Review
Re-evaluation of mammary stem cell biology based on *in vivo* transplantation

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Abstract

Over nearly half a century, transplantation methods have been employed to regenerate the mammary gland *in vivo*. Recent highly cited reports claim to have demonstrated the regeneration of an entire functional mammary gland from a single mammary epithelial cell. Nevertheless, re-examination of the literature on the transplantation biology of mammary gland regeneration reveals that a complex, combinatorial interaction between variously differentiated mammary epithelial cells and the mammary fat pad stroma is indispensable to this process. In the present article, these issues are reviewed and discussed to provide a greater understanding of the complexity of these multiplex interactions.

Early history

The experiments that demonstrate the presence of stem cells in the mammary gland are based on the pioneering studies of DeOme and his students, Les Faulkin and Charles Daniel. The approach they used was serial transplantation of normal mammary gland into the cleared mammary fat pad of syngeneic mice [1,2]. The cleared fat pad transplantation technique allowed the transplantation and growth of normal mammary cells into their normal anatomical site and under the influence of a normal physiological environment. Using this method, they demonstrated that the normal mammary gland contains cells that will grow and fill the fat pad with a normal ductal mammary tree and will respond to hormones with a normal differentiation program [3]. The progeny of the transplanted cells could be serially transplanted into the appropriate recipients multiple times; however, unlike preneoplastic cells or neoplastic cells, the normal cells always senesced after multiple serial transplants, generally five to eight transplant generations [4]. This was interpreted as indicating that the proliferative activity was a finite property of the stem cells. This finite lifespan was a fundamental difference between normal and preneoplastic/neoplastic mammary cells.

Subsequent studies demonstrated that stem cells were located along the entire mammary tree and were represented in the different developmental states of the mammary gland. These stages included primary and tertiary ducts from 6-week and 16-week virgin gland, uniparous and multiparous regressed glands, and 15-day pregnant and 10-day lactating glands [5]. Host age and reproductive history had little influence on the frequency of stem cells as measured by the percentage of successful takes and a lifespan assay [5,6]. Mammary cells from 26-month-old virgin mice had the same transplant potential as cells taken from 3-week-old mice. Both cell populations senesced after five transplant generations. Similarly, continuous hormone stimulation did not induce additional loss of ductal growth potential. These studies suggested that the mammary stem cell is a relatively quiescent cell that is only activated under conditions of gland repopulation (that is, fetal growth stage, pubertal growth phase). Under other conditions, such as pregnancy, it is probable that ductal and alveolar progenitor cells form the bulk of the increased mammary epithelial cell population [7].

These early studies demonstrated that the lifespan was intricately linked to proliferation activity. For example, the lifespan was correlated with the interval of serial transplantation. Transplanting at 12-month intervals instead of at 3-month intervals therefore prolonged the ultimate lifespan of normal cells [8,9]. Similarly, transplanting from the periphery of the ductal outgrowth (that is, such cells would have undergone more cell divisions) resulted in earlier senescence than transplanting cells from the center (that is, the original transplant site) of the outgrowth. In summary, these early studies suggested the presence of a mammary cell that could repopulate the mammary gland and could undergo a normal and complete morphogenic program (that

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ERα = estrogen receptor alpha; GFP = green fluorescent protein; MMTV = mouse mammary tumor virus; PI-MEC = parity-identified mammary cells; PR = progesterone receptor.

is, a stem cell). Such cells were spaced throughout the mammary tree, were quiescent and had a finite lifespan.

A commonly stated assumption that normal mammary stem cells are an ideal target for oncogenic transformation because they, like cancer cells, share a long lifespan (that is, replicative potential) is not supported by the transplantation results. At least for the mammary gland, the evidence to date suggests that mammary stem cells have a finite lifespan. Although untested, another possibility for the appearance of growth senescence might be due to failure of the micro-environment (niche) to provide the signals appropriate for stem cell self-renewal. This deficiency would, by necessity, involve the epithelial cell population surrounding the stem cell proper since transplantation always occurs into young mammary fat pad stroma. A corollary to this possibility would be that signals emanating from the transformed progeny surrounding the self-renewing premalignant/tumorigenic cell rather than a property intrinsic to the premalignant/tumorigenic cell are responsible for the infinite replicative lifetime of an immortalized mammary population.

