Review

The protective role of pregnancy in breast cancer

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Abstract

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. Women who gave birth to a child when they were younger than 24 years of age exhibit a decrease in their lifetime risk of developing breast cancer, and additional pregnancies increase the protection. The breast tissue of normally cycling women contains three identifiable types of lobules, the undifferentiated Lobules type 1 (Lob 1) and the more developed Lobules type 2 and Lobules type 3. The breast attains its maximum development during pregnancy and lactation (Lobules type 4). After menopause the breast regresses in both nulliparous and parous women containing only Lob 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 Lob 1 in these two groups of women might be biologically different, or might exhibit different susceptibility to carcinogenesis. Based on these observations it was postulated that 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 are therefore susceptible to undergo neoplastic transformation. These epithelial cells are called Stem cells 1, whereas Lob 1 structures found in the breast of early parous postmenopausal women free of mammary pathology, on the contrary, 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 Lob 1 of the early parous women from that of the nulliparous women by shifting the Stem cells 1 to Stem cells 2 that are 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 cells 1) has, in the past decade, reached 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 further work needs to be carried out in order to better understand the role of the Stem cells 2 and their interaction with the genes that confer them a specific signature, collectively the data presently available provide evidence that pregnancy, through the process of cell differentiation, shifts Stem cells 1 to Stem cells 2 – cells that exhibit a specific genomic signature that could be responsible for the refractoriness of the mammary gland to carcinogenesis.

The protective effect of parity in breast cancer

The incidence of breast cancer has gradually increased in the United States and in most Western countries over the past few decades [1]. 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 [1-3].

Women who gave birth to a child when they were younger than 24 years of age exhibit a decrease in their lifetime risk of developing breast cancer, and additional pregnancies increase the protection [3]. The protective effect of full-term pregnancy is a well-established concept not only in humans, but also in experimental rodent models [4-12]. 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 [9,10,13].

In rodents, maximal incidence of 7,12-dimethylbenz(a) anthracene (DMBA)-induced mammary cancer occurs when the carcinogen is administered to young virgin cycling rats, but the same carcinogen fails to induce tumors when given to rats after a full-term pregnancy [4-13]. The high susceptibility of the young virgin rat mammary gland to develop malignancies is the result of the interaction of the carcinogen with the rapidly dividing epithelium composing the terminal end buds (TEBs), which are undifferentiated ductal structures of the mammary parenchyma. Both the binding of the

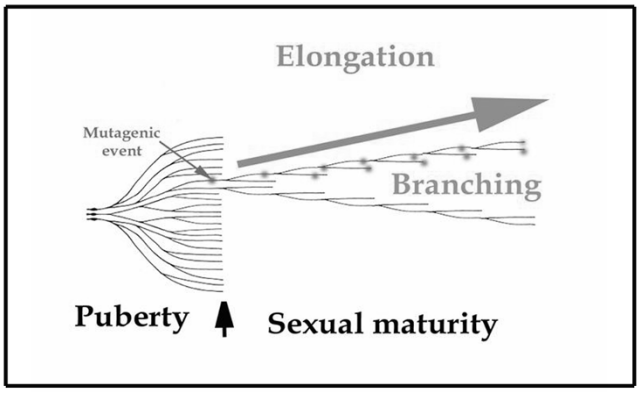
DMBA = 7,12-dimethylbenz(a) anthracene; ER = estrogen receptor; ESA = epithelial-specific antigen; PR = progesterone receptor; SP = side population; TEB = terminal end bud.

carcinogen DMBA to the DNA of rapidly proliferating epithelial cells and a low DNA repair capacity result in fixation of transformation, leading to the initiation of cancer [13-17].

There are close similarities between the pathogenesis of breast cancer in women and that induced in rodents by chemical carcinogens. Ductal carcinoma, the most frequently diagnosed breast malignancy, originates in Lobules type 1 (Lob 1), also called the terminal ductal lobular unit, an undifferentiated structure considered to be equivalent to the TEB, the site of origin of ductal carcinomas in rodents [18,19]. Importantly, under *in vitro* conditions, epithelial cells from Lob 1 can be transformed by the same chemical carcinogens that induce mammary cancer in experimental animals [20,21].

