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Evidence that an early pregnancy causes a persistent decrease in the number of functional mammary epithelial stem cells—implications for pregnancy-induced protection against breast cancer

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Abstract

A completed pregnancy at a young age reduces a woman's lifetime risk of breast cancer by up to 50%. A similar protective effect of an early pregnancy has been observed in rodent models using chemical carcinogens. However, the mechanisms responsible for this protective effect remain unclear. Stem cells have been proposed to be the cells of origin for breast cancer. We hypothesized that an early pregnancy reduces adult levels of either mammary stem cells or mammary multipotent progenitor cells. Unsorted mammary cells from adult mice that had undergone an early parity had the same mammosphere formation efficiency as cells from age-matched virgin mice. However, when we transplanted adult mammary cells in limiting dilutions into cleared fat pads of syngeneic mice, we found a significant reduction in the outgrowth potential of the cells from early parous mice as compared with age-matched virgin mice. The extent of fat pad filling in successful outgrowths did not change, suggesting that while mammary stem cells in parous mice retained their functional competence, the number of mammary stem cells was reduced. Our results provide the first direct evidence that an early pregnancy has an effect on mammary stem cells.

Keywords

Epithelial cells; Mammary glands; animal; Pregnancy; Stem cells; Stem cell transplantation

Introduction

Women completing their first pregnancy before age 20 have about half the risk of breast cancer compared to nulliparous women [1–6]. This protective effect has also been observed in rodent

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models of carcinogenesis [7–9]. The mechanisms underlying the protective effect of an early parity remain unclear, though several explanations have been proposed[10–23].

Stem cells exist in the mammary gland [24–34], and have been proposed to be the cellular origin of cancer [33, 35–42]. Although it has been speculated that changes in mammary stem cells following an early pregnancy could be responsible for reducing breast cancer risk [8, 43, 44], the effect of an early pregnancy on mammary stem cells has not been rigorously studied.

Methods

Mice and cells

Female FVB mice were allocated into age-matched pairs, with ages differing by no more than one week. One mouse of each pair was mated at five weeks of age and allowed to complete one pregnancy. Pups were weaned at 21 days, and parous mice were maintained a minimum of eight weeks after weaning to allow complete involution before being used for assays. Single cell suspensions were prepared as described [33].

Mammosphere formation & mammary transplantation

Mammosphere assays were performed essentially as reported [45]. Single cell suspensions from each parous donor mouse, serially diluted in DMEM/F12 + 5% FBS, were injected immediately after preparation into the #4 cleared fat pads of recipient virgin mice (age: 3–15 weeks; cleared at 3 weeks) as reported [46]. The contralateral fat pad received an equal number of mammary cells from an age-matched virgin donor. Eight weeks later, transplanted glands were stained in carmine alum.

Results

Pregnancy causes mammary gland alterations, including an expansion of the ductal tree and the development of alveoli, which regress during involution. We first ascertained that an early pregnancy did not alter the total number of cells, or the luminal epithelial or myoepithelial cell content in the adult mammary gland (5–15 months of age) (Figure 1A & 1B).

In non-adherent cultures, a small subset of mammary cells form mammospheres containing both epithelial and basal cell types, implying a multipotent progenitor origin [45]. Cells from both parous and age-matched virgin adult mice (8–15 months of age) formed mammospheres that were similar in appearance and cell composition (Figure 1C) to each other and to mammospheres derived from human mammary cells [45]. Cells from parous mice had the same capacity to form both primary and secondary mammospheres as cells from virgin mice (Figure 1D). Therefore, an early pregnancy does not induce lasting changes in the multipotent progenitor cell population detectable by the mammosphere assay.

The classical assay for mammary stem cells is serial dilution transplantation into epithelium-cleared fat pads to evaluate ductal tree regeneration potential. We used this assay to compare mammary cells isolated from parous and age-matched virgin mice. Donors ranged in age from 5 months (n=3 for both virgin and parous mice) to 10–15 months of age (n=8). The overall take rate from virgin donors (Figure 2A) is in general accordance with other studies using similar methods of cell preparation [33, 46]. The take rate from parous donors was significantly less than that from virgin mice (Figure 2A & 2B, $p=0.017$; main effect of parity across all dilutions). Even when only transplants from older (10–15 month) donor mice are considered, a significant parity-induced decrease in take rate was observed ($p=0.03$). Therefore, we conclude that an early age pregnancy has an adverse effect on the mammary stem cell population.

