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# Pregnancy and Stem Cell Behavior

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The identification of cancer-initiating epithelial subtypes (i.e. cancer stem cells) is important for gaining a more comprehensive understanding of the process of neoplastic transformation and tumorigenesis. Since reproductive history has a major impact on breast tumorigenesis, it is reasonable to assume that pregnancy and lactation have enduring effects on the cancer susceptibility of multipotent progenitors. Using the Cre-lox technology as a tool to genetically label pregnancy-hormone-responsive cells, we identified a mammary epithelial subtype that is abundant in parous females. These pregnancy-induced mammary epithelial cells (PI-MECs) originate from differentiating cells during the first pregnancy and lactation cycle. They do not undergo apoptosis during postlactational remodeling, and they persist throughout the remainder of a female's life. In this review, we discuss the biological relevance of PI-MECs in multiparous females and their important stem cell-like features, such as self renewal, as well as their ability to produce progeny with diverse cellular fates. Using appropriate animal models, we further demonstrate that PI-MECs are cellular targets for pregnancy-enhanced mammary tumorigenesis.

**KEY WORDS:** mammary gland; differentiation; parity-induced mammary epithelial cells; stem cells; Cre recombinase; tumorigenesis; ErbB2; MMTV.

## 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 seventeenth 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 years has approximately twice the risk of developing breast cancer

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*Abbreviations used:* BRCA1/2, breast and ovarian cancer gene 1/2, early onset; Cre, site-specific recombinase in bacteriophage P1 (catalyses recombination between *lox*P sites); DMBA, 7,12-dimethylbenz[a] anthracene; ER, estrogen receptor; IGF-1, insulin-like growth factor 1; Jak2, Janus kinase 2; LacZ, gene encoding ß-galactosidase from E. coli; loxP, locus of crossing (X-ing) over; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; NCI, National Cancer Institute; Neu, a.k.a. Her2 or ErbB2; member of the epidermal growth factor receptor family; Nkcc1, sodium, potassium, and chloride (Na+/K+/2Cl) transporter; NMU, N-nitrosomethyl urea; PI-MECs, parity-induced mammary epithelial cells; PyVMT, polyoma middle T oncogene; Rosa, transcriptionally active locus identified by gene trap in murine embryonic stem cells using a retroviral vector with Reverse-Orientation-Splice-Acceptor; Sca-1, stem cell antigen 1 or lymphocyte activation protein 6A; Stat5, signal transducer and activator of transcription 5; TEB, terminal end bud; TGF-$\beta$1, transforming growth factor beta 1; WAP, whey acidic protein; Wnt1, wingless-related MMTV integration site 1; X-Gal, 5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside.

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 [see also *(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 *(3,4)*. In contrast, most transgenic strains that express oncogenes under steroid and peptide hormone-responsive promoters exhibit pregnancy-associated mammary cancers or accelerated tumorigenesis in nonpregnant, parous females. These transgenic models are, therefore, less suitable to recapitulate the protective effects of pregnancy on breast cancer, but they are effective in modeling the transient increase in breast cancer risk following a full-term pregnancy. Like genetically engineered models, humans who carry germline mutations in tumor susceptibility genes (such as *BRCA1* and *BRCA2*) 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 *(5)*. There are, however, conflicting reports whether lactation influences the onset of breast cancer in women with *BRCA1* mutations. In a recent study, Jernstrom *et al. (6)* reported that a 1-year lactation period reduced breast cancer risk in *BRCA1* (but not in *BRCA2*) carriers. However, the authors also mention that this statistical correlation might be misleading since the reasons for not breast-feeding or for stopping breast-feeding were not recorded. Thus, women with *BRCA1* mutations who have difficulty breastfeeding could be at greater risk for breast cancer than carriers who have no trouble lactating. From controlled experiments using genetically engineered mice, we know that *Brca1*-deficiency leads to impaired mammary development *(7)*.

The current view on breast cancer as a stem cell disease is founded on compelling evidence that many breast cancers arise as clonal amplicons from epithelial progenitors with an infinite lifespan *(8–12)*. 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 *(11,12)*. This assumption is supported by several lines of evidence. First, serial transplantations of preneoplastic lesions result in the formation of hyperplastic/dysplastic ductal trees, suggesting that (a) multipotent cells are affected by transformation and (b) they pass on their neoplastic properties to their descendents *(13,14)*. Second, chemical carcinogens seem to preferentially target terminal end buds (TEBs), which are growing distal parts of ducts that contain numerous stem cells *(11,15)*. Third, the stem cell marker Sca-1 is upregulated in carcinomas, including breast cancer cell lines *(16,17)*. The identification of cancer-initiating epithelial subtypes (also known as "cancer stem cells") is, therefore, of utmost importance to understand the process of tumorigenesis. Since reproductive history has such a profound impact on breast tumorigenesis, it is reasonable to assume that pregnancy and lactation have enduring effects on the cancer susceptibility of multipotent progenitors or stem cells.

