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Parity-induced mouse mammary epithelial cells are pluripotent, self-renewing and sensitive to TGF-β1 expression

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A parity-induced mammary population, marked by β-galactosidase expression conditionally activated through cre-lox recombinase originates in WAP-Cre/Rosa-lox-STOP-lox-LacZ (WAP-Cre/Rosa-LacZ) female mice during pregnancy, lactation and involution. During subsequent pregnancies, these parity-induced mammary epithelial cells (PI-MEC) proliferated to produce new secretory acini composed of secretory luminal cells and myoepithelium. In serial transplantation assays, PI-MEC were able to self-renew over several transplant generations and to contribute significantly to the resulting mammary outgrowths. In limiting dilution transplantation, they proliferated to produce both luminal and myoepithelial cells, comprised both lobule-limited and duct-limited epithelial outgrowths, and differentiated into all the cellular subtypes recognized in murine mammary epithelium. TGF-β1 expression from the whey acidic protein promoter (WAP) in triply transgenic females did not prevent the appearance of PI-MEC after pregnancy despite the absence of full lactation or their ability to proliferate and produce progeny with diverse cellular fates *in situ* upon subsequent pregnancies. However, in transplants from triple transgenic parous females, the WAP-TGF-β1-positive PI-MEC did not contribute to the newly recapitulated mammary outgrowths, suggesting that they were incapable of expansive cellular proliferation (self-renewal). This result is consistent with our earlier publication that WAP-TGF-β1 expression in mammary epithelium induces premature stem cell senescence in mammary transplants and decreases mammary cancer risk in mouse mammary tumor virus (MMTV)-infected females even after multiple pregnancies.
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Introduction

In mice, rats and humans, a single early pregnancy provides lifelong reduction in mammary cancer risk. In rats and mice, the protective effect of pregnancy can be mimicked through hormonal application in the absence of gestation. This refractoriness to chemical induction of mammary tumorigenesis has recently been linked to the absence of a proliferative response in the parous epithelium when confronted with the carcinogen as compared with the nulliparous gland (Sivaraman *et al*., 1998, 2001). Concomitant with the reduction in proliferative response is the appearance of stable activation of p53 in epithelial cell nuclei. This suggests that in response to the hormonal stimulation of pregnancy, a new cellular population is created with an altered response to carcinogen exposure. Employing the Cre recombinase/lox P system to identify mammary cells *in situ*, which have differentiated during pregnancy and expressed Cre from the whey acidic protein (WAP) promoter, a new parity-induced mammary epithelial cell population was discovered (Wagner *et al*., 2002). This population does not persist in nulliparous females at any age, but accumulates upon successive pregnancies in the mammary glands of parous females. The evidence shows that, *in situ*, these cells are committed to secretory cell fate and contribute extensively to the formation of secretory lobule development upon successive pregnancies. In this report, we demonstrate that these cells are pluripotent, that is, capable of giving rise to all mammary epithelial subtypes and that they have an extended capacity to self-renew over multiple transplant generations. We previously reported that TGF-β1 expressed from the WAP promoter reduces mammary cancer risk by promoting premature senescence in mammary stem cells (Kordon *et al*., 1995; Boulanger and Smith, 2001). Here, we show that expression of activated TGF-β1 within the parity-induced epithelial cells severely curtails their self-renewing activity as determined by transplantation, but not their capacity to proliferate and produce epithelial progeny of diverse fates among the mammary epithelium in multigestational females when left *in situ*.

Results and discussion

Transplantation studies

In our earlier publication (Wagner *et al*., 2002), we demonstrated that the parity-induced mammary epithelial cells (PI-MEC) lineally marked by LacZ expression

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and their progeny were able to self-renew and to contribute extensively to both alveolar growth upon successive pregnancies *in situ* and to ductal growth and elongation upon transplantation to epithelium-cleared mammary fat pads. We also verified that epithelial outgrowths of mammary transplants from nulliparous bigenic females did not contain LacZ-positive cells, thereby establishing that transplantation does not cause spurious activation of WAP-Cre. In Table 1, all of the transplants that have been carried out to develop the data presented in this report are listed. As we and others (Kordon *et al*., 1995; Robinson *et al*., 1995, 1996) have reported earlier, both the endogenous WAP gene and WAP-driven transgenes are actively expressed albeit transiently during the estrus cycle in maturing and mature nulliparous female mice. Therefore, LacZ-positive cells can be transiently detected among the epithelial population of intact glands from nulliparous mice (Kordon *et al*., 1995; Robinson *et al*., 1995; Wagner *et al*., 2002). The important point in the transient appearance of WAP-Cre-activated cells in nulliparous cycling females is that LacZ expression once activated is no longer dependent upon the expression of WAP-Cre. Its expression is constitutive from the Rosa promoter in the activated cells and in any of their progeny. Therefore, the transient appearance of these estrus-activated cells indicates that they do not replicate significantly and further that they die and disappear from the population. As we reported earlier, there is no accumulation of LacZ-positive epithelial cells with age in nulliparous cycling mice (Wagner *et al*., 2002). We have transplanted dispersed cells ($1.0–5.0 \times 10^5$ per fat pad) and mammary fragments from the mammary glands of nulliparous bigenic females up to 13 months of age (Table 1). We have never found WAP-Cre-activated LacZ-positive epithelial cells in the outgrowths generated from these transplants (Figure 1a), with the exception of those transiently appearing at estrus in the cycling female hosts. Therefore, the WAP-Cre-activated cells resulting from WAP gene activation during the estrus cycle do not possess the property of self-renewal and do not proliferate upon transplantation to contribute to the development of the new epithelial ducts. These estrus-activated, LacZ-positive cells in the cycling virgin are equivalent to those found in preponderance in the lactating glands as a result of the commitment to lactogenic differentiation and milk protein gene expression. These functionally differentiated cells are dispensed following the cessation of lactation. In contrast, the cell and tissue transplants from the glands of parous bigenic females contained significant numbers of LacZ-positive cells that were present throughout the various stages of the estrus cycle even after serial transplantation (Figure 1b). This result demonstrates that the LacZ-positive epithelial cells present in the mammary glands of parous females following involution are capable of proliferation and self-renewal.