Lineage-limited progenitors among mammary epithelium

Evidence for lobule-limited and duct-limited pluripotent mammary epithelial cell activities has been established for both rats and mice by transplantation of limiting dilutions of dispersed mammary epithelial cells into hosts that were subsequently impregnated and/or treated with hormone combinations to produce alveologenesis [7,10-13]. These limited structures contain both luminal epithelial cells and luminal myoepithelial cells. Studies with retrovirally marked clonal mammary populations demonstrated that both of these lineage-limited activities were present within the clonal populations through repeated transplant generations, indicating their derivation from a single pluripotent antecedent [10,13]. In addition, serial passage of the retrovirally marked mammary epithelial clones in pregnant hosts showed that the capacity of individual outgrowths to produce lobulogenesis or ductal elongation were independently lost during the acquisition of growth senescence among individual transplants [13].

The distinction between these two progenitor-mediated activities in regenerating mammary tissue is that the lobule-limited progenitor is unable to produce cap cells, which are required for the penetration of the mammary fat pad at the tips of the growing terminal end buds. On the other hand, duct-limited progenitors fail to produce progeny capable of sustaining alveolar development and growth during pregnancy. With the development of the WAP-Cre model used in combination with the Rosa26LacZ reporter mice, evidence surfaced for a LacZ-marked lobular-limited progenitor observable in parous mouse mammary epithelium [14]. These LacZ-positive, parity-identified mammary cells (PI-MEC) were found to be pluripotent, self-renewing and capable of

maintaining their lobule-limited progenitor activities following serial transplantation in epithelium-free mammary fat pads when the hosts were subsequently impregnated [15]. During pregnancy in these hosts, the PI-MEC proliferated and gave rise to LacZ-positive luminal progeny that were progesterone receptor (PR)-positive or estrogen receptor alpha (ERα)-positive and to luminal progeny that were bereft of these steroid receptors. Further in the developing secretory acini, they contributed not only secretory progeny but also LacZ-positive myoepithelial cells.

Originally it was proposed that the LacZ-positive PI-MEC arose from de-differentiated secretory epithelial cells that had survived involution and remodeling of the mammary tissue; however, further study indicated that these cells were present in the mammary tissue of nulliparous females and that they could be detected in explant cultures after treatment of the fragments with growth factors that do not induce lactogenic differentiation [16]. These cells were shown to possess all the properties of PI-MEC, including self-renewal and pluripotency. These observations support and confirm those reported earlier [7], which indicated the presence of lobule-limited progenitor activity in limiting dilution transplants of epithelium from nulliparous donors in pregnant transplant hosts.

More recent evidence demonstrates that PI-MEC are marked by the expression of green fluorescent protein (GFP) in WAP-Cre/chicken actin gene promoter flox–stop–flox–GFP parous females. In these studies, GFP+ PI-MEC were fluorescent activated cell sorted and found to be virtually 100% present in the CD49f^{hi} population [17]. This population was shown earlier to possess essentially all of the mammary repopulating activity [18]. Subsequent transplantation of GFP+/CD49f^{hi}-positive PI-MEC and the GFP−/CD49f^{lo} epithelial cells into epithelium-divested mammary fat pads indicated that all of the repopulating activity was associated with the GFP+ fraction [17].

The foregoing data and the observations reported earlier [15] suggest strongly that PI-MEC (that is, lobule-limited progenitors) are indispensable to mammary gland ductal reconstitution in transplanted mammary fat pads.

