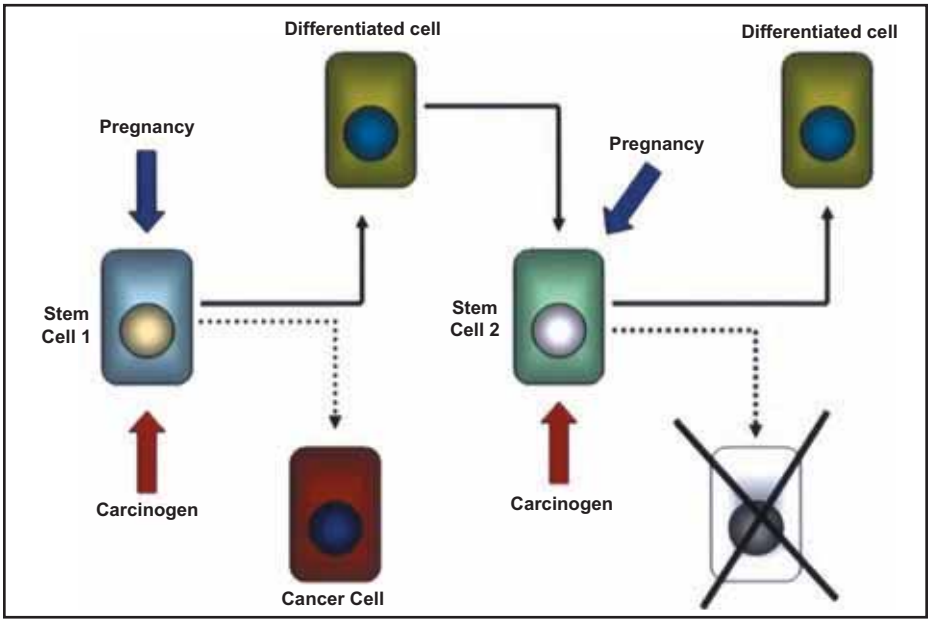

Figure 2. Breast cancer originates in undifferentiated terminal structures of the mammary gland (Lob. 1) that contain stem cells 1 which are the target of the neoplastic event. Early parity induces differentiation of the mammary gland to create stem cells 2. Although differentiation significantly reduces cell proliferation in the mammary gland, the mammary epithelium remains capable of responding with proliferation to 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, are able to metabolize the carcinogen, and repair the DNA damage more efficiently than cells of the nulliparous gland, and are less susceptible to carcinogenesis. However, if the shift from stem cell 1 to stem cell 2 has not been completed, sulficiently powerful a carcinogenic stimulus may overburden the system, thereby successfully initiating a neoplastic process.

the ductal tree, which suggested that the genes that control lobular development may have been affected in women carrying genes that predispose them to breast cancer [45,49,61].

After menopause, the breast undergoes a regressive phenomenon in nulliparous and parous women. This regression is seen 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 breasts of nulliparous and parous women contain predominantly Lob 1 (Fig. 1A,B) [54,55]. Although Lob 1 is the predominante structure in the breasts of parous and nulliparous women during the post-menopausal years, only nulliparous women have a high risk of developing breast cancer, whereas parous women remain protected [42,61]. Since ductal breast cancer originates in Lob 1 (TDLU), the epidemiological observation that nulliparous women have a higher incidence of breast cancer than parous women [18,19,22,25,78,79] indicates that in these two groups of women Lob 1 may be biologically different, or may exhibit a different susceptibility to carcinogenesis [41,43,44,47,61]. The presence of Lob 1 in the breasts of parous women has also been interpreted as a failure of the mammary parenchyma to respond to the influences of pregnancy and lactation [42,61]. The unresponsive lobules that fail to undergo full differentiation under the stimuli of pregnancy and lactation may be responsible for the development of cancer, despite the parity history of the woman. If this were the case, then this unresponsive Lob 1 would be as sensitive to carcinogenesis as the lobules found in the breasts of nulliparous women.