These observations suggest that if the human breast is exposed to a carcinogenic insult early in life, then Lob 1 would be the structure affected. It is therefore possible to postulate that genomic damage caused by radiation, environmental carcinogens, hormonal imbalances, and/or other still unidentified factors, either alone or in combination with genetic predisposition, might 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, even though the damaged cells would be clinically detectable as a neoplasm only after several years of progressing along the various stages of transformation [5]. An initial mutagenic event taking place in the primitive ductal structures of the breast before or during puberty could therefore multiply during the process of branching and ductal elongation driven by puberty and sexual maturation (Fig. 1).

Figure 1



An initial mutagenic event in the primitive ductal structures of the breast taking place early in life, such as before or during puberty, can multiply during the process of branching and ductal elongation during puberty and sexual maturation, explaining the development of a neoplastic lesion later on in life.

Breast development and differentiation during pregnancy

The breast tissue of normally cycling women contains three identifiable types of lobules: the previously described Lob 1, and the more developed Lobules type 2 (Lob 2) and Lobules type 3 (Lob 3) [21-23]. The lobular composition of the breast of sexually mature women is influenced by numerous endogenous and exogenous factors. Principal among them are age, and hence the number and regularity of menstrual cycles, endocrine imbalances, the use of exogenous hormones, environmental exposures that could act as endocrine disruptors, and the physiological condition of pregnancy.

The breast attains its maximum development during pregnancy. This development occurs in two distinctly dominant phases. The early stage, characterized by ductal lengthening and profuse branching, is sustained by active cell proliferation at the distal end of the ductal tree. The rapid increase in the number of newly formed ductules 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 Lobules type 4 (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 [21,22].

In contrast to 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. This observation suggests that a certain percentage of Lob 1 might have progressed to Lob 2, but very few of them had progressed to Lob 3 [22].

The breast regresses in both nulliparous and parous women after menopause. 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 [22]. 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 might exhibit different susceptibility to carcinogenesis [22,23].

Pregnancy and the pathogenesis of breast cancer

An important concept that emerged from the study of breast development is that Lob 1, which corresponds to a specific stage of development of the mammary parenchyma, has been

identified as the site of origin of the most common breast malignancy, the ductal carcinoma. This site is demonstrated in comparative studies of normal breasts and cancer-bearing breasts obtained at autopsy and from cancer-bearing lumpectomy or mastectomy specimens [18,19,23]. 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 indicate that Lob 1 develop preneoplastic lesions as well as neoplastic lesions [18].

More differentiated lobular structures have been found to originate tumors whose malignancy is inversely related to the degree of differentiation of the parent structure; that is, Lob 2 originate lobular carcinomas *in situ*; Lob 3 give rise to benign breast lesions, such as hyperplastic lobules, cysts, fibroadenomas, and adenomas; and Lob 4 originate lactating adenomas [18]. 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 [18,24].

The architecture of the non-tumoral breast tissues from nulliparous women's cancer-bearing lumpectomy or mastectomy specimens does not differ from that of nulliparous females free of mammary pathology, both being composed predominantly of Lob 1 [23,25]. 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 the breast tissues contain Lob 1 as the predominant structure, appearing similar to that of nulliparous women [25]. 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 [22] and therefore be hosting cells that are susceptible to carcinogenesis.

Based on these observations it was postulated by Russo and Russo in 1997 that 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 are therefore susceptible to undergo neoplastic transformation – these cells were called Stem cells 1 (Fig. 2) [26]. 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 – these cells were called Stem cells 2 (Fig. 2) – making this the postulated mechanism of protection conferred by early full-term pregnancy [22-24,26]. It was further postulated that the degree of differentiation acquired through early pregnancy has changed the 'genomic signature' that differentiates Lob 1 of the early parous women from those of the nulliparous women by shifting the Stem cells 1 to Stem cells 2 that are refractory to carcinogenesis (Fig. 2).