With respect to tumorigenesis, both lobule-limited hyperplasia and duct-limited hyperplasia have been repeatedly isolated and propagated from mouse mammary glands [19,20]. These populations do not exhibit growth senescence upon serial passage, and most exhibit an increased predilection for developing stochastic mammary tumors. Their existence strongly implicates the lobule-limited and duct-limited mammary stem/progenitor cells as targets for tumorigenic transformation. These premalignant lesions have been variously induced in the mammary epithelium by hormonal treatment, mouse mammary tumor virus (MMTV), chemical carcinogens and combinations of these agents [19-21]. The MMTV-infected populations have been

definitively demonstrated to be clonally derived, indicating that the hyperplasia and the subsequent mammary tumors have arisen from a single transformed cell [22]. Likewise, transplantation of nontransformed, normal MMTV-infected mammary tissue fragments give rise to clonal populations, indicating that all cells in the fragment capable of contributing progeny to the glandular outgrowth have derived from the same MMTV-infected antecedent. As mentioned earlier, the normal clones contain both lobule-limited progenitor/stem cells and duct-limited progenitor/stem cells and they exhibit growth senescence upon serial passage [10,13].

Dispersed cell implantation compared with fragment: clonal or combinatorial

It has been shown – both directly by retroviral tagging in serially transplanted MMTV-infected mammary outgrowths and, more recently, by implantation of visually confirmed single cells – that an entire functional mammary gland may be developed from the progeny of a single cell. On the contrary, considerable evidence exists that transplantation of dispersed mammary epithelial cells comprised of unsorted heterogeneously marked epithelial cells produce complete outgrowths that are frequently (in some cases, invariably) mixtures of the progeny derived from the variously marked donor cells [14,15,17,23,24].

In the absence of ERα expression, duct elongation and development fails in both pubertal females and parous females [23]. The amphiregulin null mouse mammary gland phenocopies this deficiency, indicating that amphiregulin is a major duct-specific growth signal mediated through ERα-positive mammary epithelial cells. Despite this indication, both ERα^{null} mammary epithelial cells and amphiregulin null mammary epithelial cells are capable of contributing progeny to all mammary epithelial subtypes when dispersed and mixed with wild-type mammary epithelium before injection into cleared mammary fat pads [23,24]. The evidence from PR null models reveals that alveologenesis cannot proceed in the absence of paracrine signals from PR-positive epithelial cells [25]. Nevertheless, dispersed PR^{null} cells marked by LacZ expression contribute alveolar progeny when mixed with wild-type epithelial cells in pregnant hosts. This observation clearly demonstrates that a complete mammary epithelial outgrowth cannot be formed without ERα-positive and PR-positive epithelial cell subtypes. These findings argue that a single mammary cell injected into an empty mammary fat pad must, at a minimum, divide asymmetrically (and remain a stem/progenitor cell) to produce an ERα-positive daughter and later again to produce cap cell progeny in order to begin ductal growth, and still later to produce a PR-positive cell to support side branching and subsequently alveologenesis.

The clear existence of lineage-limited, pluripotent duct and lobule progenitors within the nulliparous mouse mammary epithelium raises the strong probability that these cells might combine to produce mammary outgrowths comprising both ductal and lobular development when inoculated in dispersed cell populations. PI-MEC (that is, lobule-limited stem/progenitor cells) produce PR-positive and ERα-positive progeny as well as progeny negative for these receptors when contributing to mammary outgrowths in the pregnant host [15]. Similar findings were obtained when duct-limited outgrowths were tested for the presence of these steroid nuclear receptors. These results indicate that each of these lineage-limited stem/progenitors is capable of producing cell progeny shown above to be indispensable for complete mammary development. The lines between the primary antecedent and the downstream stem/progenitors therefore become blurred regarding their relative importance in producing complete mammary outgrowths in transplanted fat pads. Serial transplantation of clonal populations by fragment implantation into subsequently impregnated hosts showed that the capacity of any given fragment to produce alveologenesis and/or duct elongation was lost independently during the onset of growth senescence [13]. Serially transplanted growth senescent duct fragments were earlier shown to be able to generate lobuloalveolar growth upon impregnation of the transplant host [9]. The conclusion drawn from these observations postulates that either each lineage-limited stem/progenitor activity decays independently from the other during outgrowth development or that the primary mammary stem cell loses the capacity to produce one or the other lineage-limited downstream stem/progenitor during its own self-renewal while expanding in the previous generation.