Breast development and the pathogenesis of breast cancer

An important concept that has emerged from the study of breast development is that the TDLU, which had been identified as the site of origin of ductal carcinoma, the most common breast malignancy [42,61,83], corresponds to a specific stage in the development of the mammary parenchyma (Lob 1). This observation is supported by comparative studies of normal and cancer-bearing breasts obtained at autopsy which have shown that the non-tumoral parenchyma cancer-bearing breasts contains a significantly higher number of hyperplastic terminal ducts, atypical Lob 1, and ductal carcinomas originating in Lob 1 than do breasts of women without breast cancer. These observations indicate that Lob 1 is affected by preneoplastic and neoplastic processes [44,49,55,59,61,81]. The finding that Lob 1, which are undifferentiated structures, give rise to the most undifferentiated and aggressive neoplasm is particularly relevant since these structures are more numerous in the breasts of nulliparous women who, in turn, have a higher risk of developing breast cancer. Lob 1 in the breasts of nulliparous women never undergo differentiation and contain stem cell 1 (Fig. 2) [57,61], whereas in the breasts of postmenopausal parous women these same structures differentiate and contain stem cell 2 (Fig. 2) [57,61].

Other differentiated lobular structures have also been found to be affected by neoplastic lesions, although they produce tumors with a malignancy that is inversely related to the degree of differentiation of the parental structure, ie., Lob 2 give rise to lobular carcinomas *in situ*, whereas Lob 3 give rise to more benign breast lesions, such as hyperplastic lobules, cysts, fibroadenomas and adenomas, and Lob 4 produce lactating adenomas [42]. Each specific compartment of the breast gives rise to a specific type of lesion, and provides the basis for a new biological concept that the differentiation of the breast determines the susceptibility to neoplastic transformation [62].

The finding that the most undifferentiated structures yield the most aggressive neoplasms supports our hypothesis that the presence of Lob 1 explains the higher risk of breast cancer in nulliparous women since they represent the population with the highest concentration of undifferentiated structures in the breast [44,49,53,60]. Non-tumoral breast tissues from cancer-bearing lumpectomy or mastectomy specimens removed from nulliparous women have an architecture dominated by Lob 1, and their overall architecture is similar to that of nulliparous women without any mammary pathology [49]. Whereas the breast tissues of parous women from the general population contain predominantly Lob 3 and a very low percentage of Lob 1, the breast tissues of parous women who have developed breast cancer contains Lob 1 as the predominant structure, which is similar to that of nulliparous women [48,49,61]. All of the a parous breast cancer patients that we have studied had a history of late first full-term pregnancy or a familial history of breast cancer. The analysis of these samples indicated that the breast architecture of parous women with breast cancer differed from that

of parous women without cancer. The similarities between the breast architecture of nulliparous women and that of parous women with cancer support the hypothesis that the degree of breast development is important in the susceptibility to carcinogenesis, and that parous women who develop breast cancer may have a defective response to the differentiating influence of pregnancy hormones [48,61]. The breast tissue of the latter women contains numerous stem cells 1 that are susceptible to carcinogenesis (Fig. 2). Since ductal breast cancer originates in Lob 1 (TDLU) [42,82], the epidemiological observation that nulliparous women have a higher incidence of breast cancer than parous women [18,19,22,25,26,79,80] indicates that Lob 1 in these two groups of women may be biologically different, or have different susceptibilities to carcinogenesis [41,44,46,48,49]. Although Lob 1 is the hallmark of the postmenopausal breast, we postulate that the degree of differentiation acquired through early pregnancy produces a "*genomic signature*" that differentiates Lob 1 of early parous women from that of nulliparous women by shifting the stem cell 1 population to stem cell 2, which is refractory to carcinogenesis (Fig. 2)**.**