The extent of the fat pad filled by parous donor cells was comparable to that of virgin donor cells, even at higher dilution points (Figure 2C), suggesting that our observed decrease in take rate is due to a parity-induced loss of stem cells. By a single-hit Poisson model, the frequency of regenerative stem cells in virgin mice was 1 per 6,423 cells with a 95% confidence interval of [4,493, 9,187], similar to the estimation reported by other groups using comparable transplantation methods [33, 46, 47]. In parous mice, the frequency of regenerative stem cells was 1 per 13,221 cells [8,442, 20,708] (Figure 2A). The confidence intervals overlap only slightly, and by a Wald test the frequency difference was significant (p=0.01). Therefore, we conclude that an early age pregnancy decreases the mammary stem cell population by ~50% (Figure 2A).

Discussion

We report here a long-term decrease in the number of mammary repopulating units in the mammary glands following an early pregnancy. This decrease is most likely due to a reduction in the number of mammary epithelial stem cells. Since mammary stem cells are likely a major cellular target of breast cancer, our observation of reduced mammary stem cells may help explain why early pregnancy reduces the risk of breast cancer.

The cancer-protective effect of an early pregnancy persists throughout a woman's lifetime. However, there is a heightened risk of breast cancer in the years immediately following pregnancy [6, 48–50]. For this reason, we allowed parous mice a minimum of eight weeks between the onset of involution and analysis, so that transitory changes in this potential window of increased risk would not complicate our analysis and that our test would reveal persistent changes in mammary stem cells. Of note, most other reports on parity-induced changes in the mammary gland examined the gland only a few weeks after the initiation of involution [14, 15, 51].

Stromal, immune, and systemic changes have also been reported in animals following pregnancy [15, 17]; these changes may have an impact on mammary stem cells and their susceptibility to tumorigenesis [9, 17, 52]. Our study does not address the impact of hormonal and immune changes on mammary stem cells. We do not know if parity-induced changes in stromal cells incidentally transplanted with the mammary epithelial cells might have contributed to our observed decrease in the take rate.

Mammary stem cells have been reported to be enriched in the subset of mammary cells that are $lin^-/CD24^+/CD49f^{hi}$, $lin^-/CD24^+/CD29^{hi}$, or $CD24^{low}$ [30, 33, 34]. However, the poor extent of stem cell enrichment (up to 1 stem cell in 64 $lin^-/CD24^+/CD29^{hi}$ cells [33]) makes it unlikely that changes in the number of stem cells can be detected by examining these stem cell-enriched populations as a whole. Indeed, we did not observe a significant difference between parous and virgin mice in the $lin^-/CD24^+/CD29^{hi}$ population, nor in the $lin^-/CD24^+/CD29^{low}$ progenitor population (Supplementary Figure 1). This finding is in agreement with a recent report of no difference between virgin and primiparous mice in the percentage of $CD24^+/CD49f^+$ cells, although the age of pregnancy was not described [51]. Flow cytometry using more specific stem cell markers may unmask differences in stem cell numbers between these two groups of mice.

In conclusion, we present strong evidence that an early pregnancy reduces the number or function of mammary stem cells. Further progress in understanding an early pregnancy's protection from breast cancer requires determining the cellular targets of oncogenic transformation. It is crucial to test whether virgin mice with depleted numbers of mammary stem cells have reduced tumorigenesis upon carcinogen exposure. If so, targeting mammary