Emerging evidence suggests that cancer stem cells sustain solid neoplasms *(18,19)*. Little is known, however, about the normal cells originally transformed and the resulting cancer stem cells. A shift in the biology of targeted 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. From experiments in the rat NMU chemical carcinogenesis model, Nandi and his colleagues have argued that there is no difference in the susceptibility of the mammary epithelium to initiation (transformation toward malignancy and mutation fixation) between nulliparous and parous hosts; rather, it is a reduction in the rate of progression to frank malignancy. This refractory state is completely reversible when the parous rodents are subjected to various hormonal regimens or given growth factors such as IGF-1 *(20)*. If this view is correct, then epithelial (stem) cell targets for carcinogenesis are similar but behave differently in their respective microenvironment (niche) 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 (induction or protection depending on the genetic predisposition) is that a

gestation cycle induces a massive proliferation, but also the differentiation of epithelial subtypes that are susceptible to neoplastic transformation. Systemic changes following a full-term pregnancy (such as a decrease in circulating levels of hormones) or more probably the alteration of the mammary tissue itself might explain differences in cancer susceptibility between nulliparous and parous women. A widely accepted explanation for the protective effects of pregnancy on breast cancer, offered by Russo and Russo *(3)*, is that pregnancy induces the differentiation of the target structures for carcinogenesis, i.e. multipotent progenitors in terminal end buds and duct termini. In accordance with this model, Sivaraman and coworkers *(21,22)* suggested that the hormonal milieu of pregnancy affects the developmental state of a subset of mammary epithelial cells and their progeny. This results in persistent differences in their response to carcinogenic challenge. These changes are reflected in the muted proliferative response to carcinogen exposure by the affected cells and the appearance of a sustained expression of nuclear p53 in the hormone-treated epithelium. The proliferation block and the induction of p53 occur both in rats and mice and support the generality of this hypothesis.

A number of physiological differences between nulliparous and (multi)parous mammary epithelia have been reported in the past *(23–26)*. These differences include the dependence of nulliparous epithelia on exogenous factors for functional differentiation *(26)*, as well as DNA synthesis as a prerequisite for the responsiveness of nulliparous epithelia to lactogenic hormones *(24)*. Newer evidence suggests that pregnancy mediates persistent changes in the expression profile of a number of genes in parous females *(27,28)*. These pregnancy-induced changes can be imitated through a transient administration of hormones, in particular estrogen and progesterone or human chorionic gonadotropin *(29–32)*. Ginger *et al. (27)* 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 *(27)*. In a more comprehensive study, D'Cruz and colleagues *(28)* utilized oligonucleotide arrays to examine differences in the expression profile of approximately 5500 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 authors identified a set of 38 genes that can be applied to determine the reproductive history of females in a blinded manner. Some of the differentially expressed genes represent growth factors, such as insulin-like growth factor-1 (IGF-1), amphiregulin, or the transforming growth factor (TGF)-$\beta$3. As discussed by Ginger and Rosen *(33)*, both gene expression studies in hormone-treated or parous rats and mice revealed changes in analogous growth factor signaling pathways that might have important mechanistic implications. On the basis of persistent changes in genes of the histone methyltranferease complex, the authors further hypothesize that epigenetic factors in chromatin remodeling, DNA methylation, and histone modification might be involved in the parity-induced changes in the fate of particular epithelial subtypes. They also pointed out a limitation of these studies, such as a deregulated expression of particular genes, could be caused by systemic changes in the hormonal milieu [for example the growth hormone-IGF-1 axis]. Other limitations are that these studies do not discriminate expression profiles of genes in diverse cell types present in the mammary gland (epithelia vs. stroma), in epithelial subtypes, and, more importantly, in multipotent progenitors that are the suggested targets for neoplastic transformation. As Ginger *et al. (27)* demonstrated, not all mammary epithelial subtypes seem to undergo the same pregnancy-induced changes. The differential expression of selected genes occurs primarily at the distal ends of tertiary side branches (i.e. lobuloalveolar units) but not in major collecting ducts. Consequently, a critical step to further analyze the molecular mechanisms for pregnancy-mediated changes in gene expression is the labeling and isolation of mammary epithelial subtypes, in which these pregnancy-hormone-induced persistent changes take place.