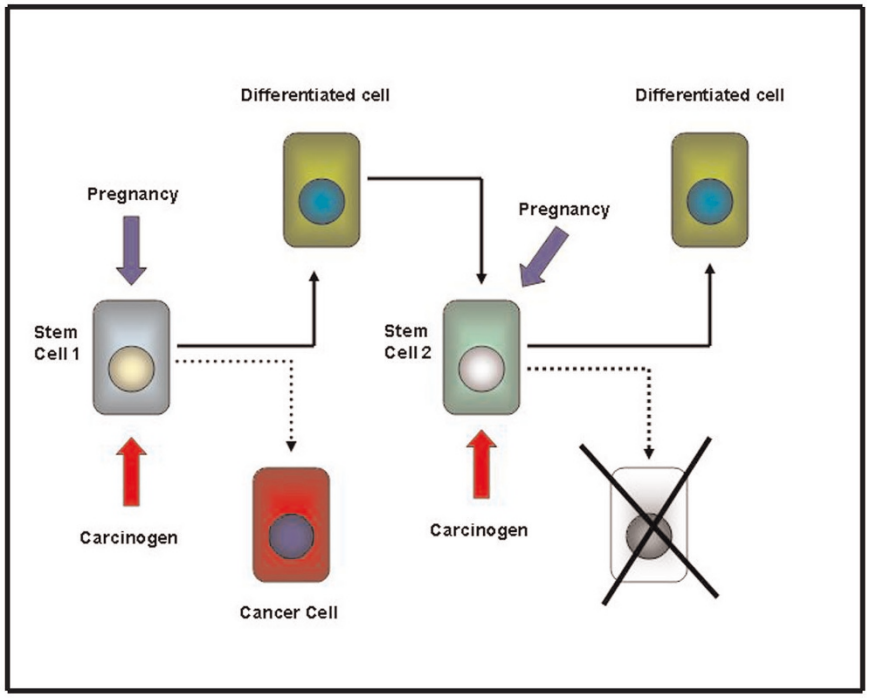
Role of the progenitor/stem cells in mammary gland development and their relationship to the cells of origin of cancer (Stem cells 1)

Morphological evidence for a stem cell 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 and colleagues demonstrated that fragments of different parenchymal portions were able to generate fully functional mammary outgrowths in mice, forming ductal and lobuloalveolar structures composed of epithelial cells and myoepithelial cells [27]. This concept was further developed by Kordon and Smith [28], who demonstrated that the progeny from a single cell may comprise the epithelial population of a fully developed lactating mammary outgrowth in mice. The development of the complete mammary tree from a small portion of a duct or from single cells therefore attests to their multifaceted potential.

It was not known, however, whether these progenitor/stem cells would be capable of initiating cancer when exposed to a carcinogenic agent. This issue was addressed by Russo and colleagues [29-31], who demonstrated that cancer started in TEBs present in the mammary gland of young virgin rats. The analysis of these structures by electron microscopy allowed one to characterize their cellular composition based upon cell and nuclear size, the nuclear–cytoplasmic ratio, the amount of chromatin condensation, the electron density of the cytoplasm, the number and distribution of organelles, and the presence or absence of Mg^{2+}-dependent and $Na^{+}K^{+}$-dependent ATPases. Based upon these criteria, in addition to myoepithelial cells, three types of epithelial cells were identified: light cells, intermediate cells, and dark cells [30,31]. Dark cells were found to be the predominant type in TEBs, intermediate cells and myoepithelial cells were present in significantly lower percentages, and light cells were only occasionally seen so 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 their type of location within the mammary gland tree. Cell proliferation was maximal in intermediate cells located in TEBs, being significantly lower in dark cells and myoepithelial cells found in the same location. High cell proliferation was associated with greater incorporation of H^3-DMBA and with a progressive dominance of intermediate cells in DMBA-

Figure 2



Breast cancer originates in undifferentiated terminal structures of the mammary gland (Lobules type 1) that contain Stem cells 1, the target of the neoplastic event. Early parity induces differentiation of the mammary gland, creating Stem cells 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 that are less susceptible to carcinogenesis. However, if the shifting of Stem cells 1 to Stem cells 2 has not been completed, a powerful enough carcinogenic stimulus may overburden the system, thereby initiating successfully a neoplastic process.

induced intraductal proliferations and in ductal carcinomas [5,31]. 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 and colleagues demonstrated that intermediate cells isolated from DMBA-induced mammary tumors originated two cell types in culture [32]: 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 cells and myoepithelial cells. Rudland and colleagues [33] 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 and colleagues [31]. Chepko and Smith [34] differentiated two 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; and '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.