stem cells becomes a new approach towards clinical interventions to replicate in nulliparous women the protective effect of an early childbirth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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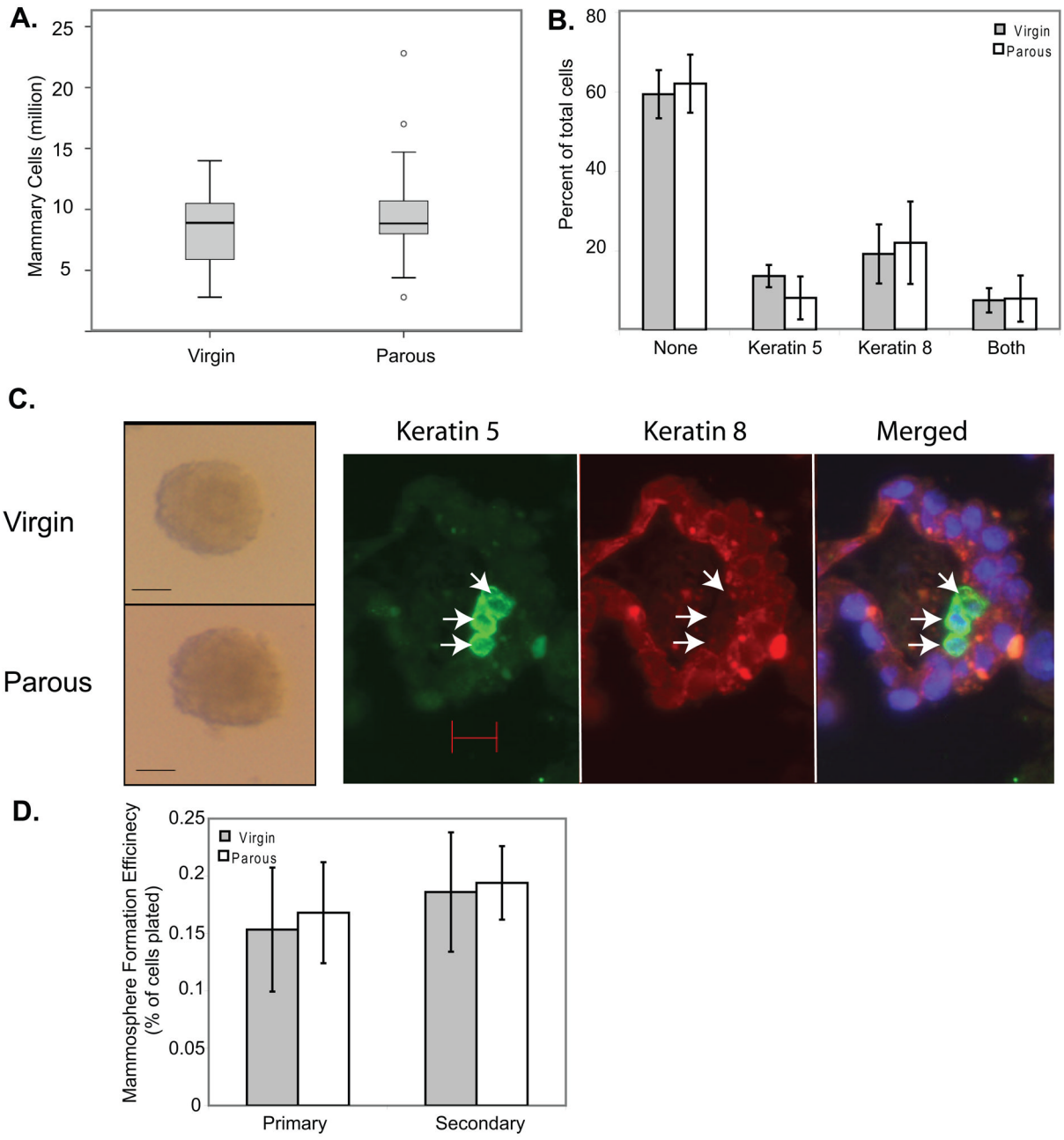


Figure 1. An early pregnancy does not induce a persistent change in total mammary cells or epithelial content, and does not cause a persistent change in mammosphere forming cells

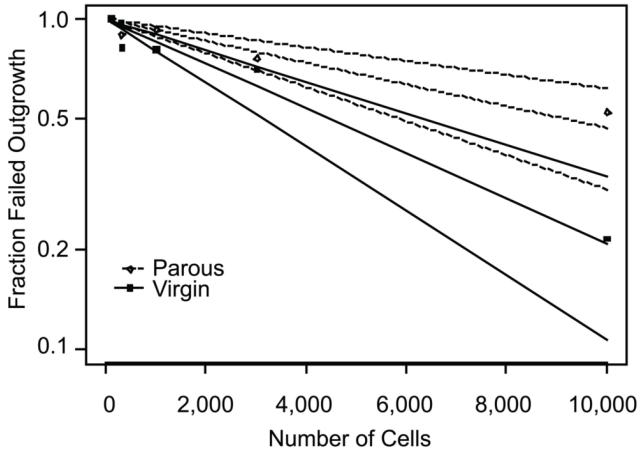
(A) Single cell suspensions were made from the pooled #2–4 mammary glands of each mouse, and the total number of viable cells recovered from each mouse was determined by FACS and hemocytometry. n=19 (virgin) or 20 (parous). (B) Single cell suspensions made from parous and age-matched virgin mouse mammary glands were spotted on slides and probed by immunofluorescence for luminal (keratin 8) and myoepithelial (keratin 5) markers. n=3. (C) Mammary cells from early parous or age-matched virgin mice were plated at 10,000 cells/well under ultra-low adherent culture conditions. Sample mammospheres that formed after 10 days in culture are shown. Scale bar = 50 μm. Primary mammospheres were fixed, sectioned, and stained for myoepithelial (keratin 5, left, arrows) and luminal (keratin 8, center) cell markers.