Morphological evidence for mammary gland stem cells

In adult structures, stem cells have been defined by their capacity for self-renewal and ability to produce a differentiated progeny. In the mammary gland, DeOme *et al.* [10] demonstrated that fragments of different parenchymal regions were able to generate fully functional mammary outgrowths in mice, with the formation of ductal and lobuloalveolar structures composed of epithelial and myoepithelial cells. This concept was further developed by Kordon and Smith [24] who demonstrated that the progeny from a single cell may form 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 clear from this work whether these progenitor/stem cells were capable of initiating cancer when exposed to a carcinogenic agent. This issue was addressed by Russo *et al.* [34,62,63], who demonstrated that cancer initiated in TEBs in the mammary gland of young virgin rats. The analysis of these structures by electron microscopy allowed the characterization of 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 the presence or absence of Mg^{2+} and $Na^{+}K^{+}$-dependent ATPases. Based upon these criteria, three types of epithelial cells (light, intermediate and dark) were identified, in addition to myoepithelial cells [34,63]. Dark cells were the predominant type in TEBs, intermediate and myoepithelial cells were present in significantly lower percentages, and light cells were seen only occasionally, so that their percentage was combined with that of intermediate cells. The index of DNA labeling revealed that all of the cell types proliferated, although at different rates, depending upon the cell type and location within the mammary gland tree. Cell proliferation was maximal in intermediate cells located in TEBs, and was significantly lower in dark and myoepithelial cells found in the same location. High cell proliferation was associated with a greater incorporation of H^3-DMBA, and a progressive dominance of intermediate cells in DMBA-induced intraductal proliferations (IDPs) and in ductal carcinomas [63,64,65]. These results indicated that intermediate cells were not only the targets of the carcinogen but were also the stem cells of mammary carcinomas.

Further work by Bennett *et al*. [2] demonstrated that intermediate cells isolated from DMBA-induced mammary tumors gave rise to two cell types in culture, namely, dark cells, representing a terminally differentiated cell or a class in transition to differentiation, and intermediate cells, which represented an undifferentiated or stem cell, a progenitor of dark and myoepithelial cells. Rudland *et al* [33] isolated and characterized epithelial cells from normal rat mammary gland and from DMBA-induced mammary adenocarcinomas. These cells were cuboidal and produced a mixture of cuboidal and spindle-shaped cells resembling fibroblasts. In confluent cultures, cuboidal cells acquired the morphology of a third type of cell, which was dark, polygonal and had many small vacuoles; ultrastructurally, these cells resembled the dark cells described by Russo *et al*. [63]. Chepko and Smith [4] differentiated three division-competent cell populations in the murine mammary epithelium that included a subset of "large light cells", structurally

and functionally compatible with the early stages of secretory differentiation, and "small light cells" that were the least differentiated; the large light cells were considered a direct precursor to terminally differentiated secretory and myoepithelial cells.

Cell markers for identifying mammary gland stem cells

The introduction of immunocytochemical and genomic markers has resulted in a shift from the traditional approach of characterizing progenitor/ stem cells by their morphology and behavior *in vitro*. Smith *et al.* [68] used the expression of keratins 6 and 14 in mouse mammary epithelium to define 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 luminal epithelium, whereas keratin 14 was expressed in basally located fusiform cells such as myoepithelial cells. These authors emphasized the usefulness of these markers for identifying mammary epithelium-specific primordial cells. Stingl *et al*. [72,73] used new molecular markers to select subpopulations of cells with distinct capacities for differentiation. These authors 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 α-integrin, in combination with the exclusion of rhodamine dye. Hebbard *et al*. [17] 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 is regulated thereafter by the estrous cycle; this protein disappears during lactation, but reappears during involution, suggesting that its expression is a suitable marker for stem cells. Novel studies in mice mammary gland [23] 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 were stem cells and their progeny (transitional cells) were arranged as transitional units (TUs) and that both expressed the proteins Zonula Occludens-1 and alpha-catenin. These findings suggest that transitional units retain stem cells.

The study of markers for other stem cells has been useful in identifying mammary stem/progenitor cells. Sca1 (stem cell antigen 1) was first described in mice as a hematopoietic stem cell antigen [70]. Welm *et al.* [84] detected a Sca1+ cell population that was enriched for functional stem/progenitor cells in the luminal epithelium of mice. These cells were labeled by BrdU did not express markers of differentiation, and were negative for the progesterone receptor. The Sca1+ population also showed "side population" (SP) properties, a characteristic first defined in bone marrow cells [69], in which cells with Hoeschst dye-releasing properties have phenotypic markers of multipotential hematopoietic stem cells. The protein responsible for this phenotype has been proposed to be breast cancer resistance protein (BCRP1), suggesting that the expression of this protein could serve as a marker for stem cells from various sources [86]. Mammary epithelial cells with SP properties have also been identified in human mammary gland. Alvi *et al.* [1] showed that 0.2-0.45% of human and mouse epithelia were formed by distinct SP cells. These SP cells generated ductal and lobuloalveolar structures when transplanted into murine cleared mammary fat pads. The SP cells had a high expression of BCRP, sca1, telomerase catalytic subunit, and low levels of differentiated markers for luminal (epithelial membrane antigen and cytokeratin 19) and myoepithelial (cytokeratin 14) cell types. 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 human stem/progenitor cells. Gudjonsson *et al.* [15] isolated a cell line derived from human mammary cells expressing epithelial specific antigen (ESA) and lacking sialomucin (MUC) that could give rise to luminal epithelial and myoepithelial cells in culture. A single ESA+/MUC- cell had the ability to generate 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 showed differentiation, and was restricted to the luminal epithelium, but had