Table 1 Transplantation studies

	Nulliparous	*Parous*	*Nulliparous* β+	*Parous* β+
Fragment	20/20[a]	88/90	20/20	67/70
Cells	29/30	65/68	18/20	36/40
Limiting dilution	ND	29/52	ND	ND

A large number of transplants of mammary tissues and cells from nulliparous and parous bigenic and trigenic (WAP-TGF-β1) females were carried out to develop evidence for PI-MEC self-renewal. Fragment implants consisted of glandular pieces bearing approximately 5–7000 epithelial cells. Dispersed cells were inoculated in a 10 μl bolus at concentrations varying from $1 \times 10^7–5 \times 10^7$ cells/ml. Limiting dilutions were 5000 and 10 000 epithelial cells/10 μl. All outgrowths were subsequently removed, fixed, stained whole length for LacZ expression (X-gal) and examined as whole mounts for the presence of PI-MEC progeny. LacZ-positive cells were not observed in any of the whole mounts or sections from outgrowths obtained after transplantation of tissues from nulliparous donors. In contrast, outgrowths from age-matched primiparous donors (60%) or multiparous females (100%) were positive for LacZ-expressing epithelial cells. These cells were evident directly in the whole mounts and in sections derived from the whole mounts. Whole mounts and sections from outgrowths developing from either nulliparous or parous WAP-TGF-β1-positive donors were devoid of LacZ-positive cells. [a]The ratio of positive takes over total implants is shown in each column

To establish an estimate of their self-renewing ability, we transplanted mammary fragments containing PI-MEC through four transplant generations. Each successful transplant results in a 400-fold increase of the implanted epithelial population, roughly a 8.65-fold doubling of the implanted cells (Kordon and Smith, 1998). We recovered activated LacZ-positive cells from all of these transplants (Figure 1b). Therefore, PI-MEC are capable of self-renewal ($4 \times 8.65 = 35$ doublings) and proliferation over several transplant generations and persist in the transplant outgrowth populations until growth senescence. No PI-MEC were found in transplants ($N = 2$) that showed growth senescence, that is, filling less than 15.0% of the available fat pad, an observation that suggests that their presence may contribute fundamentally to continued growth (not shown).

The forgoing observations demonstrate that the mere expression of LacZ by mammary epithelial cells is not reflective of their function or cell fate, only that they (or their antecedent) has expressed WAP-Cre at some earlier point and caused the activation of Rosa26-LacZ gene expression. Therefore, LacZ expression *per se* does not reveal anything about the function of the expressing cell other than its relationship to cells that have previously expressed WAP-Cre. Constitutive LacZ expression does, however, represent a lineal marker for cells derived as the progeny of a previously activated ancestor(s) and thereby provides a convenient tool for examining the developmental repertoire of WAP-Cre-activated, LacZ-positive mammary epithelial cells.

Limiting dilution transplantation

To determine to what extent the presence of neighboring LacZ-negative epithelial cells contributed to the self-renewing capacity of PI-MEC, dispersed mammary epithelial cells from multiparous WAP-Cre/Rosa26-LacZ females were inoculated at limiting dilutions into cleared fat pads. It was determined by direct examination of X-gal-stained aliquots of these cell suspensions

