Published in final edited form as:

Adv Exp Med Biol. 2008 ; 617: 69–78.

Stem Cells, Hormones and Mammary Cancer

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Keywords

stem cell; MMTV; mammary; cancer; transplantation

Pregnancy and breast cancer

The incidence of breast cancer is influenced by age, genetics, ethnicity, diet, socioeconomic status, and reproductive history. The latter is the strongest and most reliable risk factor besides age and genetic susceptibility [1]. Reproductive factors have been associated with risk for breast cancer since the 17th century, when the disease was noted to be more prevalent among Catholic nuns. It is now a well-established fact that a full-term pregnancy early in life is associated with a long-term risk reduction for developing breast cancer. A woman who has her first child after the age of 35 has approximately twice the risk of developing breast cancer as a woman who has a child before age 20 (see current NCI Cancer Fact Sheet on Pregnancy and Breast Cancer Risk). Despite this long-term reduction in breast cancer risk in parous women, epidemiologists agreed at a recent NCI-sponsored workshop on “Early Reproductive Events and Breast Cancer” (<http://nci.nih.gov/cancerinfo/ere>) that each gestation *increases temporarily* the likelihood for developing breast cancer [2]. This transient increase in breast cancer risk lasts for a few years after a full-term pregnancy.

Pregnancy has a very similar dual effect on the etiology of mammary cancer in animal models. Like humans, parous rats and mice have a greatly reduced susceptibility to chemically induced mammary tumorigenesis compared to their nulliparous siblings [3]. Humans who carry germ line mutations in tumor susceptibility genes do not benefit from the protective effects of pregnancy, but have a significantly greater risk of developing the disease following one or multiple gestation cycles [4]. There are, however, conflicting reports whether lactation influences the onset of breast cancer in women with *BRCA1* mutations. The current view on breast cancer as a stem cell disease is founded on compelling evidence that many breast cancers may arise as clonal expansions from epithelial progenitors with an infinite lifespan [5]. It has been hypothesized that unique properties of mammary stem cells, such as self-renewal, make this population a prime target for transformation and tumorigenesis. Several experimental breast cancer models support this hypothesis. The most venerable is the mammary tumor virus [6] model in mice, where MMTV proviral insertions produce mutated mammary cells, which attain immortality (escape from growth senescence) and produce clones of mammary cells with increased propensity to develop mammary cancer. Serial transplantations of these preneoplastic lesions result in the formation of hyperplastic/dysplastic ductal trees, suggesting that multipotent cells are affected by MMTV transformation and that they pass on their neoplastic properties to their descendants [7]. Morphologically undifferentiated cells,

reminiscent of stem/progenitor cells are present in both premalignant and malignant mammary populations (Fig.1). Reproductive history has a profound impact on breast tumorigenesis, thus it is reasonable to assume that pregnancy and lactation have enduring effects on the cancer susceptibility of multipotent stem/progenitor cells.

Evidence that cancer stem cells sustain solid neoplasms has recently emerged [5,8]. Whether these “cancer stem cells” arise *de novo* or result from mutations within normal tissue stem/progenitor cells is presently unknown. A shift in the microenvironment of mammary epithelial cells as the result of pregnancy is a plausible mechanism by which to explain the greater refractivity of mammary tissue after early parity to cancer induction or progression. In a rat chemical carcinogenesis model, Nandi and his colleagues have argued that there is no difference in the susceptibility of the mammary epithelium between nulliparous and parous females to initiation (malignant transformation) by NMU; rather, it is a reduction in the incidence of progression of the “initiated” cells to frank malignancy. This difference in “progression” is completely reversible when the parous rodents are subjected to various hormonal regimens or given growth factors such as IGF-1 [9]. If this is correct, then epithelial (stem/progenitor) cell targets for carcinogenesis are the same but behave differently in their respective microenvironments (niches) during homeostatic tissue maintenance in the parous female.

Pregnancy mediates permanent changes in mammary epithelial cells

The basic principle for the dual phenomenon of pregnancy and breast cancer is that a gestation cycle induces massive proliferation and an endpoint differentiation of epithelial subtypes. Either permanent systemic changes following a full-term pregnancy (such as a decrease in circulating levels of hormones) or the alteration of the mammary tissue itself could explain the difference in breast cancer risk between nulliparous and parous women. Sivaraman and coworkers [10] suggested that the hormonal milieu of pregnancy affects the developmental state of a subset of mammary epithelial cells and their progeny.