no stem cell properties. Wicha *et al*. [11] 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 into three mammary epithelial lineages and to clonally generate complex functional structures in 3D culture systems. Cytological and immunocytochemical analyses of secondary mammospheres revealed that these structures contained cells positive for α 6 integrin, cytokeratin 5, which was widely expressed, and CD10; ESA-positive and cytokeratin 14-positive cells occurred less frequently, Muc 1, α-smooth muscle antigen (ASMA), and cytokeratin 18 were not detected. In addition to cells, mammospheres contained extracellular material (ECM). However, immunostaining for fibronectin and collagen IV, the classic components of adult gland ECM, was 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 [11]. Moreover, comparison of the genomic profile of undifferentiated cells from mammospheres with that of differentiated cells cultured on collagen identified candidate genes for stem/progenitor cell markers. Some of these genes have already reported to be involved in stem/progenitor cell-specific functions or in the regulation of self-renewal, and the abnormal expression of some of them has been correlated with the development breast cancer (cell proliferation, survival and invasion).

Role of steroid hormone receptors as markers for mammary gland stem cells

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 (ERα) in these processes. The importance of the role played by ERα in mammary gland development has been highlighted by the development of the αERKO mouse [8]. At birth, the mammary gland of normal mice 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 αERKO 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 [64]. This period is characterized by rapid ductal growth and active proliferation of the mammary epithelium of Lob 1. These structures are composed of a rapidly proliferating epithelium that has a high content of ERα and progesterone receptor (PR)-positive cells. With the progressive maturation of Lob 1 to Lob 2, Lob 3, and Lob 4, there is a 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 transformation by chemical carcinogens [40]. These data indicate that the stem cells that give rise to the mammary tree and cancerous lesions are located in a specific compartment of the mammary parenchyma, namely the Lob 1 (TDLU); or stem cell 1, as identified by Russo and Russo [57].

Work by Petersen *et al.* [31] has 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 that form terminal ductal lobular unit (TDLU)-like structures, can be distinguished by the presence of cytokeratin 19. The suprabasal population of breast stem cells consists of undifferentiated "intermediate" cells with Hoechst dye-releasing "side population" (SP) properties. These cells do not express myoepithelial and luminal apical membrane markers such as CALLA and MUC1, but are rich in ERα-positive cells and express several fold higher levels of ERα, p21 (CIP1) and Msi1 genes than non-SP cells. These cells also form branching structures in matrigel that includes cells of luminal and myoepithelial lineages. These data suggest a model in which scattered steroid receptor-positive cells are stem cells that self-renew through asymmetrical cell division and generate patches of transit-amplifying cells and differentiated cells [5,6]. ERα/PR+ breast cancers show a loss of Musashi-1 and Notch-1, the two key regulators of asymmetrical cell division, and may therefore arise from the symmetrical division of ERα/PR+ stem cells [5]. These data are supported by the observations of Russo *et al.* [40] that epithelial cells of Lob 1 co-express, ERα, PR and the proliferation marker Ki67, suggesting that these cells could give rise to ERα-positive tumors. However, these cells represent less than 1% of the total cell

population. The observation that the majority of ERα/PR+ cells do not express Ki67 suggests that cells containing these receptors are unable to proliferate. The finding that proliferating cells differ from those that are ERα- and PR-positive supports data indicating 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 their ERα-positive cells, indicating that only proliferating cells that are also ERα-negative can survive; the latter type of stem cell may give rise to ER negative tumors [40]. The fact that the majority of proliferating breast epithelial cells do not express ERα and PgR could explain the findings of Clayton *et al.* [7] who showed that human mammary stem cells, expressed ESA, did not take up Hoechst dye had low levels of MUC-1 and CALLA, and had no detectable expression of ER alpha and beta. Cells with this phenotype had a high cloning efficiency when cultured from a single cell, and generated mixed colonies containing luminal and myoepithelial cells.