## THE ORIGINATION OF PARITY-INDUCED MAMMARY EPITHELIAL CELLS DURING LATE PREGNANCY AND LACTATION

Using the Cre-lox technology (Fig. 1), we have recently identified a mammary epithelial subtype, which is abundant in nonlactating and nonpregnant, parous mice *(34)*. This epithelial subpopulation originates from differentiating cells during the first pregnancy. These parity-induced mammary epithelial cells (PI-MECs) then permanently reside at the terminal ends of ducts (i.e. lobuloalveolar units)

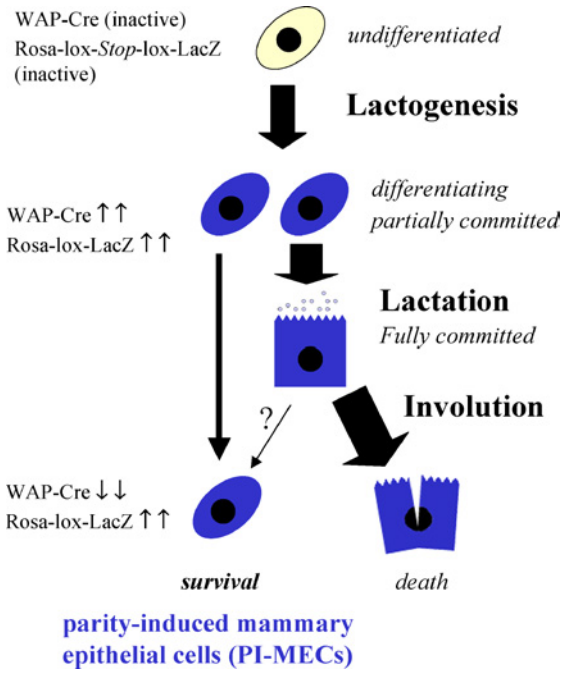


**Fig. 1.** The Cre-lox system as a technology to genetically label a mammary epithelial subpopulation, which is specific for nonpregnant, parous females. The transient upregulation of Cre recombinase expressed by the WAP promoter in differentiating epithelial cells during late pregnancy permanently activates an inactive reporter transgene (Rosa-lox-STOP-lox-LacZ→Rosa-lox-LacZ) due to the Cre-mediated excision of the floxed transcriptional STOP sequence between the ubiquitously expressed Rosa promoter and the beta-galactosidase (LacZ) coding sequence. Hence, the cell-type specific activation of the Rosa-LacZ gene is not dependent on the differentiation status of a given cell, and therefore, the permanent activation of the reporter transgene labels differentiating cells that are apoptosis resistant during involution. The reporter remains active in cells that change their fate during development whether they still express Cre recombinase or not. Thus, the labeled cells in the remodeled gland represent an epithelial subtype that is not abundant in the nulliparous gland. X-Gal staining can be used to label individual beta-galactosidase expressing cells in fixed tissues or in culture.

after postlactational remodeling. The expression of the *whey acidic protein* (Wap) gene is very often used to monitor an advanced differentiation status of alveolar cells during the second half of pregnancy. The transient upregulation of Cre recombinase expressed by the *Wap* gene promoter *(35)* permanently activates a Rosa-LacZ reporter transgene *(36)* at mid-gestation, due to the Cre-mediated excision of a floxed transcriptional STOP sequence between the *Rosa* promoter and the *β-galactosidase* (*LacZ*) coding sequence. The ubiquitously expressed Rosa-LacZ reporter transgene remains active in cells that change their fate during development regardless of Cre recombinase expression. Consequently, the activation of the reporter transgene permanently labels pregnancy-hormone–responsive cells with an advanced differentiation profile that are apoptosis resistant during the postlactational involution stage. A standard X-Gal staining technique can be utilized to visualize these β-galactosidase expressing cells. Nulliparous Wap-Cre/Rosa-LacZ double transgenic females exhibit between 0.8–4% of X-Gal-positive cells, depending on the stage of the estrus cycle. The number of labeled cells increases to approximately 20–30% in nonpregnant, multiparous mice, and, therefore, we named this epithelial subtype the parity-induced mammary epithelial cell population (PI-MECs) *(34)*. In unpublished studies, we observed that this epithelial subtype remains present in the mammary gland throughout the remainder of the life of a parous female (Matulka and Wagner, unpublished data).