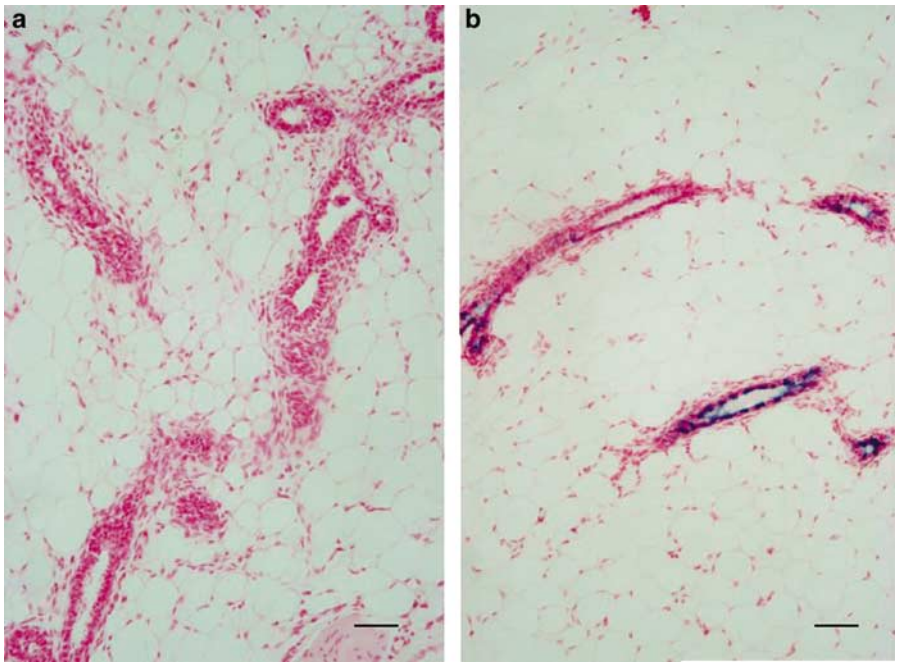
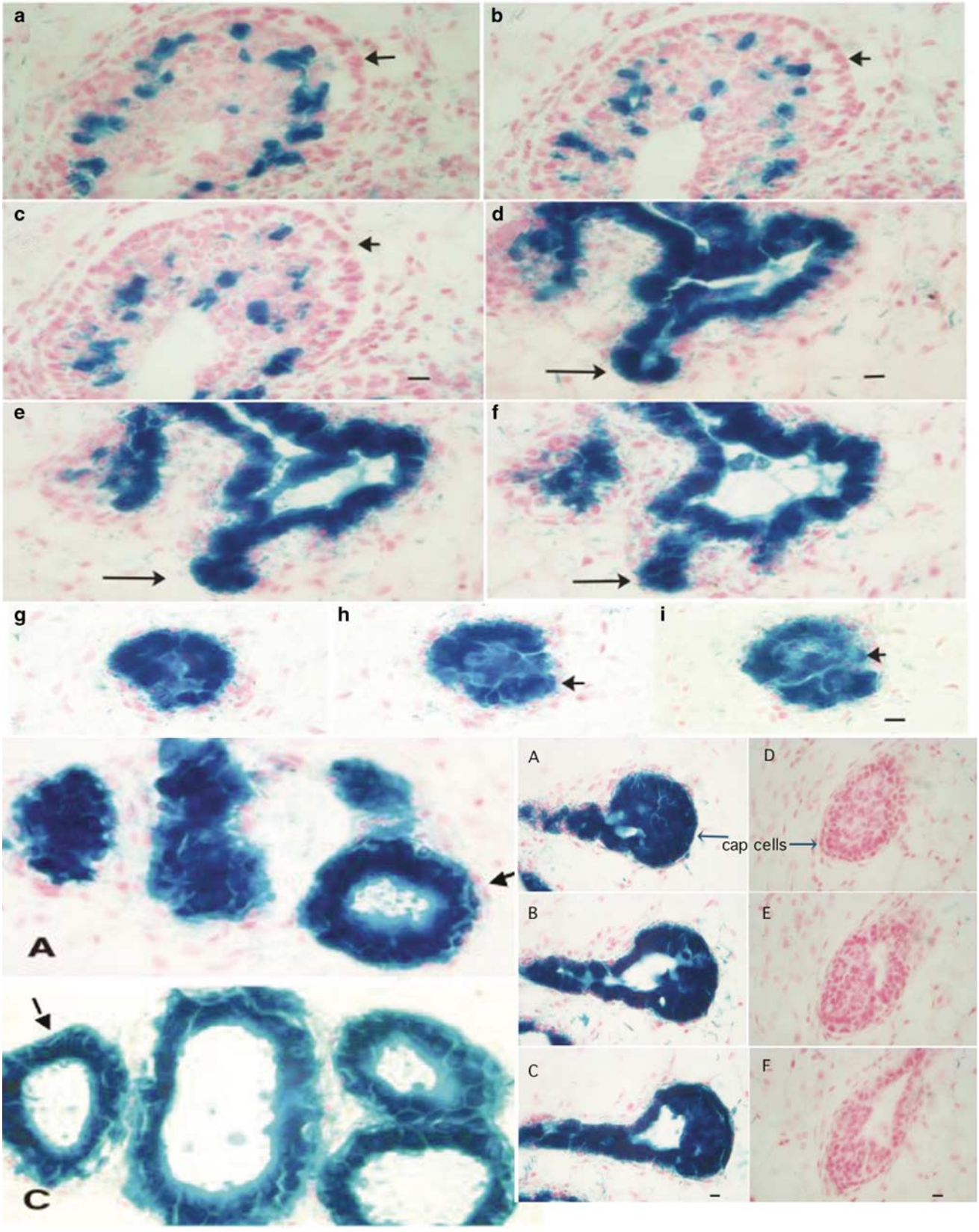


