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Models of Breast Morphogenesis Based on Localization of Stem Cells in the Developing Mammary Lobule

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SUMMARY

Characterization of normal breast stem cells is important for understanding their role in breast development and in breast cancer. However, the identity of these cells is a subject of controversy and their localization in the breast epithelium is not known. In this study, we utilized a novel approach to analyze the morphogenesis of mammary lobules, by combining one-dimensional theoretical models and computer-generated 3D fractals. Comparing predictions of these models with immunohistochemical analysis of tissue sections for candidate stem cell markers, we defined distinct areas where stem cells reside in the mammary lobule. An increased representation of stem cells was found in smaller, less developed lobules compared to larger, more mature lobules, with marked differences in the gland of nulliparous versus parous women and that of *BRCA*1/2 mutation carriers versus non-carriers.

INTRODUCTION

The mammary gland differs from other organs in that it continues to undergo morphogenesis postnatally, with paramount changes in tissue structure and cell population dynamics occurring during developmental windows such as puberty, pregnancy, and menopause. These processes are likely supported by a population of mammary stem cells that resides within the tissue. Regeneration of the entire gland from one single cell in multiple passages together with lineage-tracing experiments in vivo constitute compelling evidence for the existence of stem cell populations in the mouse mammary gland (Prater et al., 2014; Rios et al., 2014; Shackleton et al., 2006). However, owing to obvious experimental limitations, there is no direct definitive proof for the existence of a bona fide stem cell population active in the adult human mammary gland. Xenotransplantation of human mammary epithelium in cleared humanized mammary fat pads or under the renal capsule of immunodeficient mice allows in vivo outgrowths equivalent in size to one human mammary lobule. There has been no evidence of the generation of large mammary ducts in any in vivo or in vitro model. Moreover, only surrogate assays for self-renewal of putative human mammary stem cells are available for experimentally testing cell functions (Dontu et al., 2003; Eirew et al., 2008).

Given these limitations, the current knowledge regarding the identity of normal human mammary stem cells is based on markers that associate with the highest enrichment in stem-like functional properties, such as the ability to differentiate along both luminal and myoepithelial lineages, branching morphogenesis in 3D culture, and generation of outgrowths in xenotransplantation experiments. Combinations of cell surface markers that have been used to detect cell populations enriched in these properties include $CD49f^{high}EpCAM^{low}$ (Eirew et al., 2008; Lim et al., 2009), CD73+CD90– (Roy et al., 2013), CD10+ (Keller et al., 2012), and CD49f+DLL1+DNER+ (Pece et al., 2010). Functional properties used to identify stem cells are high aldehyde dehydrogenase (ALDH) activity (Ginestier et al., 2007) and the ability to survive and proliferate in anchorage-independent conditions (Dontu et al., 2003; Pece et al., 2010). Some of these markers (i.e., ALDH+ and CD49f+) correlate with poor clinical outcome when highly expressed in breast tumors (Ali et al., 2011; Ginestier et al., 2007), possibly because they also identify a cancer stem cell population. Other stem cell markers validated in in vitro assays include SSEA4+ and CK14+CK19+ (Villadsen et al., 2007). All these phenotypes identify heterogeneous cell populations that contain more differentiated cells in addition to stem cells.

The combination of assays and markers listed above have not led to a consensus regarding the identity and

localization of human mammary stem cells (Visvader and Stingl, 2014). To address this issue, we adopted an alternative, theoretical approach based on modeling mammary morphogenesis. We utilized 1D cell-replacement rules as well as computer-generated 3D fractals for modeling the human mammary lobule. This approach allowed us to formulate hypotheses for the localization of stem and progenitor cells within the branching structure of the gland. We compared predictions of these theoretical models with the pattern of marker expression in situ, as determined by immunostaining of sections of normal breast. Several proposed stem cell markers were co-expressed and their localization in situ coincided with the predictions of one of the models put forward in this study, in which stem cells are primarily present in clusters at the growing ends of intralobular branching ductules.