Cell markers identifying the stem cells in the mammary gland

A shift from the pioneering work performed for characterizing, by morphology and by *in vitro* behavior, the progenitor/stem cells started with the search for immunocytochemical and genomic markers. Smith and colleagues [35] utilized the expression of keratin 6 and keratin 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 and colleagues [36,37] utilized new molecular markers (Table 1) 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 α-integrin, in combination with exclusion of rhodamine dye. Hebbard and colleagues [38] 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. CD44 expression in rodents is first detected at puberty, and thereafter it is regulated by the estrous cycle; the expression disappears during lactation and reappears during involution, suggesting that the expression of this protein is a marker of a stem cell. Novel studies in the mouse mammary gland have identified stem cells in TEBs and ducts by pulse-labeling primary mammary epithelial cells with fluorescent TRITC-cell linker membrane label and with BrdU [39]. 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 in mammary ducts that were actively growing or static. That study demonstrated that label-retaining mammary epithelial cells are stem cells and that their progeny (transitional cells) are arranged as transitional units, and also demonstrated that both cells express Zonula Occludens-1 and α-catenin proteins, data suggesting 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. Stem cell antigen 1 (Sca1) (Table 1) was first described in mice as a hematopoietic stem cell antigen [40]. Welm and colleagues detected in the luminal epithelium of mice a $Sca1^+$ cell population that is enriched for functional stem/progenitor cells [41]. 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 [40], as cells with Hoechst 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 (BCRP1), suggesting that the expression of this protein could serve as a marker for stem cells from various sources [42]. Mammary epithelial cells with SP properties were also identified in the human mammary gland. Alvi and colleagues [43] showed that 0.2–0.45% of both human epithelia 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 BCRP, Sca1, and telomerase catalytic subunit, and had low levels of differentiated markers for luminal cell types (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 that may be specific for the human stem/progenitor cells. Gudjonsson and colleagues isolated a cell line derived from human mammary cells expressing ESA and lacking MUC expression that could give rise to both luminal epithelial cells and myoepithelial cells in culture [44]. 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 and luminal epithelial-restricted without stem cell properties (Table 1).

Wicha and colleagues developed a system to enrich the population of human mammary progenitor/stem cells by culturing them in suspension, where they formed 'non-adherent mammospheres' [45]. These structures were able to differentiate along all three mammary epithelial lineages and to clonally generate complex functional structures in three-dimensional culture systems. Cytological and immunocytochemical analysis of secondary mammospheres revealed that these structures contained cells positive for α6-integrin, cytokeratin 5 (which was widely expressed), and CD10; ESA-positive and cytokeratin 14-positive cells were less frequently found. Muc1, α-smooth muscle antigen, and cytokeratin 18 were not detected. In addition to cells, mammospheres contained extracellular material. However, immunostains for fibronectin and collagen IV, the classical components of adult gland extracellular material, were negative – although ~20% of the mammospheres stained positive for laminin. In contrast, abundant expression of the embryonic extracellular material components tenascin and decorin, was detected in mammospheres [45]. Moreover, the comparison of the genomic profile of undifferentiated cells from mammospheres with that of differentiated cells cultured on collagen identified gene candidates for stem/progenitor cell markers. Some of these genes were already described as involved in stem/progenitor cell-specific functions or in regulation of self-renewal, and abnormal expression of some of the genes has been correlated with breast cancer development such as proliferation, cell survival, and invasion (Table 1).

Role of steroid hormone receptors as markers 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 senescence 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 the ERα in mammary gland development has been highlighted by the development of the αERKO mouse [46]. 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

Table 1

Genes attributed to the stem cells of the mammary gland (Stem cells 1)