Figure 1 Each panel contains an image taken from sections cut from mammary whole mounts that had been stained directly for LacZ expression after removal from the transplant hosts. Panel a shows a section from an outgrowth from 100 000 dispersed mammary epithelial cells isolated from a 13-month-old nulliparous WAP-Cre/Rosa26-LacZ female. No LacZ staining was observed in the whole mount or in sections taken at 200 μm intervals throughout the entirety of the gland. Panel b contains a representative section from a third-generation serial transplant of mammary tissue taken from a primiparous WAP-Cre/Rosa-LacZ female. LacZ-positive epithelial cells were found throughout the outgrowth indicating continuing self-renewal of PI-MEC progeny, Bar = 20 μm

that one in five (19.4%) epithelial cells were positive for LacZ expression. A total of 5000 dispersed mammary cells plus 1.0×10^5 Nu/Nu mouse mammary fibroblasts were injected in 10 μl of saline into the cleared inguinal fat pads of nude mice. The hosts were placed with males 4 weeks later. The implants were collected between 4 and 10 days of pregnancy prior to *de novo* activation of Rosa26-LacZ by expression from WAP-Cre (Wagner *et al*., 2002). The term of pregnancy was confirmed by microscopic examination of the embryos. From 20 inoculated pads, 12 (60%) were found to contain positive epithelial growth. Seven implants were branching ducts with side branches composed of developing secretory acini filling 50–95% of the fat (Figure 2a–i) The remaining positive fat pads contained multiple sites of growth. In these fat pads, the growths were small (1–3 mm^3) lobule-limited or duct-limited structures. All outgrowths contained LacZ-positive cells (Figure 2a–f), even though PI-MEC represented only 20% of the inoculated epithelial cells. No epithelial outgrowths were comprised entirely from LacZ-negative cells. In contrast, the lobule-limited (Figure 2 lower left A and C) and duct-limited (Figure 2 lower right A–F) outgrowths were entirely comprised from PI-MEC and their LacZ-positive progeny as determined by serial sections through these structures (Figure 2 bottom two panels). The morphology of the duct-limited and lobule-limited structures strongly indicates that all luminal, myoepithelial and terminal bud cap cells were derived from LacZ-positive progenitors. The results demonstrate that PI-MEC may recombine with LacZ-negative epithelial cells to generate branching ductal structures with developing secretory acini. In these structures, PI-MEC progeny (LacZ-positive cells) are found among the body cells of growing terminal end buds, along the subtending ducts and in actively developing secretory acini along the ducts. PI-MEC display the capacity to proliferate and form secretory lobular structures exclusively comprising LacZ-positive progeny (Figure 2 lower left A and C) either by recombining together or by

Figure 2 These images were developed from X-Gal-stained outgrowths from limiting dilutions studies with dispersed mammary epithelial cells from a multiparous female. X-Gal-positive cells represented roughly 20% of the injected cells. Panels **a–c** represent serial sections from a terminal end bud of a branched ductal/lobular outgrowth (arrows indicate X-Gal-negative cap cells. Panels **d–f** are longitudinal serial sections from a developing secretory acinus (long arrows) in the same outgrowth. Panels **g–i** are serial cross-sections of a developing acinus (arrows indicate myoepithelial cells). Two sections (**A** and **C**) taken from the same lobule-limited outgrowth are shown in the lower left panel. These contain myoepithelial cells, which are stained positively for β-galactosidase expression (arrows). The lower right panel contains serial sections from the terminus of a duct-limited structure (A–C), demonstrating that all cells were derived from PI-MEC including the cap cells (arrow). For comparison, serial sections from an unstained terminus are shown in (D–F). Cap cells indicated by arrow. Bars represent 10 μm

clonal expansion. Likewise, occasional duct-limited outgrowths were entirely comprised of their LacZ+ progeny (Figure 2 lower right A–F). In the latter instance, PI-MEC progeny also include the specialized cap cells of the terminal end bud. We conclude from these results that PI-MEC are not capable of continued



self-renewal when clonally expanding or in recombinants comprised entirely of PI-MEC and their immediate progeny. Therefore, in structures formed in the absence of unactivated epithelium, they do not exhibit the capacity to completely repopulate the cleared fat pad that might be expected from primary mammary epithelial stem cells. Nevertheless, PI-MEC appear to be capable of giving rise to both luminal, myoepithelial and cap cell progeny and have the capacity to self-renew extensively when combined with their LacZ-negative epithelial partners. An additional 32 fat pads were inoculated with 10 000 mammary epithelial cells. The resulting outgrowths (21) were examined as whole mounts after staining with X-gal to reveal LacZ expression. The results were equivalent for LacZ staining and all three types of growth patterns, duct-limited (3), lobule-limited (4) and mixed (10), were observed (Table 1). Demonstration of the presence of the βGeo sequence (present in the Stop DNA in the Rosa26 reporter transgene) by PCR provided evidence that the mixed outgrowths (both LacZ-positive and -negative cells) were comprised of both activated and unactivated epithelial cells and not the result of Rosa26-LacZ gene silencing (not shown). With regard to the presence of LacZ-positive myoepithelial cells in the lobule-limited outgrowths, duct-limited outgrowths and in developing acini along the ducts in pregnant hosts, it should be noted that duct-associated myoepithelial cells are derived from the cap cells of the terminal end bud during duct morphogenesis (Williams and Daniel, 1983), whereas those in the alveoli are not. Therefore, since PI-MEC do not form the cap cells in the terminal end buds of the mixed outgrowths, the duct myoepithelium is LacZ negative.