This analysis of adult stem cell localization in the context of 3D architecture of the mammary lobule establishes consensus regarding the identity of adult mammary stem cell markers, and it proposes a model of lobule morphogenesis with implications for the cellular origin of breast cancer.

RESULTS

Theoretical Models of Mammary Lobule Development

We set out to model mammary lobule development to clarify the contribution of stem cells to breast morphogenesis. The models generated may have additional applications in histological studies of branched epithelia.

The tree-like structure of the human mammary gland consists of lobules and extralobular ducts that collect into big galactophore ducts (Figure S1A). Lobules are formed of dichotomically branched ductules, the ends of which form the alveoli filled with milk during lactation. Both ducts and lobules are delineated by two layers of epithelial cells: an inner layer of luminal cells and an outer layer of myoepithelial cells. Mammary lobules are the dynamic units of the normal adult breast, with a much higher cellular turnover than the ducts. It is universally recognized that the vast majority of breast cancers originate within the lobule rather than in the large extralobular ducts (Gusterson et al., 2005; O'Malley et al., 2011). For these reasons, we focused on modeling the developing mammary lobule.

We initially generated 1D cell-based models of lobule development with replacement rules for each dividing cell. We assumed that cellular de-differentiation is not a common phenomenon in the normal adult breast tissue, that cellular differentiation is accompanied by a progressive reduction in proliferative potential, and that terminally differentiated cells do not proliferate. For simplicity, we neglected naturally arising noise in cell division and performed a deterministic parallel replacement of each cell with its two daughters at each generation. Under these assumptions, several theoretical models of lobule development can be formulated based on the type of cell divisions that stem cells undergo and based on the spatial orientation of cell progeny relative to the mother cell and the parental duct. We use the term stem cell for the most undifferentiated cell type in the lobule, although we recognize that this may be a primitive type of progenitor cell. All other cells that can proliferate are termed progenitor cells. The fate choices taken into account for stem cell divisions were as follows: (1) asymmetric self-renewal or differentiation, (2) high or low rate of entering the cell cycle from quiescence, and (3) distal or proximal orientation of the more undifferentiated progeny relative to mother cell and parental duct (Figure S1C; Supplemental Experimental Procedures).

Combinations of these fate choices generated eight different models for lobule growth that differed in rate of growth and differentiation, as well as in localization and representation of stem cells within the developing lobule. In Figure 1, we show two examples of different outcomes in cell disposition within the lobule generated by different combinations of cell fate decision. All of the eight models are shown in Figure S1D. Two additional cell fates were also modeled, i.e., symmetric self-renewal of stem cells accompanied by asymmetric division of progeny (example shown in Figure S1E) and symmetric cell divisions of progenitor cells (example shown in Figure S1F). Other combinations including these cell fates are not presented here because the outcome cannot be distinguished from the models shown in Figure S1D, being different only in growth rate. For the simplicity of diagrams, only the luminal cell layer is shown. The myoepithelial layer is supposed to be generated from stem cells in the same direction as luminal cells. It is formed of fewer, longer cells with uniform morphology and marker expression.

We compared the predictions of the models shown in Figures 1 and S1 with observations of distribution of markers for proliferation and lineage differentiation, as well as the estrogen receptor (ER) in mammary lobules in sections through normal breast tissue. ER+ cells contain early progenitor cells according to several studies (Honeth et al., 2014; Keller et al., 2012; Shehata et al., 2012). Whereas lineage differentiation markers (e.g., CD10, EpCAM, SMA, and cytokeratins 18 and 19) have a uniform distribution in the lobule, proliferation markers (e.g., MCM2 and Ki67) and ER are present in scattered cells or in clusters of cells across lobules (Figure S2; Santagata et al., 2014). The majority of the models we generated predicted a continuous gradient of proliferation and differentiation along the growing lobule (see examples in Figures 1A and S1D). If lobule development would follow one of