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Breast Cancer Subtypes: Two decades of Journey from Cell Culture to Patients

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Abstract

Breast cancer remains the second leading cause of cancer-related deaths among women. Clinically breast cancer patients present with distinct diseases with vastly different outcomes. Recent molecular profiling has identified five major subtypes of breast cancers. Importantly, survival analyses have shown significantly different outcomes for patients belonging to various subgroups. These studies strongly support the idea that breast tumor subtypes may represent malignancies of biologically distinct cell types producing distinct disease entities that may also require different treatment strategies. Alternatively, different types of breast cancers may arise from a common precursor based on oncogene-driven reprogramming. Experimental systems that clearly define cancer cell heterogeneity and link this process to cancer stem/progenitor cells have not been developed. It is also unclear if oncogenic transformation of committed progenitors drives them along their committed pathway, and hence the cell of origin determines the histological features of breast cancer, or if different oncogenic pathways can transform the same precursor along distinct phenotypes. One major hurdle to addressing these fundamental questions about the origin and heterogeneity of human breast cancer is the lack of immortal human stem/progenitor cells that could be interrogated with breast cancer-relevant oncogenesis protocols.

We have now identified, isolated and immortalized (using hTERT) such mammary stem/progenitor cells that are immortal and still maintain their progenitor/stem cell properties (self-renewal and differentiation into myoepithelial and luminal cells). Our research using these progenitor/stem cells that are highly susceptible to oncogenesis and various models of mammary cell immortalization has allowed us to define several novel cellular pathways and demonstration of their involvement in oncogenesis and breast cancer progression. Given the emerging evidence that stem/progenitor cells are precursors of cancers and distinct subtypes of breast cancer have different survival outcome, these studies are timely and carry the potential of developing novel therapeutics in the future as well as provide potentially novel markers for diagnostic/prognostic use in breast cancer.

Keywords

breast cancer; stem cells; mammary epithelial cells; immortalization; transformation; breast cancer subtypes; basal cells; luminal cells; myoepithelial cells

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Introduction

Despite the enormous progress made in understanding biology of cancer over past two decades, breast cancer remains the second leading cause of cancer-related deaths among women in USA [1]. The major problem remains that although the majority of breast cancers first respond to therapy, they subsequently develop resistance to chemo-, radio- as well as biological therapies [2]. Moreover, the recent evidence that breast cancer is not a single disease but rather a group of diseases is making oncologists re-think their therapeutic strategies for breast cancers. Recent advancement in technology has made it possible for researchers to perform molecular profiling, which led to the identification of five major subtypes of breast cancers: a basal epithelial-like group, an ErbB2-overexpressing group, a normal breast epithelial-like group and two luminal subtypes [3, 4]. The significance of these studies became evident by analyses that showed different subtypes of breast cancers respond differentially to therapy; as basal subtype of breast cancer presents the most difficult disease with poor prognosis and poor disease-free and overall survival of these patients. On the other hand luminal A subtype of breast cancer presents a favorable outcome after conventional therapies [3, 4]. Although initially these studies were unexpected and particularly the nature of basal subtypes of breast cancers, our analyses of studies published using *in vitro* cell cultures of human mammary gland confirmed presence of a population of basal subtype of cells in human breast tissue [5]. More importantly, *in vitro* studies have shown high susceptibility of basal subtype of breast cells to oncogenic transformation [6]. Together, these studies strongly support the idea that breast cancer subtypes either represent malignancies of biologically distinct cell subtypes producing distinct disease entities that may require different treatment strategies, or different subtypes of breast cancers may arise from a common precursor based oncogene-driven reprogramming. With our newfound knowledge of breast cancer subtypes, increasing evidence that breast cancer may arise from breast cancer initiating or breast stem cells, and the notion that these cells may account for therapy resistance, there is a new hope for cancer researchers to define stem cell based therapeutics.

Our laboratory has been investigating these questions for over two decades and has developed cellular models with the goal to i) identify and define cancer initiating cells in human mammary gland that may represent the precursors of different subtypes of breast cancers and ii) identify novel cellular pathways whose disruption leads to transformation of cells. Below we will briefly focus on the work conducted in our laboratory over past twenty years.

Evidence that human breast tissue contains various epithelial cell subtypes

The human mammary gland consists of fatty and fibrous tissue along with a branching ductal system ending as terminal ducts and acinar structures called terminal ductal-lobular units (TDLUs) [7]. Although, not clearly demonstrated, it is believed that most breast cancers arise in TDLUs. It is unclear if a linear model of breast cancer progression similar to colon cancers exists, however different stages of benign breast cancers (such as typical and atypical hyperplasia), non-invasive cancers (such as carcinoma in situ - ductal or lobular), and invasive cancers (such as invasive ductal or lobular carcinomas) do exist. It is generally accepted that hyperplasia and/or carcinoma in situ represent earlier stages of invasive carcinomas. Additionally, multiple types of in situ carcinomas, such as solid, cribriform, papillary or comedo types have been reported and it is possible that these represent tumors originating from different epithelial subtype [7].

Histological examination of TDLU shows two major types of cells, luminal cells (that surround the lumen) and myoepithelial cells (that line the basement membrane) [7, 8]. The question we have been addressing is, if there are only two types of cells in breast, then what

is the origin of the different subtypes of breast cancers? Recent studies from our laboratory and that of others have shown existence of stem/progenitor cells in human tissues, which begins to answer these questions (discussed below).