Further considerations and perspectives on mammary gland stem cells

As discussed above the identification of a putative breast stem cell has advanced significantly in the last decade, and several markers reported for other tissues have been found in the mammary epithelial cells of rodents and humans. There are, however four main issues that require further investigation. The first issue is to determine whether the stem or progenitor cells that give rise to a complete mammary gland are the same as those that are affected by a carcinogenic process. The second important point is the role of ERα as a marker for stem cells. The third aspect is the need for extreme care in validating conclusions drawn from studies *in vitro* by confirming them with data, obtained *in vivo,* in which factors such as donor age, reproductive history, number of samples studied, and consideration of the intrinsic sample-to-sample variability can exert an important influence, but are seldom considered in publications dealing with the mammary gland stem cells. The fourth consideration is that the data reported in the literature tend to support the concept that mammary gland contains a stem cell 1 that could be the progenitor of the differentiated breast, or the site of origin of a neoplastic process. In support of this concept is the fact that all of the genes ascribed to stem cells in the mammary gland are involved in more than one function in normal and malignant breast tissue.

The evidence for stem cell 2 in the post-pregnancy mammary gland

Epidemiological studies in humans and models of experimental carcinogenesis have provided extensive evidence of the protective effect of pregnancy against the development of breast cancer [22,25-27,32,51,64,66,74,84,85]. Russo *et al.* [35,51,64,75,76] postulated that the pregnancy-induced protection was 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. Alternative explanations attributed the protective effect of pregnancy to changes in the environmental milieu [77] and/or to alterations in the immunological profile of the host [66]. A further refinement of the hypothesis of how pregnancy could influence the susceptibility cancer by inducing differentiation of the mammary gland was first proposed by Russo and Russo [57], who postulated that Lob 1 and TEB in the breasts of nulliparous women or of young virgin rats, respectively, had not completed their differentiation into Lob 2, Lob 3 and Lob 4, and retained a high concentration of stem cells 1, which are susceptible to neoplastic transformation when exposed to a carcinogenic agent (see previous section and Fig. 2). After the postmenopausal involution of the mammary gland, the architecture of the parous breast is similar that of the nulliparous breast, and contains predominantly Lob 1 composed of stem cells 2, that are refractory to transformation (Fig. 2). It was further postulated that the degree of differentiation acquired through early pregnancy permanently changes the "*genomic signature*" that differentiated the Lob 1 of early parous women from that of nulliparous women, with a shift from stem cells 1 to stem cell 2, which is refractory to carcinogenesis (Fig. 2).

After post-lactational involution, the mammary epithelium remains capable of responding with proliferation and differentiation to the stimulus of a new pregnancy. However, stem cells 2 are refractory to carcinogenesis, even though they are stimulated to proliferate and to regenerate the whole mammary gland. Stem cells 2 are characterized by having a

genomic signature that has been induced by the first cycle of differentiation (Fig. 2). During the last eight years, supporting evidence for this hypothesis has been provided by Russo *et al.*, as well as other researchers. Recent studies by Smith *et al.* [3,20,82] using transgenic WAP-driven Cre and Rosa 26-fl-stop-fl-LacZ mice have 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, the percentage of these cells increases to reach 60% of the total epithelium in multiparous females. The parity-induced mammary epithelial cells (PI-MEC) are equivalent to the stem cells 2 postulated by Russo *et al*. [57] since these cells are capable of 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 may contribute to differences in the response to hormonal stimulation and carcinogenic agents observed between nulliparous and parous females [35,39-41,44,47,51].

Several authors have investigated molecular changes as a mechanism for pregnancy-induced protection [9,12,13,27,28,67,71]. Russo and coworkers found that the post-pregnancy involuted mammary gland had a genomic signature characterized by elevated expression of the genes involved in apoptotic pathways, such as testosterone repressed prostate message 2 (TRPM2), interleukin 1β-converting enzyme (ICE), bcl-XL, bcl-XS, p53, p21, and c-myc, which are upregulated by 3 to 5 fold [61,70,71]. The activation of programmed cell death genes occurs through a p53-dependent process, is modulated by c-myc and shows partial dependence on the bcl2-gene family. In addition, inhibin A and B, heterodimeric non-steroidal secreted glycoproteins with tumor suppressor activity are also upregulated [61,70,71]. Genes for which the level of expression progressively increases with the duration of pregnancy to reach their highest levels 21-42 days post-partum include those coding for a fragment of glycogen phosphorylase, AMP activated kinase, bone morphogenetic protein 4 and vesicle-associated protein 1. The expression of a 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 DNA repair is part of the mammary gland signature induced by pregnancy. These observations confirm previous findings that the ability of these cells to repair carcinogen-induced damage by unscheduled DNA synthesis and adduct removal is more efficient in the parous and animal mammary gland [76].