Gene	Abbreviation	Accession number	Cytoband	References
A disintegrin and metalloproteinase domain 12 (meltrin alpha)	ADAM12	NP_067673	10q26.3	[45]
Actin alpha 1, alpha smooth muscle antigen	ACTA1/ASMA	NP_001091	1q42.13-q42.2	[45]
Apolipoprotein E	APOE	NP_000032	19q13.2	[45]
ATP-binding cassette, subfamily A, member 1	ABCA1	NP_005493	9q31.1	[45]
ATP-binding cassette, subfamily G, member 2	ABCG2/BCRP	NP_004818	4q22	[42,43]
Catenin (cadherin-associated protein), alpha 1 (102 kDa)	CTNNA1	NP_001894	5q31	[39]
CD44 antigen	CD44	NP_001001392	11p13	[38]
Chemokine (C–C motif) ligand 2	CCL2	NP_002973	17q11.2-q21.1	[45]
Cyclin D_2	CCND2	NP_001750	12p13	[45]
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	NP_510867	6p21.2	[49,50]
Decorin	DCN	NP_598014	12q13.2	[45]
Epithelial membrane protein 3	EMP3	NP_001416	19q13.3	[45]
Estrogen receptor 1	ESR1	NP_000116	6q25.1	[47,49-51]
Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	FGFR1	NP_075599	8p11.2-p11.1	[45]
Flotillin 2 (epithelial-specific antigen)	FLOT2/ESA	NP_004466	17q11-q12	[36,37,44,51]
Four and a half LIM domains 1	FHL1	NP_001440	Xq26	[45]
Frizzled homolog 2 (Drosophila)	FZD2	NP_001457	17q21.1	[45]
FYN oncogene related to SRC, FGR, YES	FYN	NP_694593	6q21	[45]
Glycoprotein (transmembrane) nmb	GPNMB	NP_002501	7p15	[45]
Glypican 4	GPC4	NP_001439	Xq26.1	[45]
Growth-associated protein 43	GAP43	NP_002036	3q13.1-q13.2	[45]
Growth hormone receptor	GHR	NP_000154	5p13-p12	[45]
Insulin-like growth factor 2 receptor	IGF2R	NP_000867	6q26	[45]
Insulin-like growth factor binding protein 4	IGFBP4	NP_001543	17q12-q21.1	[45]
Insulin-like growth factor binding protein 7	IGFPP7	NP_001544	4q12	[45]
Integrin, alpha 6	ITGA6	NP_000201	2q31.1	[36,37,45]
Keratin 19	KRT19	NP_002267	17q21.2	[48-50]
Lymphocyte antige 6 complex, sca-1	LY6A/SCA-1	NP_034868	15	[40,41,43]
Membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	MME/CALLA/CD10	NP_009220	3q25.1-q25.2	[36,37,45, 49,51]
Mucin 1 (transmembrane), epithelial membrane antigen	MUC1/EMA	NP_877418	1q21	[36,37,43-45, 49,51]
Musashi homolog 1 (Drosophila)	MSI1	NP_002433	12q24.1-q24.31	[49,50]
Nidogen (enactin)	NID	NP_002499	1q43	[45]
Notch homolog 1, translocation-associated (Drosophila)	NOTCH1	NP_060087	9q34.3	[49]
Notch homolog 3 (Drosophila)	NOTCH3	NP_000426	19p13.2-p13.1	[45]
Notch homolog 4 (Drosophila)	NOTCH4	NP_004548	6p21.3	[49]
P53 target zinc finger protein	WIG1	NP_689426	3q26.3-q27	[45]
Periostin, osteoblast specific factor	POSTN	NP_006466	13q13.3	[45]
Platelet-derived growth factor receptor, beta polypeptide	PDGFRB	NP_002600	5q31-q32	[45]
Polycystic kidney disease 2 (autosomal dominant)	PKD2	NP_000288	4q21-q23	[45]
Progesterone receptor	PGR	NP_000917	11q22-q23	[41,47,49,50]
Prostate tumor overexpressed gene 1	PTOV1	NP_059128	19q13.33	[45]
Retinoic acid receptor responder (tazarotene induced) 3	RARRES3	NP_004576	11q23	[45]
Stem cell growth factor; lymphocyte secreted C-type lectin	SCGF	NP_002966	19q13.3	[45]
Telomerase reverse transcriptase	TERT	NP_937986	5p15.33	[43]
Tenascin C (hexabrachion)	TNC	NP_002151	9q33	[45]
Tight junction protein 1 (zona occludens 1)	TJP1	NP_783297	15q13	[39]
Tissue inhibitor of metalloproteinase 3	TIMP3	NP_000353	22q12.1-q13.2	[45]
Wiskott–Aldrich syndrome protein interacting protein	WASPIP	NP_003378	2q31.1	[45]

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 [5]. 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 ERα-positive 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 [47]. 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 Lob 1 or the terminal ductal lobular unit; these are the cells that were called Stem cells 1 by Russo and Russo [26].

Supporting studies by Petersen and colleagues [48] have shown that a subset of suprabasal breast luminal epithelial cells that are able to generate themselves, as well as differentiated luminal epithelial cells and myoepithelial cells, and are able to form terminal ductal lobular unit-like structures are distinguished by expression of cytokeratin 19. The suprabasal population of breast stem cells consists of undifferentiated 'intermediate' cells with Hoechst dye-effluxing SP properties. These cells lack expression of myoepithelial and luminal apical membrane markers such as CALLA and MUC1. The cells are rich for ERα-positive cells and express several-fold higher levels of the ERα, p21 (CIP1), and Msi1 genes than non-SP cells (Table 1). These cells also form branching structures in matrigel that 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 [49,50].