Evidence of multiple cell fates for LacZ-positive progeny

The morphologic evidence of multiple LacZ-positive mammary epithelial subtypes in developing acinar buds both *in situ* and in transplants growing in pregnant hosts was bolstered by coexpression of cell type-specific markers and LacZ in the epithelial cells. Tissue sections from intact mammary tissues of 8-day pregnant, primiparous females and from mammary outgrowths in pregnant hosts were doubly stained for LacZ expression and for the mammary epithelial subtype-specific epitopes, smooth muscle actin (SMA-myoepithelial), progesterone receptor (PR) and estrogen receptor (ER). Spindle-shaped LacZ-positive cells in the basal portions of developing secretory alveoli were costained with SMA confirming their myoepithelial identity (Figure 3a–d). Some LacZ-positive cells both within the body of terminal end buds and in the subtending ducts stained for PR and ER indicating that both steroid receptor-positive and -negative luminal epithelial cells were derived from LacZ-positive antecedents (Figure 3 ER and PR). It is to be noted that not all PR- and ER-positive cells are also positive for LacZ, indicating that LacZ-negative epithelial cells also contribute to the steroid receptor-positive epithelium. PCNA staining confirmed the mitotic activity of PI-MEC within the body of terminal end buds (Figure 3 PCNA). Contrary to the duct-limited structures described above, cap cells in the termini of the mixed (both LacZ-positive and -negative cells) outgrowths were negative for LacZ, but positive for SMA and in this structure apparently were not derived from PI-MEC in the body of the end bud (Figure 3 SMA). This most likely explains why the myoepithelial cells along the subtending ducts of the mixed outgrowths, in contrast to those in the developing secretory acini, stain negatively for LacZ. Cap cells differentiate into this epithelial subtype as they migrate from the growing terminal end bud to the basal surface of the subtending duct (Williams and Daniel, 1983), whereas the myoepithelial cells associated with secretory lobules in pregnant mice are derived in a different manner, presumably from lobular progenitors (sic PI-MEC), as there are no cap cells or terminal end buds involved in the process of lobulogenesis.

Effects of TGF-β1 expression in WAP-Cre-activated epithelial cells

The expression of TGF-β1 from the WAP promoter results in a mammary phenotype characterized by incomplete development of secretory lobules and a failure in lactation (Jhappan *et al*., 1993). Lactation failure results from the early apoptotic death of differentiating alveolar cells in pregnant females (Kordon *et al*., 1995), resulting in the absence of sufficient secretory lobule development to maintain the pups. Analysis of mammary cell proliferation during pregnancy in these mice indicates that epithelial mitotic activity continues at a significantly higher rate throughout pregnancy than in the wild-type glands. Therefore, the paucity of alveolar development is the result of extensive apoptosis and not from suppression of epithelial cell proliferation. In addition to this phenotype, transplants of WAP-TGF-β1-positive mammary tissue show an early growth senescence, which in the case of mouse mammary tumor virus-infected females results in a significant reduction in mammary cancer risk (Boulanger and Smith, 2001). Therefore, it was of interest to us to determine what effect TGF-β1 expression might have on WAP-Cre induction of LacZ-positive cells in mice bearing both WAP-driven transgenes and the LacZ reporter and on the subsequent behavior of PI-MEC both *in situ* and in transplants. Postpartum females bearing all three transgenes, WAP-Cre, WAP-TGF-β1 and Rosa26-LacZ ($N = 20$), were unable to nurse their pups because of sparse secretory lobule development, as expected. Despite this, the existing lobules were populated with LacZ-positive epithelial cells (Figure 4a, b). Several weeks after parturition, WAP-Cre-activated, LacZ-positive cells were still present (Figure 4c). Their number and location were similar to those observed in the involuted glands of primiparous WAP-Cre/Rosa26LacZ females (Figure 4c). Upon a second round of pregnancy, the surviving LacZ-positive cells could proliferate and produce small acinar structures, which included both LacZ-positive luminal