The labeling technique to visualize PI-MECs has two shortcomings that are innate to the Cre-lox technology. First, the methodology cannot distinguish cells that continue to express Cre recombinase from those that completely silence the WAP-Cre transgene after postlactational remodeling. Second, the technology does not discriminate cells with high or low levels of WAP-Cre transgene expression. To address the first issue, we performed an immunohistochemical staining of WAP and Cre recombinase to determine the expression of the endogenous Wap protein and a residual activity of the WAP-Cre transgene in PI-MECs *(37)*. As shown in Fig. 2, we were unable to detect expression of endogenous Wap or nuclear staining of Cre recombinase in X-Gal positive PI-MECs. These observations suggest that the regulatory elements of the *Wap* gene (endogenous and transgene, respectively) are not constitutively active in these cells. The second limitation of the Cre-lox technique is more difficult to assess experimentally due to the lack of more advanced differentiation markers than WAP. While the overall expression of WAP correlates clearly with advanced differentiation of the entire mammary gland, it is currently unknown whether a difference in endogenous *Wap* gene expression from one differentiating cell to another truly represents a scale in a more or less advanced differentiation stage of a given cell. Therefore, the Cre-lox technology cannot provide experimental proof whether PI-MECs originate from partially committed cells expressing WAP that are not terminally differentiated, or whether they arise from differentiated cells that bypass apoptosis and

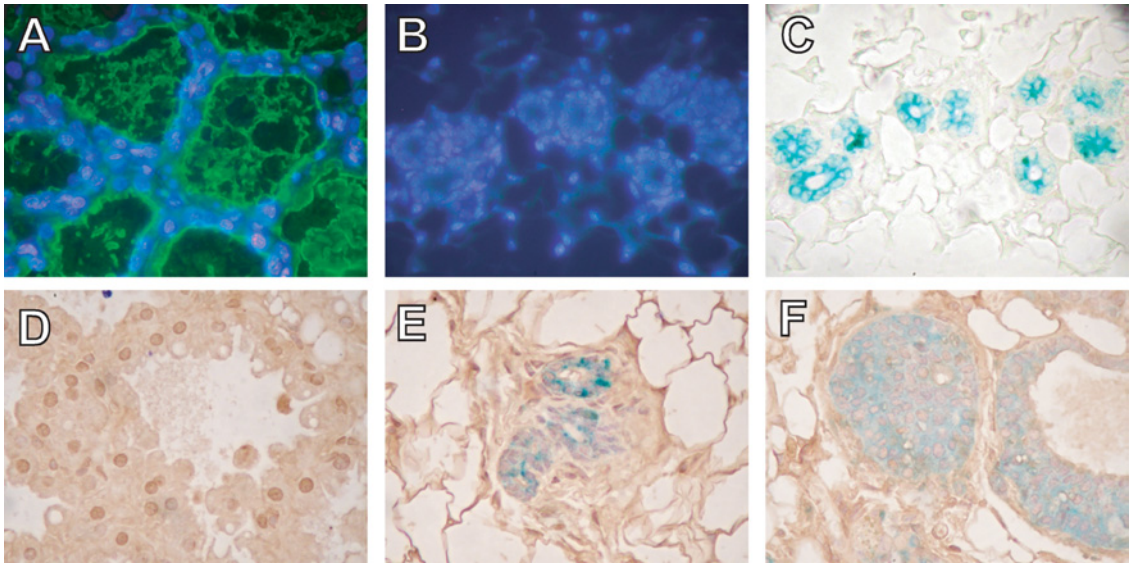


**Fig. 2.** Immunostaining of the whey acidic protein, WAP (A–C), and Cre recombinase (D–F) in formalin-fixed mammary sections from lactating (A, D) and nonpregnant, parous (B, C, E) females that carry the WAP-Cre (A, D) and the WAP-Cre/Rosa-LacZ transgenes. Panel F represents a section through an early dysplastic lesion in a parous female that carries the MMTV-neu transgene in addition to the double transgenic reporter. The blue X-Gal staining in panels C, E, and F indicates the location of parity-induced epithelial cells or their derived neoplastic descendents (F). Note that the whey acidic protein is abundant in the milk and the apical membrane of secretory epithelial cells (A) whereas this protein is not present in detectable levels in PI-MECs in the involuted gland (B; C is the corresponding bright-field image to B). Cre recombinase can be visualized in nuclei of secretory epithelial cells in lactating females carrying the WAP-Cre transgene (D), but WAP-Cre expression cannot be detected in labeled PI-MECs in nonpregnant, parous females or their derived MMTV-neu transformed progeny. Slides were counterstained with DAPI (A–C) or hematoxylin (D–F). Magnification 640×.