ERα/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 ERα/PR$^+$ stem cell [49]. These data are supported by the observations of Russo and colleagues that epithelial cells of the Lob 1 co-express ERα, PR, and the proliferation marker Ki67 [47], suggesting that these cells could originate ERα-positive tumors. However, these cells represent less than 1% of the total cell population whereas the majority of ERα/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 ERα-positive and PR-positive cells support data that indicate 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 ERα-positive cells, indicating that only proliferating cells that are also ERα-negative can survive, representing a type of stem cell that may originate ER-negative tumors [47]. The fact that the majority of proliferating breast epithelial cells do not express ERα and PgR could explain Clayton and colleagues' [51] 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 ERα and ERβ. Cells expressing that phenotype had high cloning efficiency in culture from a single cell, generating mixed colonies containing luminal cells and myoepithelial cells.

Further considerations and perspectives on the stem cells of the mammary gland

As discussed in the previous sections, the identification of a putative breast stem cell has in the past decade reached a significant impulse, and several markers also reported for other tissues have been found in the mammary epithelial cells of both rodents and humans (Table 1). There are, however, four main issues that require further investigation. The first is to determine whether the stem cells or progenitor cells that give origin to a complete mammary gland are the same cells that are affected by a carcinogenic process. Another important point that needs further clarification is the role of ERα as a marker of the stem cells. The third issue is the need to be extremely careful in validating conclusions drawn from *in vitro* studies by properly confirming them with *in vivo* data, in which numerous factors (such as age of the donor, reproductive history, number of samples studied, and consideration of intrinsic variability from sample to sample) are so important, but seldom considered in the major publications dealing with the stem cells in the mammary gland. Finally, the data reported in the literature tend to support the concept that the mammary gland contains Stem cells 1 that could be the progenitor of the differentiated breast or could be the site of origin of a neoplastic process. Supporting this concept is the fact that all the genes ascribed to the stem cells in the mammary gland are involved in more than one function of the normal breast as well as the malignant breast.

The evidence for Stem Cells 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 [2-12]. Russo and colleagues [5,10,17,52] 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. Alternative explanations attributed the protective effect of pregnancy to changes in the environmental milieu [53] and/or alterations in the immunological profile of the host [7]. 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 [26], who postulated that 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 (Stem cells 1), which are susceptible to undergo neoplastic transformation when exposed to a carcinogenic agent (see previous section and Fig. 2) [26]. After the postmenopausal involution of the mammary gland, the architecture of the parous breast is similar to the nulliparous breast, containing predominantly Lob 1 composed of Stem cells 2, an epithelial cell population that is refractory to transformation (Fig. 2).

It was further postulated that the degree of differentiation acquired through early pregnancy permanently changes the 'genomic signature' that differentiate Lob 1 of early parous women from that of nulliparous women, shifting the Stem cells 1 to Stem cells 2 that are refractory to carcinogenesis (Fig. 2). These cells were called Stem cells 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. Stem cells 2 are characterized by having a genomic signature that has been induced by the first cycle of differentiation (Fig. 2).

Supporting evidence to this hypothesis has been generated during the past 8 years by Russo and colleagues as well as by other researchers. Recent studies by Smith and colleagues [54-56] using transgenic whey acidic protein-driven Cre and Rosa 26-fl-stop-fl-LacZ mice provided evidence of a new mammary epithelial cell population that originates from differentiating cells during pregnancy; 5–10% of this parity-induced epithelium survives post-lactational involution after the first pregnancy. With successive pregnancies the population percentage increases, reaching 60% of the total epithelium in multiparous females. The parity-induced mammary epithelial cells (PI-MEC) are equivalent to Stem cells 2 as postulated by Russo and Russo [26] 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 females and parous females [54-56].

Several authors have focused on finding molecular changes as a mechanism of pregnancy-induced protection [57-64] (Table 2). Russo and colleagues 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-1β-converting enzyme, bcl-XL, bcl-XS, p53, p21, and c-*myc*, which can be from threefold to fivefold upregulated [57,58,65] (Table 2). 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 inhibin B, heterodimeric non-steroidal secreted glycoproteins with tumor suppressor activity, are also upregulated [57,58,65,66]. 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. The G/T mismatch-specific thymine DNA glycosylase gene is also increased fivefold in this model (Table 2).