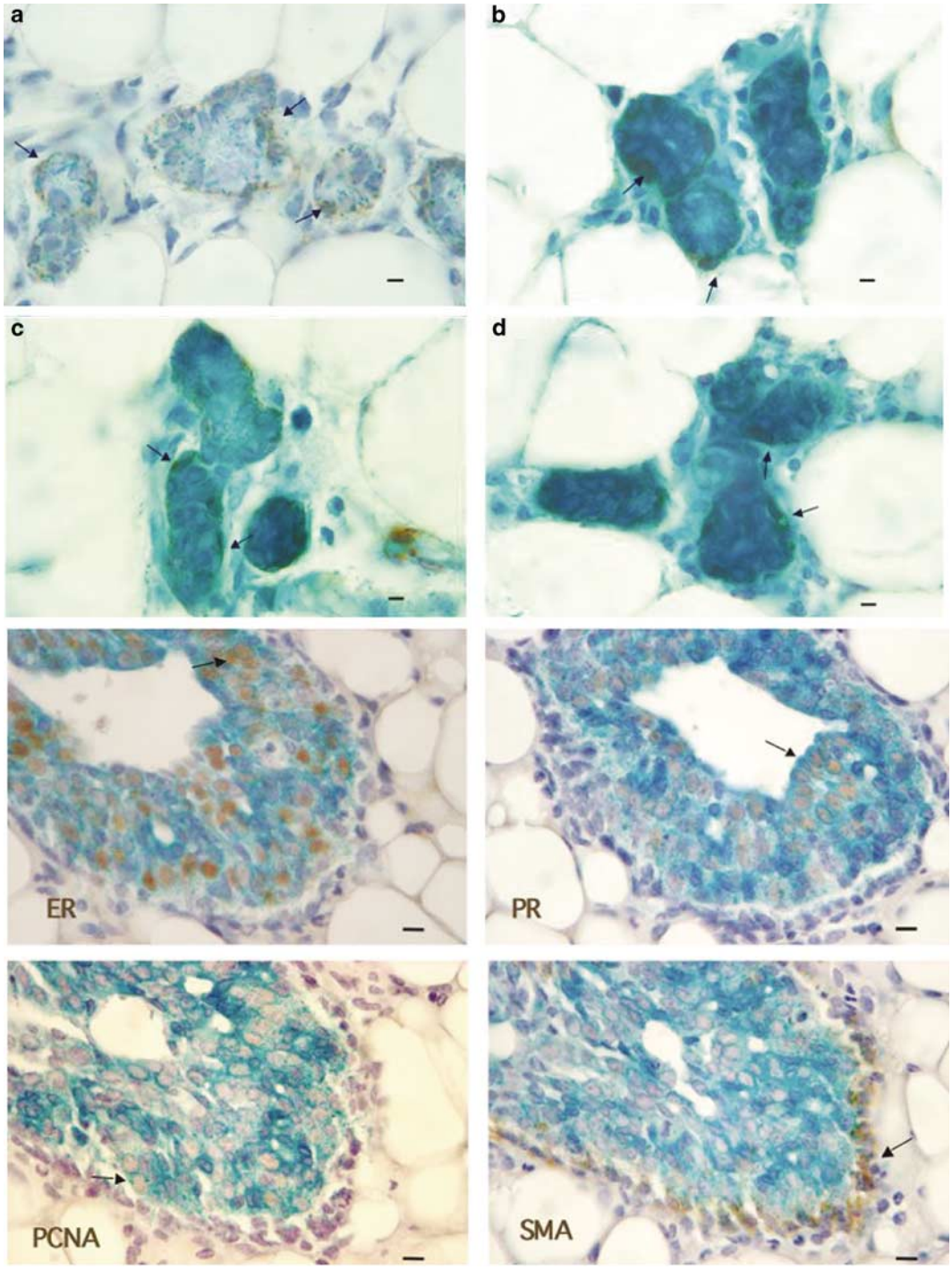


Figure 3 To confirm that PI-MEC give rise to myoepithelial cells during early pregnancy in primiparous females, glands were doubly stained for β-galactosidase and for smooth muscle actin (SMA). In these slides, nuclei were counterstained with hematoxylin. (**a**) X-Gal-negative acini stained with anti-SMA (brown), which marks the myoepithelial cell cytoplasm (arrows). (**b–d**) X-Gal-positive acini with myoepithelial cells coexpressing SMA (brown) and β-galactosidase (blue) in their cytoplasm (arrows). Below are serial sections through a terminal end bud costained for the expression of β-galactosidase and estrogen receptor (ER), progesterone receptor (PR), PCNA and smooth muscle actin (SMA). The arrows indicate coexpressing cells in ER, PR and PCNA. The cap cells stained for SMA only (arrow) in the last panel. Bars represent 10 μm

and myoepithelial cells (Figure 4d). In prolactin receptor (PrlR) heterozygous null mice bearing the WAP-Cre, Rosa26-LacZ transgenes, the parity-induced LacZ-positive population increases upon successive pregnancies rescuing the capacity of these females to nurse after several gestations (Wagner *et al*., 2002). This resumption of lactation is not found in WAP-Cre, Rosa26-LacZ, WAP-TGF-β1 female after multiple litters even though