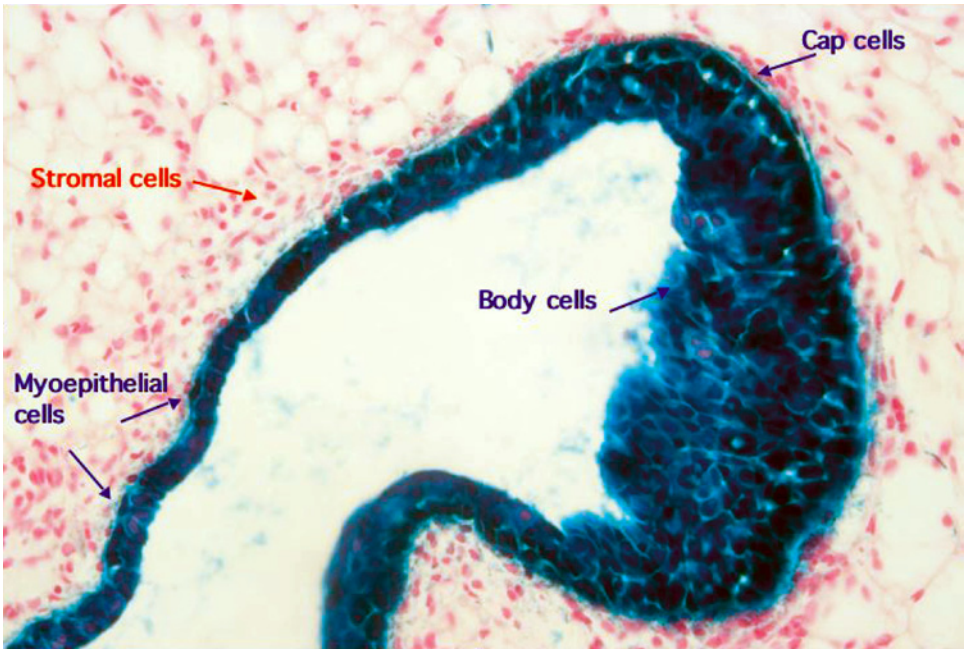


**Fig. 3.** Section through an X-Gal-stained terminal end bud (TEB) of a duct-limited outgrowth after transplantation of limiting dilutions of PI-MECs from parous, nonpregnant WAP-Cre/Rosa-LacZ double transgenic mice into the cleared fat pad of wildtype recipients. Note that all epithelial subtypes, including myoepithelial cells and cap cells of the TEB, appear to be derived from LacZ-expressing PI-MECs.

revert into a less differentiated state, or both (Fig. 1). Our current understanding of mammary development might favor the first hypothesis whereas experimental evidence (lack of endogenous WAP gene expression and undetectable nuclear accumulation of Cre recombinase in PI-MECs) might indicate that cells are able to revert into a "less differentiated" state under changing physiological conditions during postlactational remodeling.

Despite the gap in our understanding about the precise origin of PI-MECs, this unique epithelial population can be clearly defined as a pregnancy-hormone-responsive cell type that originates during late gestation and exhibits, at least transiently, an advanced differentiation profile. 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 transgene expression closely follows the activation of the endogenous WAP locus *(34,35)*. Second, Ludwig and coworkers *(38)* 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). Finally, it is incorrect to assume that PI-MECs originate at different time points during ontogenesis or mammogenesis (i.e. embryonic or postnatal mammary development in virgin mice). As stated above, the basic principle of the Cre-lox-based labeling method is that any descendants from proliferating cells that activate Cre recombinase remain tagged whether they still express Cre or not after differentiation or even neoplastic transformation (see last paragraph). Since primary or secondary side branches in WAP-Cre/Rosa-LacZ double transgenic virgins are X-Gal negative, a transient activation of WAP-Cre or the survival of labeled epithelial cells during earlier stages of mammogenesis is unlikely. We previously reported that very few X-Gal positive cells can be detected at the terminal end of a limited number of ducts *(34)*. We determined that these cells are being transiently amplified from less than 1% to about 4% during estrus, and they decline to about 1% during metestrus, suggesting that these cells are not a permanent population in the nulliparous gland. A similar observation about WAP expression in the virgin gland as a result of fluctuating levels of hormones during the estrus cycle has been previously reported *(39,40)* Clearly, these transient LacZ-expressing cells in the virgin do not serve as alveolar progenitors during the first pregnancy cycle, since developing alveoli are X-Gal negative until around mid-gestation, when WAP-Cre and endogenous *Wap* become active. Therefore, PI-MECs originate from certain specific differentiating cells during pregnancy and lactation, and they are not simply descendants of a small number of cells that transiently express WAP-Cre in the nulliparous gland. If these transiently X-Gal positive cells were maintained after each estrus cycle then, after several months, glands would contain significantly higher numbers of labeled cells; this was not observed, even in nulliparous females surviving up to two years of age (Smith, G.H. unpublished observation).