Microarray evidence suggests that pregnancy mediates persistent changes in the gene expression profile in parous females [11,12]. These pregnancy-induced changes can be imitated through a transient administration of hormones, in particular estrogen and progesterone or human chorionic gonadotropin. Ginger et al. [12] used subtractive hybridization as a method to identify differentially expressed genes between hormone-treated Wistar-Furth rats and their untreated controls. Twenty-eight days after the last treatment, they identified approximately 100 differentially expressed loci. In a more comprehensive study, D’Cruz and colleagues [11] utilized oligonucleotide arrays to examine differences in the expression profile of approximately 5,500 genes between parous mice and their nulliparous controls. These initial results were verified by more laborious methods (northern blot analysis and *in situ* hybridization) and across several mouse strains as well as in two rat models.

The origination of parity-induced mammary epithelial cells during late pregnancy and lactation

Using the Cre-lox technology, a mammary epithelial subtype, which is abundant in non-lactating and non-pregnant, parous mice, was recently described [13]. These parity-induced mammary epithelial cells (PI-MEC) then permanently reside at the terminal ends of ducts (i.e. lobuloalveolar units) after post-lactational remodeling.

Two lines of evidence exist that the presence of PI-MECs in the involuted mammary gland is not an artifact caused by a deregulated activation of the promoter of our randomly integrated WAP-Cre construct. First, the WAP-Cre transgenic expression closely follows the activation

of the endogenous WAP locus and Ludwig and coworkers [14] have reported similar observations in genetically engineered mice that express Cre recombinase under the endogenous *Wap* gene promoter (i.e. WAP-Cre knock-in mutants). Second, limiting dilution transplantation assays with dispersed epithelial cells from nulliparous female mice demonstrate the existence of lobule-limited and duct-limited progenitors [15]. These studies were carried out with epithelial cells from WAP-LacZ transgenic mice where LacZ is expressed from the whey acidic protein promoter in late pregnant mice. Lobule-limited outgrowths positive for LacZ expression were observed in the implanted fat pads at parturition. Similar lobule-limited outgrowths were developed when PI-MEC were inoculated in limiting dilution into the cleared mammary fat pads of subsequently impregnated hosts (Fig.2). These structures like those described earlier [15] comprised both secretory luminal cells and myoepithelial cells and were 100% positive for LacZ activity indicating that they were developed entirely from PI-MEC. Therefore it is likely that PI-MEC arise from the lobule-limited progenitor population discovered by Smith [15] among the mammary epithelial cells present in nulliparous unbred females. In addition, to luminal and myoepithelial progeny, PI-MEC produced both small (SLC) and large undifferentiated light cells (ULLC) in the lobules. SLC and ULLC have essential roles in mammary stem/progenitor cell function [7]. The existence of committed mammary alveolar precursors in mice and rats has been proposed earlier [16,17].

PI-MEC are self-renewing and pluripotent

When fragments of gland containing PI-MEC were transplanted to gland-free fat pads in nulliparous hosts, PI-MEC contributed to ductal elongation in a very significant manner. The vast majority of resulting outgrowths contained X-Gal positive cells, and in >75% of the transplants, PI-MEC-derived cells were present throughout the *entire* ductal tree. These results clearly demonstrated that PI-MEC exhibit two important features of multipotent stem cells: self-renewal and contribution to diverse epithelial populations in ducts and alveoli. We demonstrated, for the first time, that the progeny from cells previously expressing an *alveolar* differentiation marker (i.e. WAP) could contribute to the formation of primary and secondary *ducts*. When the transplanted hosts were impregnated, the self-renewed PI-MEC at the tips of duct side branches proliferated during early pregnancy to form the new secretory acini. The transplantation procedure itself had no effect on the activation of the WAP-Cre and Rosa-LacZ transgenes because mammary fragments from nulliparous double transgenic donors never produced outgrowths with uniformly distributed X-Gal-positive cells [13].

To establish an estimate of the self-renewing ability of PI-MECs, mammary fragments containing X-Gal positive cells were transferred through four transplant generations [13]. Each successful transplant resulted in a 400-fold increase of the implanted epithelial population, which represents roughly an 8 to 9 (8.65)-fold doubling of the implanted cells.

To determine to what extent the presence of neighboring X-Gal-negative epithelial cells contributed to the self-renewing capacity of labeled PI-MEC, dispersed mammary epithelial cells from multiparous WAP-Cre/Rosa-LacZ females were inoculated at limiting dilutions into cleared fat pads, and the hosts were subsequently impregnated. All outgrowths contained LacZ-expressing cells, even though PI-MEC represented only 20% of the inoculated epithelial cells. Notably, no epithelial outgrowths were comprised entirely from unlabeled (LacZ-negative) cells. Both lobule-limited and duct-limited outgrowths were, however, entirely comprised from PI-MEC (and their LacZ-expressing descendants), as determined by serial sections through these structures. These results indicate that all luminal, myoepithelial, and cap cells of terminal buds may be derived from PI-MEC and their progeny. This conclusion was confirmed by demonstrating that the X-gal positive cells in these structures could be doubly stained for mammary cell lineage markers for myoepithelium (smooth muscle actin, Fig. 3), estrogen receptor alpha (ER- α), or progesterone receptor (PR).