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 pregnancy. These observations confirm previous *in vivo* findings that 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 [17]. In concordance with the studies of Srivastava and colleagues [57], Sivaraman and colleagues [61] 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 and Kittrell [59,60] 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 (Table 2).

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 the modulation of the transforming growth factor beta signaling pathway were upregulated in the post-treatment involuted mammary gland [62]. In that study, an unknown non-coding RNA (designated G.B7) and RbAp46, which has been implicated in a number of complexes involving chromatin remodeling, were found to be persistently upregulated in the lobules of the regressed glands (Table 2). Using gene profile analysis, D'Cruz and colleagues [64] also observed downregulation of growth factors potentially involved in epithelial proliferation, as well as persistent upregulation of transforming growth factor beta 3 and several of its transcripts targets in the involuted gland of parous rats and mice (Table 2).

Table 2

Genes found after pregnancy or pregnancy-like conditions in the mammary gland (Stem cells 2)

Gene	Abbreviation	Accession number	Cytoband	References
Adenosine deaminase	ADA	NP_000013	20q12-q13.11	[64]
Adipose differentiation-related protein	ADFP	M93275		[64]
B-cell CLL/lymphoma 2	BCL2	NP_000648	18q21.33	[57]
bcl-XL	BCL-XL	NP_612815	20q11.21	[57]
bcl-XS	BCL-XS	NP_001182	20q11.21	[57]
Bone morphogenetic protein 4	BMP4	NP_570912	14q22-q23	[57,58,66]
Carbonic anhydrase 2	CAR2	NP_000058	8q22	[64]
Carboxyl ester lipase	CEL	NP_001798	9q34.3	[64]
Casein alpha	CSNA	NP_001881	4q21.1	[62,64]
Casein beta	CSNB	NP_001882	4q21.1	[57,64]
Casein gamma	CSNG	D10215		[64]
Casein kappa	CSNK	NP_005203	4q21.1	[64]
Caspase 1, apoptosis-related cysteine protease (interleukin-1, beta, convertase)	CASP1	NP_150637	11q23	[57]
Catechol-*O*-methyltransferase	COMT	NP_009294	22q11.21-q11.23	[57]
Cell division cycle 42	CDC42	NP_426359	1p36.1	[62]
Chitinase 3-like 1	CHI3L1	NP_001267	1q32.1	[64]
Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	CLU	NP_976084	8p21-p12	[57,64]
c-*myc* proto-oncogene (myelocytomatosis viral oncogene homolog, avian)	MYC	NP_002458	8q24.12-24.13	[57]
Collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	COL2A1	NP_149162	12q13.11-q13.2	[57]
Collagen, type III, alpha 1	COL3A1	NP_000081	2q31	[57]
Cyclin D_1	CCND1	NP_444284	11q13	[64]
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	NP_510867	6p21.2	[57]
Decorin	DCN	NP_598014	12q13.2	[62]
Extracellular proteinase inhibitor	EXPI	X93037		[64]
Fatty acid binding protein 3 (mammary-derived growth inhibitor)	FABP3	NP_004093	1p33-p32	[57]
Folate-binding protein 1		ET63126		[64]
Follistatin-related protein	FSTL1	NP_077345	2	[62]
G/T mismatch-specific thymine DNA glycosylase	MTDG/TDG	NP_003202	12q24.1	[57,58,66]
Glycogen phosphorylase		NP_005600	11q12-q13.2	[57,58,66]
Heme-binding protein	PRDX1	NP_476455	5q36	[62]
Heterogeneous nuclear ribonucleoprotein A1	HNRPA1	NP_112420	12q13.1	[62]
Immunoglobulin A heavy chain	IgA	J00475		[64]
Immunoglobulin G heavy chain	IgG	ET61798		[64]
Immunoglobulin M heavy chain	IgM	ET61785		[64]
Inhibin, alpha	INHA	NP_002182	2q33-q36	[58,66]
Inhibin, beta B (activin AB beta polypeptide)	INHBB	NP_002184	2cen-q13	[58,66]
Inhibitor of DNA binding 2	IDB2	NP_002157	2p25	[64]
Kappa-immunoglobulin light chain	KIG LC	X16678		[64]
Lactalbumin, alpha	LALBA	NP_002280	12q13	[57,64]
Lactotransferrin	LTF	NP_002334		[64]
Lipocalin 2 (oncogene 24p3)	LCN2	NP_005555	9q34	[64]
Lipopolysaccharide binding protein	LBP	NP_004130	20q11.23-q12	[64]
Lysozyme P	LYP	NP_000230	12q15	[64]
Macrophage expressed gene 1	MPEG1	XP_166227	11q12.1	[64]
Matrix metalloproteinase 12	MMP12	NP_002417	11q22.3	[64]
Nap 1, Cdc42guanine exchange factor 9	ARHGEF9	NP_076447	Xq31	[62]
Phosphorylase, glycogen; muscle (McArdle syndrome, Glycogen storage disease type V)	PYGM	NP_005600	11q12-q13.2	[57,58,66]