To summarize, both dispersed cell and fragment implantation led to mammary epithelial outgrowths comprised of progeny produced by independently self-renewing stem/progenitor populations. These facts do not in any way dispute the existence of a primary mammary stem cell antecedent. They do indicate, however, the persistence of multiple pluripotent stem/progenitor cell activities within the mammary epithelial population that are capable of independently contributing diverse epithelial progeny during mammary gland growth and regeneration.

What is relevance of immortal lineage-limited transplantable populations to the cancer stem cell debate?

A massive scientific literature exists that identifies and characterizes the presence of premalignant mammary epithelial lesions in rodents. These lesions are transplantable to epithelium-divested mammary fat pads, as in normal mammary epithelium, and grow maintaining their hyperplastic phenotype [19,20]. Unlike normal mammary outgrowths, these populations do not exhibit growth senescence upon serial transplantation. Similar to normal mammary outgrowths, they fail to grow in ectopic sites, do not overgrow normal mammary outgrowths within the same fat pad and cease to grow when the confines of the mammary fat pad are reached. These populations exhibit a greater tendency to develop focal mammary tumors than normal mammary outgrowths.

Two major phenotypic premalignant immortal populations have been isolated, those populations that maintain a lobular–alveolar morphology and growth pattern in the absence of pregnancy and those that grow with a ductal morphology. It is tempting to ascribe the origins of these premalignant populations to immortalized lobule-limited progenitors and duct-limited progenitors, respectively, which would mean these lineage-limited progenitors are targets for malignant transformation. Both of these populations comprise luminal and myoepithelial cell types in keeping with the pluripotent nature of the lineage-limited progenitors. Many of the ductal hyperplasia lines (that is, EL-12) can be induced with hormonal stimulation to form secretory alveoli. Others (that is, EL-11) are refractory to alveolar development upon hormonal treatment or even in full-term pregnant hosts [26,27]. These latter populations in all probability contain only duct-limited stem/progenitors. Alveolar hyperplastic outgrowths are unable to produce terminal end buds with their characteristic cap cells and are therefore unable to produce ducts; instead they expand radially in a manner that is presently poorly understood. The hypothesis is that these populations are supported entirely by transformed lobule-limited stem/progenitors. This has not yet been definitively proven.

The *in situ* lesions, hyperplastic alveolar nodules and hyperplastic ductal lesions that give rise to the immortalized outgrowths described above may be considered analogous to early-stage hyperplastic lesions described in the human breast (for example, hyperplastic enlarged lobular units and proliferative disease without atypia), and as such may reveal important clues to the etiology of these human breast lesions upon further study. We suggest these models indicate that self-renewing pluripotent mammary cells other than the primordial mammary stem cell can be targets for neoplastic transformation.

What are long label-retaining cells: unique to stem cells or mixed?

Long DNA label retention has repeatedly been ascribed as a property of stem cells due to their supposed absence of mitotic activity during tissue homeostasis. Recent studies have indicated that long label-retaining cells in a variety of tissues actually cycle and retain their original labeled DNA template strands. This has been demonstrated in the intact mouse mammary gland and in outgrowths from transplants of mammary epithelium into cleared mammary fat pads [28]. In both instances, label-retaining epithelial cells following prolonged chase periods were labeled by a second DNA analogue and were shown to transmit the second label (associated with newly synthesized DNA strands) to their immediate progeny. This property was demonstrated for ERα-positive, PR-positive mammary epithelium as well as those not expressing these receptors [29]. In addition, lobule-limited alveolar stem/progenitors (PI-MEC) were shown by Smith to adopt this method of asymmetric division (that is, selective template DNA segregation) in growing transplants in nonpregnant hosts [28].