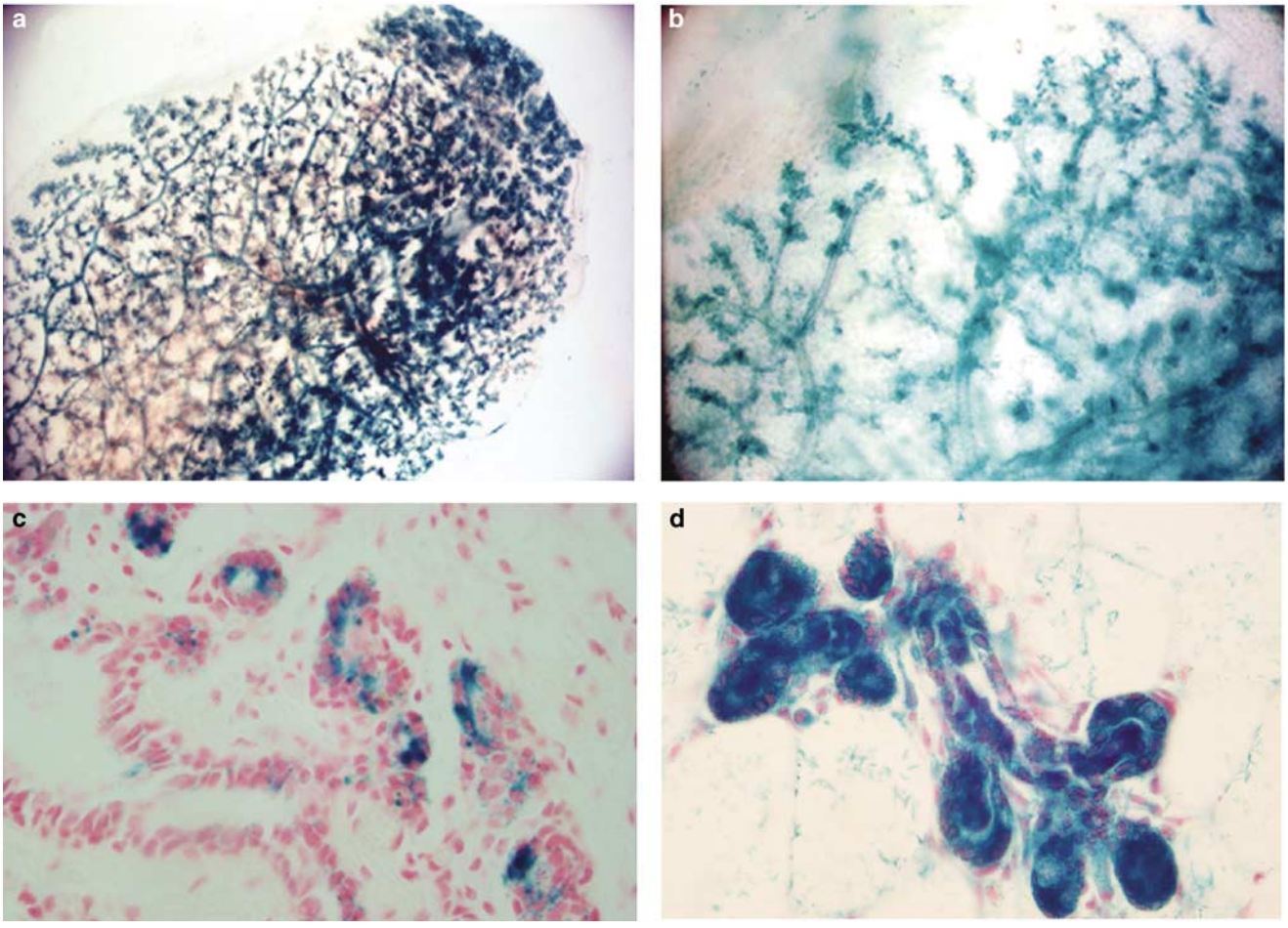


Figure 4 Panels **a** and **b** are whole mounts of glands from postpartum WAP-Cre, Rosa26ST, WAP-TGF-β1-positive females. Acinar development is sparse; nevertheless, WAP-Cre-activated X-Gal-positive cells are prevalent. Following involution, PI-MEC (blue) persist in the primiparous female (**c**) and at 8 days into a subsequent pregnancy these cells proliferate robustly to contribute to the development of new acini (**d**). Bars represent 10 μm

at each successive pregnancy the surviving LacZ-positive cells appeared competent to proliferate. This failure to lactate may be attributed to the apoptotic death of the differentiating secretory cells in the developing lobules during each successive pregnancy.

The percentage of LacZ-positive cells in the triple transgenic glands after a single parity was indistinguishable from that observed in WAP-Cre/Rosa26-LacZ females. This was also true for the dispersed epithelial cell preparations from these glands. As expected, mammary tissue implants and dispersed cells from the triple transgenic females after either a single gestation or multiple gestations failed to produce full lobular development in full-term pregnant hosts (Table 1). Perhaps, more importantly, LacZ-positive cells were not present among the ducts in these transplant outgrowths either in nulliparous or early pregnant hosts ($N \geqslant 100$). Nevertheless, newly activated cells did appear in the transplant population and were present in the lobular structures indicating the presence of WAP-Cre activation at late pregnancy in these transplants (after 15 days to parturition, $N = 8$, not shown).

These results demonstrate that the PI-MEC that develop during pregnancy and survive subsequent tissue remodeling in the absence of lactation in WAP-TGF-β1 females were incapable or severely limited in their ability to self-renew in transplants and could not contribute to duct development in subsequent transplant outgrowths. Therefore, self-renewal and proliferation competence appear to be independent properties in the PI-MEC that survive involution. By definition, self-renewal of stem cells occurs by two different processes, for example, in asymmetric divisions, the most common activity of stem cells residing in a niche (Lin, 2002), 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 daughters, which 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; for example, during ductal growth and expansion in the postpubertal female or when the mammary epithelial implant is growing in the transplanted mammary fat pad. This distinction between self-renewal of pluripotent cells through asymmetric cell division *in situ versus* expansive symmetric cell division in growing transplants is compatible with our observation of the absence of LacZ-positive cells in the outgrowths from these glands.