## PI-MEC'S ARE SELF-RENEWING AND PLURIPOTENT

The permanent labeling of PI-MECs and their decedents allowed us to study their unique growth properties in greater detail. Based on the location of the X-Gal positive cells at the extremity of the ductal side-branches, we hypothesized that these cells might serve as progenitors for proliferation and differentiation of alveolar cells during subsequent pregnancies in multiparous mice. We, therefore, examined the role of X-Gal positive PI-MECs during early stages of the second pregnancy (day 8 of gestation) in WAP-Cre/Rosa-LacZ double transgenic females. Newly committed alveolar cells proliferate highly at this stage of mammogenesis, but *Wap* gene expression remains low. This initial experiment revealed that in the developing alveoli, cells proximal to existing PI-MECs were X-Gal positive, indicating that these cells were descendants of PI-MECs. We concluded that PI-MECs serve as committed alveolar progenitors among the luminal epithelium of multiparous animals. Collecting ducts and large side branches do not contain PI-MECs after multiple pregnancy, lactation, and involution cycles.

The existence of committed alveolar precursors has been postulated earlier *(41–43)*. Unlike undifferentiated alveolar progenitors, the unique developmental route that PI-MECs undergo during each pregnancy cycle is the basis for a general mechanism of selection and adaptation of alveolar cells to accomplish optimal functional differentiation and normal milk production. This concept about a universal mechanism of selection is founded on the existence of a cell population, such as PI-MECs, that "knows" how to differentiate (expression of advanced differentiation markers), that survives apoptosis during postlactational remodeling, and that serves as an alveolar progenitor pool during subsequent

pregnancies. We named this developmental phenomenon the "functional memory" of the mammary epithelium after demonstrating that PI-MECs were selectively amplified in normal lactating, multiparous females that were unable to support a litter during the first and second lactation period *(34)*. Conversely, the selective inhibition of PI-MEC multiplication and apoptosis mediated by TGF-$\beta$1 expression from the *whey acidic protein* promoter during pregnancy leads to a lactation failure in multiparous animals *(44)*. This appears to be the combined result of early apoptosis of differentiating PI-MEC progeny and the loss of the capacity of PI-MECs to expand numerically in symmetric divisions. The second point was confirmed by the observation that WAP-TGF-$\beta$1-expressing PI-MECs were unable to self-renew and contribute to ductal outgrowth upon transplantation into epithelium-divested mammary fat pads *(44)*.

When fragments of gland containing PI-MEC's were transplanted to gland-free fat pads in nulliparous hosts, PI-MECs contributed to ductal elongation in a very significant manner *(34)*. 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-MECs 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 cells previously expressing an *alveolar* differentiation marker (i.e. WAP) can contribute to the formation of primary and secondary *ducts*. When the transplanted hosts were impregnated, the self-renewed PI-MECs 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 *(44)*.

Unlike the few LacZ-expressing cells in the nulliparous gland, X-Gal positive PI-MECs present in the mammary glands of parous females following involution are capable of proliferation and self-renewal. To establish an estimate of the self-renewing ability of PI-MECs, we transferred mammary fragments containing X-Gal positive cells through four transplant generations *(44)*. Each successful transplant resulted in a 400-fold increase of the implanted epithelial population, which represents roughly an 8–9 (8.65)-fold doubling of the implanted cells *(42)*. We recovered LacZ-expressing cells from all of these transplants. The presence of the floxed transcriptional STOP sequence within the Rosa-LacZ reporter was confirmed by PCR, indicating that the mixed outgrowths with both X-Gal-negative and X-Gal-positive cells were the result of the proliferation of both cell types and not due to gene silencing of a Cre-lox activated Rosa-LacZ sequence. Therefore, PI-MECs are capable of self-renewal and proliferation over several transplant generations for at least 35 ($4 \times 8.65$) doublings. PI-MECs were not present in transplants ($N = 2$) that exhibited growth senescence (i.e. filling less than 15% of the available fat pad). This observation suggests that the presence of PI-MECs may contribute fundamentally to continued growth.

To determine to what extent the presence of neighboring X-Gal-negative epithelial cells contributed to the self-renewing capacity of labeled PI-MECs, 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 *(44)*. All outgrowths contained LacZ-expressing cells, even though PI-MECs 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-MECs (and their LacZ-expressing descendents), 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-MECs and their progeny (Fig. 3). 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), estrogen receptor alpha (ER-$\alpha$), or progesterone receptor (PR). Thus, PI-MECs 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.