These observations indicate that the property of selective segregation of DNA strands by asymmetrically dividing cells is not only a property of stem cells but also of lineage-limited stem/progenitors, and perhaps specific transit amplifying committed epithelial cells as well. Much more study of this important asymmetric mitotic event is required and necessary to establish both the mechanism for this selective segregation and to understand its role in tissue development, differentiation, repair and maintenance.

Immortality versus senescence: role of stem cells versus niche

One of the primary findings made through transplantation of mammary tissue into the cleared mammary fat pads was the recognition that growth senescence was reached during serial transplantation of normal mammary epithelium but was not observed when premalignant mammary tissue was proliferated in the same way [1]. This paradox remains a mystery. This property of proliferative immortality is a property of premalignant mammary epithelium irrespective of the initiating treatment, whether it is viral, hormonal or chemical. Proliferatively immortal lines were also selected from mammary epithelial cells propagated *in vitro*. It was shown in selected lines that immortality during transplantation and the increased propensity to develop focal neoplastic lesions were unlinked [26]. Some of these lines showed no trend to develop focal mammary tumors.

The immortality phenotype may be considered linked to self-renewal, as molecular markers (such as retroviral insertions) specific for any specific immortalized premalignant mammary population are stable and reproducible through successive generations. It is commonly held that growth senescence during serial transplantation of normal gland results from the loss of self-renewal capacity of the mammary stem cell. As discussed above, however, steroid receptor-expressing epithelial cells are essential for ductal and lobular growth and development. The absence of one or other of these essential paracrine-mediating epithelial cell types could therefore explain the absence of continued epithelial growth. The appearance of growth senescence, then, could result without loss of the stem cell proper, and could instead result from an alteration in the microenvironment supporting stem cell function. No specific experimental approach to the resolution of this issue has been addressed in the mammary scientific literature.

CD49f, CD24, CD29, and so forth: meaning and/or markers for stem cell activities

There seem to be conflicting views regarding the importance of these surface markers and their relevance to the prospective isolation of populations of epithelial cells enriched for their ability to produce competent mammary epithelial reconstitution in transplanted mammary fat pads. Two groups have claimed that CD49f^{hi}/CD24^{pos} or CD29^{hi}/CD24^{pos} cells constitute populations highly enriched for mammary stem cell activities competent for regeneration

of a complete and functional mammary gland and capable of self-renewal [18,30]. Reports from another group indicate that the bulk of *in vivo* reconstituting activity resides in the CD24^{lo} population and practically none is associated with CD24^{hi} in cells isolated from mammary tissue using this single cell surface marker [31]. The CD24^{hi} group was further segregated into two separate fractions by sorting with prominin^{hi} (CD133) and by the presence or absence of ERα in CD24^{hi} cells. These fractions also lacked *in vivo* regenerating activities [32].

In another study, it was shown that Pl-MEC (that is, lobule-limited stem/progenitor cells) from WAP-Cre/chicken actin gene promoter–GFP mammary glands were virtually all found within the CD49f^{hi} sorted population [17]. These studies provide clear evidence that CD49f^{hi}/CD24^{pos} cell fractions are not homogeneous for primary mammary stem cells; nevertheless, the current literature continues to exhort the high expression/content of these cell surface markers as indicative of the presence of the mammary stem cell, when in fact these markers are also highly expressed upon lineage-limited lobular stem/progenitors.

What have we learned recently about mammary stem cell biology from transplantation studies and where shall we go from here?