WAP-TGF- β 1 expression aborts self-renewal of PI-MEC in transplants

The reproductive capacity of the mammary epithelial stem cell is reduced coincident with the number of symmetric divisions it must perform. In a study using WAP-TGF- β 1 transgenic mice, it was observed that mammary epithelial stem cells were prematurely aged due to ectopic expression of TGF- β 1 under the regulation of the *WAP* gene promoter [18]. To assess whether TGF- β 1 expression in PI-MEC abolishes their capacity to self-renew, mammary epithelia from WAP-TGF- β 1/WAP-Cre/Rosa-LacZ triple transgenic mice were transplanted into wild type recipients. It is important to note that the percentage of labeled cells in the triple transgenic glands after a single parity was indistinguishable from that observed in WAP-Cre/Rosa-LacZ double transgenic controls. As expected, mammary tissue implants and dispersed cells from the triple transgenic females, after either a single pregnancy or multiple gestation cycles, failed to produce full lobular development in full-term pregnant hosts. Perhaps more importantly, X-Gal positive cells were not observed in the ducts in these transplant outgrowths either in nulliparous or early pregnant hosts. LacZ-expressing cells did appear in the transplant population and were present in the lobular structures during late pregnancy in these transplants (after 15 days to parturition). In summary, the results of these studies demonstrate that the PI-MEC that develop during pregnancy and survive subsequent tissue remodeling in the absence of lactation in WAP-TGF- β 1 females were incapable or severely limited in their ability to self-renew in transplants and could not contribute to ductal development in subsequent transplant outgrowths. Therefore self-renewal (expansion outside of a stem cell niche) and proliferation competence (asymmetric divisions within a niche) appear to be properties independently affected by autocrine TGF- β 1 expression in the PI-MEC.

By definition, the self-renewal of stem cells occurs by two different processes. In asymmetric divisions, the most common activity of stem cells residing in a niche, the stem cell is preserved and one daughter becomes committed to a particular cell fate. Alternatively, a stem cell may divide symmetrically and expand to produce two or more stem cell daughters that retain stem cell properties. This latter form of self-renewal is essential for expansion of the stem cell population during allometric growth of the tissue (i.e. during ductal growth and expansion in the post-pubertal female or when the mammary epithelial implant is growing in the transplanted mammary fat pad). The negative effect of TGF- β 1 on the expansive self-renewal of PI-MEC supports our earlier observation regarding protection from mouse mammary tumor virus (MMTV)-induced mammary tumorigenesis in WAP-TGF- β 1 transgenic females [18]. This might suggest that the cellular targets for MMTV-mediated neoplastic transformation are PI-MEC because multiple pregnancies accelerate MMTV-induced oncogenesis [7].

PI-MEC and mammary tumorigenesis

Pregnancy has a dual effect on human breast cancer (protection or promotion), depending on the age of an individual, the period after a pregnancy, and the genetic predisposition. In genetically engineered strains that are highly susceptible to mammary tumorigenesis and exhibit accelerated tumor development in postpartum or parous females, one might expect that PI-MEC serve as targets for neoplastic transformation. The unique growth properties of PI-MEC (i.e. responsiveness to pregnancy hormones, survival during involution, and ability to self renew) make this epithelial subtype a potential target for pregnancy-associated tumorigenesis. Transgenic mice expressing the wild type *Her2/neu* (*ErbB2*) oncogene under transcriptional regulation of the MMTV-LTR seem to be suitable for studying the involvement of PI-MEC in pregnancy-associated mammary tumorigenesis since this animal model exhibits a relatively long latency of tumorigenesis (T50 of 205 days). Using this animal model, we demonstrated that: a) multiparous females consistently exhibited accelerated tumorigenesis compared to their nulliparous littermate controls in a mixed genetic background and b) PI-MEC were, indeed, primary targets of neoplastic transformation in this model [19]. The *de*

novo generation and amplification of a large number of hormone-responsive and apoptosis-resistant epithelial cells (i.e. PI-MEC) during the first and subsequent reproductive cycles might, therefore, account for the significantly increased cancer susceptibility of parous MMTV-neu transgenic females.