## WAP-TGF-$\beta$1 EXPRESSION ABORTS SELF-RENEWAL OF PI-MEC'S 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-$\beta$1 transgenic mice, it was observed that mammary epithelial stem cells were prematurely aged due to ectopic expression of TGF-$\beta$1 under the regulation of the *WAP* gene promoter *(40)*. To assess whether TGF-$\beta$1 expression in PI-MECs abolishes their capacity to self-renew, mammary epithelia from WAP-TGF-$\beta$1/WAP-Cre/Rosa-LacZ triple transgenic mice were transplanted into wildtype recipients *(44)*. It is important to note that the percentage of labeled cells in the triple transgenic glands after a single parity was indistinguishable from that observed in WAP-Cre/Rosa-LacZ double transgenic controls. As expected, mammary tissue implants and dispersed cells from the triple transgenic females, after either a single pregnancy or multiple gestation cycles, failed to produce full lobular development in full-term pregnant hosts. Perhaps more importantly, X-Gal positive cells were not observed in the ducts in these transplant outgrowths either in nulliparous or early pregnant hosts. LacZ-expressing cells did appear in the transplant population and were present in the lobular structures during late pregnancy in these transplants (after 15 days to parturition). In summary, the results of these studies demonstrate that the PI-MECs that develop during pregnancy and survive subsequent tissue remodeling in the absence of lactation in WAP-TGF-$\beta$1 females were incapable or severely limited in their ability to self-renew in transplants and could not contribute to ductal development in subsequent transplant outgrowths. Therefore self-renewal (expansion outside of a stem cell niche) and proliferation competence (asymmetric divisions within a niche) appear to be properties independently affected by autocrine TGF-$\beta$1 expression in the PI-MECs.

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 *(45)*, 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 postpubertal female or when the mammary epithelial implant is growing in the transplanted mammary fat pad). The negative effect of TGF-$\beta$1 on the expansive self-renewal of PI-MECs supports our earlier observation regarding protection from mouse mammary tumor virus (MMTV)-induced mammary tumorigenesis in WAP-TGF-$\beta$1 transgenic females *(46)*. This might suggest that the cellular targets for MMTV-mediated neoplastic transformation are PI-MECs because multiple pregnancies accelerate MMTV-induced oncogenesis *(47)*.

## PI-MEC'S AND MAMMARY TUMORIGENESIS

We discussed earlier that 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. We hypothesized that *in normal* mammary epithelia, PI-MECs might mediate the long-term protective effects against cancer development since (a) these cells initiate a differentiation program during pregnancy, (b) they are located at the terminal end of ducts where neoplasms are suggested to originate *(48)*, and (c) they are present the entire life following just one full-term pregnancy and lactation period. We are currently testing this assumption by studying DMBA-treated nulliparous and parous WAP-Cre/Rosa-LacZ females to see whether or not differentiating, hormone-responsive cells that activate (or had activated) the *WAP* promoter are able to serve as cancer stem cells.

In genetically engineered strains that are highly susceptible to mammary tumorigenesis and exhibit accelerated tumor development in postpartum or (multi)parous females, one might expect that PI-MECs serve as targets for neoplastic transformation. The unique growth properties of PI-MECs (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 wildtype Her2/neu (ErbB2) oncogene under transcriptional regulation of the MMTV-LTR *(49)* seem to be suitable for studying the involvement of PI-MECs in pregnancy-associated mammary tumorigenesis since this animal model exhibits a relatively long latency of tumorigenesis (T50 of 205 days). It is, therefore, possible to generate primiparous and multiparous females many weeks or months before the first tumor becomes palpable. MMTV-neu mice generate ER-negative lesions that exhibit histopathological features similar to a subset of human breast cancers *(50)*. More importantly, the overexpression of Her2/neu has been observed in a significant subset of pregnancy-associated breast cancers in humans

*(51)*. Using this animal model, we recently demonstrated that (a) multiparous females consistently exhibited accelerated tumorigenesis compared to their nulliparous littermate controls in a mixed genetic background and (b) PI-MECs were, indeed, primary targets of neoplastic transformation in this model *(37)*. Interestingly, the significantly fewer lesions that arose in nulliparous controls originated from hormone-responsive cells that transiently activated WAP-Cre (i.e. an epithelial subpopulation that represents only 1–4% of all epithelial cells in the virgin gland; see above). The de novo generation and amplification of a large number of hormone-responsive and apoptosis-resistant epithelial cells (i.e. PI-MECs) 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-MECs are primary targets for neoplastic transformation in MMTV-neu transgenic mice, we eliminated or greatly impaired the growth of PI-MECs 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 *(52–54)*. In multiparous MMTV-neu females, impaired genesis or elimination of PI-MECs resulted in a significantly reduced tumor onset *(37)*, suggesting that restraining the growth and survival of differentiating alveolar cells during pregnancy (and, therefore PI-MECs in parous mice) eliminates the cellular basis for transformation in this model. This assumption is currently being verified again by deleting the *Jak2* gene from the entire mammary ductal tree in MMTV-neu transgenic mice (Sakamoto and Wagner, unpublished). Using a conditional knockout approach, we and others have recently shown that the interference of Jak2-Stat5 signaling during ductal elongation leads to impaired proliferation and specification of alveolar progenitor cells during pregnancy *(55, 56)*. We expect that the selective growth inhibition of alveolar precursors (and, therefore, PI-MECs in multiparous females) will significantly lower the incidence of neoplastic transformation in MMTV-neu transgenic mice.