In agreement with the studies of Srivastava *et al*. [70,71], Siveraman *et al.* [67] observed that p53 was involved in the protective effect of parity, and could be mimicked by treating virgin rats with estrogen and progesterone. Studies by Medina *et al.* [27,28] in the same hormonal model showed that a functional p53 was required for the hormone-mediated protection against 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 in modulation of the transforming growth factor beta (TGF-α) signaling pathway were upregulated in the post-treatment involuted mammary gland [12]. 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 *et al*. [9] also observed the downregulation of growth factors potentially involved in epithelial proliferation, as well as the persistent upregulation of TGF-α3 and several of its transcript targets, in the involuted gland of parous rats and mice.

The proposed model of parity-induced specific changes [57] has been further confirmed by Ginger and Rosen [13], who reported that pregnancy induces multiple changes in mammary epithelial cells, including the 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 carcinogens. Pregnancy would induce epigenetic changes, such as chromatin remodeling, DNA methylation/demethylation, and histone modifications, thereby affecting cell fate in

the parous mammary gland. All of the genes that have been attributed to stem cells 2 appear to act by functional pathways different from those described for stem cells 1.

Although more work is needed to improve our understanding of the role of stem cells 2 and their interaction with the genes that confer a specific signature, the data discussed here nevertheless show that pregnancy, can stimulate the differentiation, of stem cells 1 into stem cells 2, with the latter having a specific genomic signature that could account for the refractoriness of the mammary gland to carcinogenesis.

Unifying concepts

Breast cancer originates in undifferentiated terminal structures of the mammary gland. The terminal ducts of Lob 1 of the human female breast, where ductal carcinomas originate, 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 neoplastic transformation has been confirmed by studies *in vitro* showing that this structure has the highest proliferative activity and rate of carcinogen binding to DNA [46,64]. More importantly, when treated with carcinogens in vitro, the epithelial cells express phenotypes indicative of cell transformation [41,47]. These studies indicate that in the human breast the target cells of carcinogens occur in a specific compartment, the characteristics of which are the determining factors in the initiating event (Fig. 2). These target cells will become the stem cells (stem cell 1 in Fig. 2) of the neoplastic event, depending upon: (a) their topographic location within the mammary gland tree, (b) the age at exposure to a known or putative genotoxic agent, and (c) the reproductive history of the host. The higher incidence of breast cancer seen in nulliparous women supports this concept because it parallels the higher incidence of cancer elicited by carcinogens in rodents when exposure occurs at a young age. In addition, early parity is associated with a pronounced decrease in the risk of breast cancer, with additional live births confering an even greater reduction in the risk [25].

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 etiological agent or agents act.

Although differentiation significantly reduces cell proliferation in the mammary gland, the mammary epithelium remains capable of responding with proliferation to certain stimuli, such as a new pregnancy (Fig. 2). 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 or from stem cells 2 (stem cells 2 of Fig. 2) that are able to metabolize the carcinogen and repair the DNA damage more efficiently than cells of a virginal gland, and are less susceptible to carcinogenesis, as has been demonstrated in rodents. However, if the shift from stem cell 1 to stem cell 2 has not been completed, a sufficiently powerful carcinogenic stimulus may overburden the system, thereby successfully initiating a neoplastic process. Such conditions may explain the small fraction of women who develop breast cancer after an early first full-term pregnancy, because they have not fully completed the first cycle of differentiation.

The relevance of our work lies in the *vis-à-vis* comparison of *in vivo* and *in vitro* studies in the human breast that validate experimental data and allow extrapolation to humans. The finding that differentiation is a powerful inhibitor of the initiation of cancer provides a strong rationale for pursuing the identification of the genes that control this process. The knowledge gained will provide novel tools for developing rational strategies for breast cancer prevention**.**

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