To further substantiate that PI-MEC are primary targets for neoplastic transformation in MMTV-neu transgenic mice, we eliminated or greatly impaired the growth of PI-MEC by deleting the *Tsg101* gene in cells that transiently activated WAP-Cre (i.e. females that carry two transgenes, MMTV-neu and WAP-Cre, in a homozygous *Tsg101* conditional knockout background). The complete deletion of *Tsg101* can serve as an excellent negative “selection marker” for WAP-Cre expressing cells since this gene is indispensable for the survival of normal, immortalized, and fully transformed cells [20]. In multiparous MMTV-neu females, impaired genesis or elimination of PI-MEC resulted in a significantly reduced tumor onset, suggesting that restraining the growth and survival of differentiating alveolar cells during pregnancy (and therefore PI-MEC in parous mice) eliminates the cellular basis for transformation in this model.

Some PI-MEC are asymmetrically dividing long-label retaining cells

It was proposed over 30 years ago that somatic stem cells avoid accumulation of genetic errors resulting from DNA synthesis prior to dividing by selectively retaining their template DNA strands and passing the newly synthesized strands to their committed daughters [21]. Therefore somatic epithelial stem/progenitor cells labeled by DNA analogs during their inception will become long-label retaining epithelial cells (LREC). Label retention has long been considered to be a characteristic of somatic stem cells and this propensity to retain DNA label has been explained by postulating that somatic stem cells seldom divide and are mainly proliferatively quiescent. Recent studies have however shown that in multiple tissues long-label-retaining cells are actively dividing and asymmetrically retain their labeled template DNA strands while passing the newly synthesized DNA to their differentiating progeny [22,23]. Interestingly, PI-MEC that have proliferated extensively in transplants give rise to LacZ-positive progeny which retain the original DNA label for long periods and when pulsed with a second alternative DNA label prove to be actively traversing the cell cycle and thus become doubly labeled incorporating the second label into new DNA strands. Subsequent to a short chase period the second label is transferred along with the new DNA strands to LacZ-positive progeny (Fig 4). This evidence demonstrates that during self-renewal PI-MEC produce progeny (in addition to luminal and myoepithelial offspring) that behave as asymmetrically dividing stem/progenitor cells responsible for the steady-state maintenance of the diverse LacZ-positive mammary epithelial population in the resulting outgrowth.

Conclusion

Stem cells are defined by how they act physiologically in the context of heterologous cells, i.e. the microenvironment or stem cell niche that balances protecting stem cells from exhaustion and protecting the host from unregulated stem cell growth. In addition to this complex model, it has been demonstrated recently that not only normal tissues, but also neoplastic lesions contain heterogeneous (hierarchical) types of stem cells [24]. The discovery and genetic labeling of a parity-induced mammary epithelial cell population that is specific for parous females makes it possible to further examine the concept of stem cell hierarchy in the mammary gland and the homeostasis of mammary stem cells within the niche. In addition, our study of this progenitor population provides direct evidence for the proof of principle that a stem/progenitor cell may be the target of carcinogenic events and also that progression to frank malignancy is dependent upon the continued ability of the affected cell to expansively proliferate.

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Figure 1.

This electron micrograph depicts an ultra thin section through one of the acini in an MMTV-induced alveolar hyperplasia. There is evidence of virus replication (MMTV), of secretory activity leading to secretory granule formation in the apical cytoplasm of the luminal cells and release into the lumen. An undifferentiated suprabasal cell (SLC) is present and proximal to it, a differentiated myoepithelial cell (arrow). Bar equals 1.0 μ M.

Figure 2.

The image shows a section through a lobule-limited LacZ-positive outgrowth in full term pregnant host composed entirely of progeny from PI-MEC. The growth comprises both luminal and myoepithelial (long arrows) cells and small-undifferentiated light cells (short arrows). Bar equals 20 μ M.

Figure 3.

This composite shows LacZ-negative acini stained for SMA in the upper left panel and LacZ-positive acini in the remaining three panels. The arrows indicate the myoepithelial cells demonstrated by positive SMA-staining. The LacZ-positive cells appear as dark gray in this grayscale figure. Bars equal 5 μ M. Thus, PI-MEC are not only self-renewing, but they are pluripotent as well, giving rise to progeny that differentiate along all the epithelial cell lineages of the mammary gland.

Figure 4. PI-MEC long-label-retaining (^3H -thymidine) with autoradiographic grains were doubly labeled with 5BrdU (darker nuclei) in transplant labeled with thymidine 7 weeks earlier following a 2 day pulse with 5BrdU and produced 5BrdU-labeled-only (arrows) daughters after a 6 day chase.