Continued overleaf

Table 2 (continued)

Genes found after pregnancy or pregnancy-like conditions in the mammary gland (Stem cells 2)

Gene	Abbreviation	Accession number	Cytoband	References
Pleckstrin homology-like domain, family A, member 1	PHLDA1	NP_031376	12q15	[64]
Podocalyxin		AF109393.1	7	[62]
Protein kinase, AMP-activated, alpha 1 catalytic subunit	PRDAA1/AMPK	NP_996790	5p12	[57,58,66]
Protein kinase, AMP-activated, alpha 2 catalytic subunit	PRKAA2	NP_006243	1p31	[57,58,66]
Protein phosphatase-1, delta	PPP1CB	NP_037197	6q13	[62]
Retinoblastoma binding protein 7 (RbAp46)	RBBP7	NP_114004	Xq21	[62]
Retinol binding protein 1, cellular	RBP1	NP_002890	3q23	[64]
Ring zinc finger protein	RZFP	AF037205.1	3	[62]
Secreted phosphoprotein 1	SPP1	NP_000573	4q21-q25	[64]
Sp3 transcription factor	SP3	NP_003102	2q31	[62]
Stearyl-CoA desaturase (acyl-CoA desaturase, fatty acid desaturase, delta(9)-desaturase)	SCOAD	NP_005054	10q23-q24	[62]
Thymosin beta 4	TMSB4X	NP_066932	Xq21.3-q22	[57]
Transferrin	TNSF	NP_058751	8q32	[62]
Transforming growth factor, beta 3	TGFB3	NP_003230	14q24	[64]
Tumor protein p53 (Li–Fraumeni syndrome)	TP53	NP_000537	17p13.1	[57,59-61,63]
Tyrosine phosphatase Prl-1	PTP4A1	NP_113767	9q21	[62]
Vesicle-associated membrane protein 1 (synaptobrevin 1)	VAMP1	NP_954740	12p	[57,58,66]
Whey acidic protein	WAP	J00544		[64]
Zinc finger protein	ZFP	U90919		[62]
Zinc finger protein, Pzf	PZF	U05343.1		[62]

The proposed model of parity-induced specific changes [26] has been further confirmed by Ginger and Rosen [63], who reported that pregnancy induces multiple changes in the mammary epithelial cells, including nuclear accumulation of p53 and induction of whey acidic protein. 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 depicted in Table 2, all the genes that have been attributed to Stem cells 2 seem to work in different functional pathways than those described for Stem cells 1 (Table 1).

Although further work needs to be carried out in order to better understand the role of Stem cells 2 and their interaction with the genes that confer their specific signature, collectively the data described present evidence that pregnancy, through the process of cell differentiation, shifts Stem cells 1 to Stem cells 2 – cells that exhibit a specific genomic signature that could be responsible 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, 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 the highest rate of carcinogen binding to the DNA [5]. More importantly, when treated with carcinogens *in vitro*, Lob 1 epithelial cells express phenotypes indicative of cell transformation [20]. 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 (Fig. 2). These target cells will become the stem cells (Stem cells 1; Fig. 2) of the neoplastic event, depending upon the topographic location within the mammary gland tree, the age at exposure to a known or putative genotoxic agent, and the 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, and additional live births confer greater risk reduction [3]. The protection afforded by early full-term pregnancy in women could thus be explained by the higher degree of differentiation of the mammary gland at the time at which an etiologic agent, or etiologic 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 (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, becoming Stem cells 2 (Fig. 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 has been demonstrated in the rodent experimental system. However, if the shift of Stem cells 1 to Stem cells 2 has not been completed, a powerful enough carcinogenic stimulus 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.

Competing interests

The author(s) declare that they have no competing interests.

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