All mammospheres contained keratin 8^+ cells; approximately one in three also contained keratin 5^+ cells. Scale bar = 10 μm. (D) Primary mammospheres larger than 50 μm were quantitated at 10 days, dissociated to single cells (by digestion in 0.05% trypsin EDTA followed by pipetting), and replated at 5000 cells/well to measure secondary mammosphere formation. The number of mammospheres formed as a percentage of cells plated is shown. n=9 (primary mammospheres) or 5 (secondary mammospheres).

A.

	Number of cells injected	Estimated epithelial cells	Outgrowths/Transplants	
			Virgin donor	Parous donor
a	100	40	0% (0/9)	0% (0/9)
b	300	120	18% (2/11)	10% (1/10)
c	1,000	400	19% (6/32)	7% (2/28)
d	3,000	1,200	30% (8/27)	24% (6/25)
e	10,000	4,000	78% (18/23)	48% (11/23)
f	100,000	40,000	100% (4/4)	100% (2/2)
Estimated stem cell frequency				
Among total mammary cells			1 per 6,423	1 per 13,221
Among epithelial cells			1 per 2,608	1 per 5,050

B.



C.

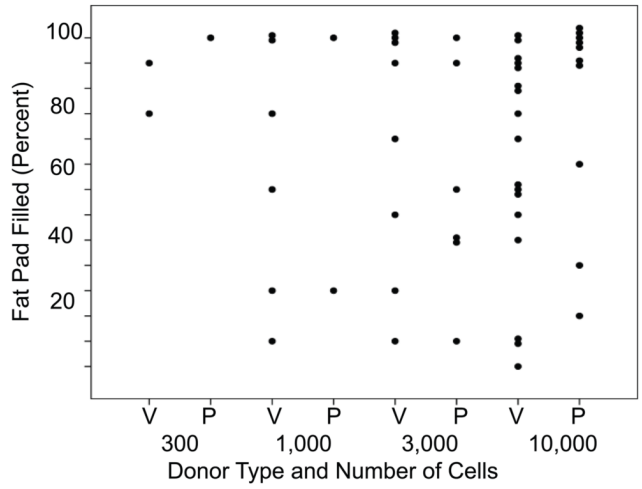


Figure 2. An early pregnancy reduces the number of mammary stem cells

(A) The indicated number of mammary cells isolated from parous (n=11) or age-matched virgin mice (n=11) was injected into the cleared #4 fat pads of recipient mice. The number of successful outgrowths (>5% fat pad-filling) after 8 weeks and total number of transplants performed are shown. These data were analyzed by using Generalized Estimating Equations in a Generalized Linear Model with a logit link function (PROC GENMOD, SAS V9.1, Cary, NC)[53]. This ANOVA-like analysis, which tests for overall effects of parity while accounting for dilution and the paired nature of the outgrowth data, found that an early parity significantly reduced the take rate compared to virgin (p=0.017). Limiting dilution analysis was conducted to estimate the frequency of mammary stem cells per total mammary cells by fitting the single-

hit Poisson model (SHPM) to the limiting-dilution data from (A) using a complementary log-log generalized linear model[54]. The fit of the model was checked using the method proposed by Bonnefoix et al [55]. (B) Regression lines of the estimated outgrowth frequency and 95% confidence intervals are graphed. The Wald confidence intervals were calculated via delta method for the frequency of regenerative stem cells. Limiting-dilution statistical analyses were performed using the limdil function in the Statmod package [56] in the software R [57]. (C) The extent of the cleared fat pad filled by outgrowths in (A) is shown.