To highlight the influence of diverse mammary epithelial cell types in bringing about successful regeneration, near-limiting dilutions of dispersed mammary epithelial cells were comingled with testicular cells isolated from adult WAP-Cre/Rosa26R mice. The resulting mixtures were inoculated into cleared fat pads, and mammary ductal morphogenesis was allowed to proceed for 6 to 8 weeks. Subsequently, a fraction of the transplant hosts were maintained as virgins and the rest were mated and permitted to complete a full pregnancy, lactation and involution cycle. To determine whether testicular cell progeny were present among the involuted mammary epithelial cell population, X-gal whole-mount staining was carried out. Only male cells possess the WAP-Cre and Rosa26 LacZ reporter gene. LacZ-positive cells among the regenerated mammary epithelium therefore indicate the presence of testicular cell progeny. Of 22 successful mammary outgrowths, 18 outgrowths possessed LacZ-positive mammary epithelium. The mammary nature of these LacZ-positive cells was confirmed by staining for mammary-specific markers for milk protein synthesis, cytokeratins K5/K14 and smooth muscle actin. Fluorescent-label *in situ* hybridization analysis confirmed that these cells were male and indicated the absence of fusion between male and female cells. LacZ-positive cells were found in all second-generation transplants from the male/female chimeric outgrowths, indicating their capacity for self-renewal.

These experiments demonstrate the overarching importance of the signals provided by mammary epithelial cells for the development of a microenvironment(s) capable of sustaining stem cell activity and differentiation. Experiments have also demonstrated that neural stem cells and lineage negative bone marrow cells isolated from WAP-Cre/Rosa26 LacZ reporter mice responded in the same manner as the testicular cells in this mammary niche assay (unpublished observations).

In the human breast, little transplantation biology is available due to technical difficulties in establishing mammary out-growths *in vivo*. Progress has been made recently in this area through humanization of the mouse mammary fat pad with human-derived stromal cells [33]. The results of successful implantations of normal human organoids indicate that independent ductal, lobular and acinar structures may be generated within humanized mouse mammary fat pads by human mammary epithelial cells. This result and those demonstrating the association of bi-potency with individual mammary epithelial cells [34,35] suggest that a similar stem/progenitor cell hierarchy exists in human breast epithelium.

The present discussion seeks to heighten the awareness of those interested in studying the unresolved complexities involved in mammary developmental biology and mammary neoplastic progression, and further to suggest biological events wherein our current level of ignorance could be addressed through thoughtful investigation. Four critical issues that require further study in mammary gland stem cell biology and that are approachable through transplantation study are as follows. First, what is the basis for growth senescence in regenerating mammary epithelial populations? This problem has only been examined for fragment transplantation and little is known regarding this issue via dilution studies of mammary cells isolated and dispersed from serially transplanted populations. Second, how is growth senescence avoided in premalignant populations? Alterations in the expression of some genes (for example, p53 and p19ARF [20,36,37]) result in escape from growth senescence in mammary transplantation studies. What role do these genes play in mammary stem cells or their immediate progeny (niche)? Third, what are the relative roles of the lineage-limited stem/progenitor populations in these two foregoing issues? Finally, are the lineage-limited stem/progenitor cells themselves *bonafide* targets for oncogenic transformation?

From the foregoing it must be clear that none of the markers used for concentrating mammary stem cell activities, as determined by successful mammary outgrowths *in vivo* (CD49f^{pos}, CD29^{pos}, CD24^{pos}), produces a uniform population of mammary stem cells. Rather, a mixture of stem cells, lineage-limited stem/progenitors and differentiated epithelial cells are essential for completion of a functional mammary stem cell niche and are likely to be represented among this population. Our challenge is not to sort out from this mixture the primal mammary stem cell, but instead to comprehend the interaction among these components that allows the long-term maintenance of mammary stem cell activity. We wish to

emphasize that focusing our primary deliberations upon the primordial mammary stem cell deflects our attention from the important issue of extending our understanding of how stem/progenitor cells and their progeny interact to maintain mammary homeostasis and how this may be disturbed during neoplastic transformation.

Competing interests

The authors declare that they have no competing interests.

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