Since the *Wap* gene promoter and the *MMTV-LTR* are synchronously upregulated during pregnancy, it is logical to assume that the expression of the Her2/neu oncogene might be higher in hormone-responsive PI-MECs compared to other epithelial subtypes. This raises the possibility that tumorigenesis in virally initiated (e.g. MMTV-induced) models (see previous section) or other MMTV-promoter-driven transgenics originate from the same epithelial subtype (i.e. PI-MECs). Indeed, we and others have shown that neoplasms in females expressing the polyoma middle T oncogene (MMTV-PyVMT) are comprised of labeled PI-MECs *(37,57)*, and it can be hypothesized that this might also be the case for other MMTV-driven oncogenes that act in a cell autonomous fashion. Since a variety of oncogenes, such as TGF$\alpha$ and Wnt1, act as autocrine as well as paracrine growth factors *(58,59)*, however, it would be incorrect to assume that PI-MECs are the sole targets for cellular transformation in all MMTV-driven tumor models. It was recently suggested that the MMTV-wnt1 oncogene targets undifferentiated progenitors or mammary stem cells *(60,61)*. This could be a reason why MMTV-wnt1 mice exhibit a greater variety of histopathologically distinct lesions compared to the MMTV-neu model *(50)*. In support of these observations, various subtypes of MMTV-wnt1-induced tumors express the ductal differentiation marker Nkcc1 that is absent in MMTV-neu derived lesions *(37)*. Using immunohistochemistry, we reported that a number of MMTV-wnt1-derived tumors were comprised of different epithelial subtypes while only a fraction of the cells maintained the expression of Nkcc1. Whether this observation is the result of the suggested paracrine function of Wnt1 or the transformation of pluripotent progenitors that differentiate into various subtypes remains to be determined. It also remains to be seen whether PI-MECs are present in a subset of MMTV-wnt1-derived tumors, which could help to distinguish the origin of specific lesions with particular histopathological features in this model.

## 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 *(62)*. 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 *(63)*. Also, the adult skin posses at least two distinct stem cell populations that coexist in the stem cell niche of the hair follicle. The first population maintains contact to the basal lamina, and the second suprabasal population arises after the start of the first postnatal hair cycle *(64)*. 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.

PI-MECs coexist with undifferentiated stem cells in mammary epithelia of parous females. Due to their location at the extremity of ducts or alveolar units, they act primarily as alveolar progenitors in multiparous females. Here, they might protect undifferentiated stem cells from exhaustion during rapid cell proliferation and differentiation during subsequent gestation cycles. Furthermore, they act as a "functional memory" of the mammary gland to achieve optimal growth, differentiation, and milk secretion to nourish the young. PI-MECs are cellular targets (i.e. cancer stem cells) in selected pregnancy-enhanced breast cancer models, such as MMTV-neu and MMTV-PyVMT transgenic mice, in which cell autonomously acting oncogenes are expressed under pregnancy hormone-induced regulatory elements. However, PI-MECs may not be the sole targets for cellular transformation in all MMTV-driven transgenic models, in particular not in those models, in which the transforming oncogene can act in a paracrine fashion. The combined studies of using stem cell markers (Sca-1 and Keratin 6), the ductal differentiation marker Nkcc1, and the labeling of multipotent PI-MECs support the idea that tumors might originate from distinctly different epithelial subtypes in selected MMTV-promoter driven cancer models expressing diverse types of oncogenes *(37,60)*. In contrast to viral and transgenic mouse models of breast cancer, pregnancy renders the mammary gland refractory to chemical carcinogens in normal females that are not genetically predisposed to mammary tumorigenesis. Whether PI-MECs are the cellular basis for the pregnancy-mediated protection against chemically induced carcinogenesis needs to be addressed in the future.

Upon transplantation into an epithelium-free fat pad, PI-MECs are challenged with a different microenvironment. Here, they behave like multipotent stem cells. They self-renew and produce diverse epithelial subtypes. Together with undifferentiated stem cells, PI-MECs may play an important role within the stem cells niche. Their elimination from a transplant, through limiting dilutions or through inhibition of their capacity to self-renew by overexpressing TGF-$\beta$1, seems to have unfavorable consequences on the resulting outgrowth. There are many open questions about the origin and biology of PI-MECs that need to be addressed in the future. For example, it needs to be determined whether these cells originate during pregnancy and lactation from partially differentiated cells or secretory epithelial subtypes that de-differentiate during involution. Furthermore, double-labeling studies might reveal whether PI-MECs express known stem cell markers, such as Sca-1, that can be utilized to classify, select and purify multipotent progenitors from the mammary gland.

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