It is also consistent with our original conclusion that these cells represent committed lobule-specific progenitors, whose function, *in situ*, is to generate, through asymmetric division, new secretory alveoli during subsequent pregnancies along the mammary ducts. Expression of TGF-β1 from the WAP promoter leads to the loss of the ability of WAP-Cre-activated cells to contribute to branching ductal morphogenesis upon transplantation. This is a process that requires both proliferation and self-renewal. In keeping with this conclusion, outgrowths from WAP-Cre, Rosa26-LacZ, WAP-TGF-β1 second-generation transplants showed a growth senescent phenotype and were devoid of LacZ-positive cells. The effect of TGF-β1 on the self-renewal of PI-MEC supports our earlier observation regarding protection from MMTV-induced mammary tumorigenesis in WAP-TGF-β1-positive females (Boulanger and Smith, 2001) compared to their wild-type sisters and suggests that the cellular targets for MMTV may be PI-MEC because multiple pregnancies accelerate MMTV-induced oncogenesis (Callahan and Smith, 2000). The recent finding (Henry *et al*., 2004) that PI-MEC may represent the targets for MMTV-neu tumorigenesis, which is also accelerated in multiparous females, further supports a potential role for these cells as targets for transformation in models where multiple pregnancies enhance cancer risk.

Materials and methods

Mice

The transgenic WAP-Cre/Rosa 26 mice were engineered and typed according to Wagner *et al*. (2002). Female Nu/Nu/NCR mice were used as hosts for the transplantation studies. To generate WAP-Cre/Rosa26S-LacZ/WAP-TGF-β1 female mice with WAP-TGF-β1, males bearing this transgene were crossed withWAP-Cre/Rosa26S-LacZ females. All mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The National Cancer Institute Animal Care and Use Committee approved all experimental procedures.

Single cell dissociation procedure

Preparation of dissociated mammary epithelial cells directly from freshly excised mammary tissue has been described in detail earlier (DeOme *et al*., 1978). In brief, mammary glands were aseptically removed from each donor mouse, rinsed in DMEM (Gibco/BRL Gaithersburg, MD, USA) and weighed. Glands were minced and placed in 250 ml Erlenmeyer flasks containing 20 ml DMEM with 0.1% collagenase (Worthington Biochemical Corp., Freehold, NJ, USA; Type III approximately 100 U/ng) and 0.1% hyaluronidase (Sigma Chemical Co., St Louis, MO, USA; Type I 360 U/ng) per gram of tissue. The flasks were then swirled at 120 r.p.m. at 37° for approximately 90 min. Samples were then placed in 50 ml conical tubes and spun for 5 min at 1200 r.p.m. The fatty top layer and pellet were collected in DMEM with 1.25% pronase (Calbiochem B grade 45 000 proteolytic U/g), 10 ml/g of original tissue and shaken for an additional 15 min at 4°C and then centrifuged. The pellet was washed twice and resuspended in 10 ml DMEM and filtered through 40um nylon cell strainers (Falcon 2340). Viability was determined by staining with trypan blue. The number of live epithelial cells was typically 70% of the total suspension.

Cell and tissue transplantation

The surgical techniques used to clear the mammary epithelium from the #4 fat pad of 3-week-old host mice, and the subsequent transplantation of tissue fragments or cell suspensions have been described in detail (DeOme *et al*., 1959; Daniel *et al*., 1968; Smith *et al*., 1991; Smith, 1996). Briefly, the mice were anesthetized and the clearing procedure was performed immediately prior to insertion of the transplanted fragment or cell suspension. Transplanted tissues were approximately 1–2 mm in size. Cell suspensions were implanted in 10 μl volumes with a Hamilton syringe equipped with a 30-gauge needle. The implanted females were maintained as nulliparous mice for 7–12 weeks to determine the contribution of WAP-Cre-activated cells to duct morphogenesis. Alternatively, they were placed with males 4 weeks following implantation to initiate pregnancy and secretory development.

X-Gal and Immunostaining of mammary gland whole mounts

Whole mounts of the entire inguinal gland were fixed and stained as described earlier (Daniel *et al*., 1968). Briefly, the gland was spread on a glass slide, fixed in paraformaldehyde (4.0%) for 1–2 h, permeabilized in 0.01% NP-40 in phosphate-buffered saline (PBS) overnight at 4°C and subsequently processed for X-Gal as described (Wagner *et al*., 1997). Stained glands were repeatedly rinsed in PBS, then postfixed in Carnoy's fixative, cleared in 100% ethanol and the placed in xylene before whole mount analysis. For histological examination, X-Gal-stained whole mounts were embedded in paraffin, sectioned at 6.0 μm and counterstained with nuclear fast red. Immunocytochemistry was carried out on deparaffinized sections following antigen retrieval in 0.01 M sodium citrate in a microwave set at 80% power for 3 min. The antibodies used were against smooth muscle actin (Dako #M0635, 1 : 200); ER (Santa Cruz # sc-542, 1 : 300) and PR (Dako #A0098, 1 : 100). Known positive and negative tissue controls fixed similarly were used to confirm the specificity of staining.

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