Isolation of stem cells from normal and tumor breast tissues

Stem cells have an enormous replicative potential with the characteristics of self-renewal and multi-lineage differentiation. Although still a controversial notion, many cancers are thought to originate from cancer stem cells [9]. This idea has also generated a great interest in the field of breast cancer and researchers have begun to examine the existence of mammary stem cells. The cellular milieu of the mammary gland undergoes significant changes during pregnancy, lactation and involution [7]. These include bursts of proliferation of existing cells during pregnancy, continued differentiation during lactation and apoptosis during involution at the end of the cycle. This cyclical behavior strongly supports the idea of a stem cell-like population in the mammary gland, which would meet the demand of a pregnancy cycle. The existence of adult mammary epithelial stem cells has therefore been proposed. Direct evidence for the existence of such cells has come from clear fat-pad transplantation, retroviral tagging and X-chromosome inactivation studies in rodent model [8-14].

Recently, using various stem cell markers, attempts have been made to identify the human mammary epithelial stem cells [11-14]. Using immunomagnetic cell sorting based on surface antigen markers (Muc and ESA), Gudjonsson et al. were able to separate Muc-/ESA +/Ker-19+ cells that were able to self renew and give rise to Muc-/ESA+ cells, thus exhibiting characteristic of breast stem cells [13]. Dontu et al. isolated undifferentiated mammospheres from single cell suspensions of mammary epithelial cells (MECs) obtained by mechanical and enzymatic dissociations of mammary tissue [14]. Primary mammospheres can be further passaged to generate secondary mammospheres. Primary as well as secondary mammospheres were highly enriched in early progenitor or stem cells capable of differentiating along multiple lineages and of self-renewal. Immunostaining of these mammospheres showed the presence of CD10, $\alpha 6$ integrin and cytokeratin 5 on early progenitors, and ESA and cytokeratin 14 on late progenitor cells [14].

Apart from normal mammary stem cells, the possible existence of a breast cancer stem cell has been reported in the literature [15, 16]. In a NOD/SCID xenotransplants model, Al-Hajj et al. used four cell surface markers, CD44, CD24, ESA and B38.1 (a Breast/ovarian cancer specific marker) [16], and lineage markers to sort different populations of breast cells from breast tumor tissues. All mice injected with Lin-, CD44+, B38.1+, CD24-/low generated tumors while none of the mice injected with CD44-, B38.1-cells showed tumors. Lin-, CD44+, B38.1 fractions were further subdivided based on the expression of ESA. When used in xenotransplants, Lin-, ESA+, CD44+, CD24-/low cells generated tumors [16]. The presence of such a population in breast tumor tissues suggests the possible existence of breast cancer stem cells.

Establishment of culture conditions to isolate and propagate human mammary progenitor/stem cells

For more than twenty years, our laboratory developed techniques and culture conditions to establish normal human mammary epithelial cell (hMEC) [17]. In our laboratory, we defined a medium DFCI-1 that allows proliferation of hMECs for 3-4 passages (about 15-20 population doublings) and then most of the cells senesce (unable to proliferate) [17, 18]. However, after about a month of non-proliferating heterogeneous cell population, a more homogenous cell population emerges that continue to proliferate further for 50-60 population doublings, prior to senescence [17, 18]. This process of emergence of cells

capable of proliferating for extended period is also known as self-selection; prior to selection, the cells are termed pre-selection cells whereas those that emerge after selection are called post-selection cells. Several years ago, using two dimensional gel electrophoresis we had shown that hMECs cultured in DFCI-1 medium express keratin markers (K5, K6, K7, K14, K17, K18 and K19) that would characterize these cells as progenitors rather than a particular lineage i.e. luminal (K8, K18, K19) or myoepithelial (K5, K14). Furthermore, these cells expressed markers of basal (K5 and K6) subtypes of cells [5]. Although at that point it seemed a curious finding, recent direct cDNA microarray profiling of human breast cancers has led to similar insights i.e. identification of multiple subtypes of human breast cancers that present distinct outcomes. Importantly, phenotypic and gene expression characteristics of these breast cancer subtypes point to their possible origin from specific subtypes of MECs, such as basal or luminal cells [3, 4].

It is important to mention that hMECs exhibiting stem cell markers still underwent cellular senescence [5]. They entered into senescence either because i) currently available *in vitro* culture conditions developed by us and others for hMECs do not allow mammary stem cells to self renew indefinitely or ii) these hMECs are not stem cells but are uncommitted progenitor cells and after several population doublings they lose their ability to self renew. Unavailability of markers to distinguish stem vs. progenitor cells in mammary tissue makes it difficult for researchers to ascertain if hMECs cultured under these conditions are stem or progenitor cells.

In order to study these cells for further biological pathways, we have devised methods to isolate and immortalize (using hTERT-human telomerase reverse transcriptase) mammary stem/progenitor cells [19] and recently have been successful in defining culture conditions for their self renewal and differentiation (discussed below) [20].

hTERT immortal hMECs exhibit stem/progenitor cell properties and express markers for well-known stem cell signature pathways

As described above, we have successfully isolated immortal stem cell that uniformly expressed various markers, such as basal (K5, K14, p63, vimentin) and luminal (K8, K18, E-cadherin) cell markers. One cell strain (70N) also expressed K19 (a known luminal cell marker), CD29 and CD49f (two known stem cell marker) [11-14], but did not express detectable levels of differentiated luminal cell marker MUC1 [13 and 20 and references therein] or differentiated myoepithelial cell markers (such as α -smooth muscle actin (α -SMA), CD10 or Thy-1) [20 and references there in]. Notably, despite expression of several luminal cell markers, the primary and hTERT-immortalized hMECs lacked the expression of estrogen receptor alpha (ER-alpha) providing further support for the idea that stem cells lack ER expression [21]. Immunofluorescence analyses confirmed that the same cells expressed different lineage markers (Fig. 1). We also demonstrated the presence of such progenitor/stem cells in normal reduction mammoplasty tissue (Fig. 2). Importantly, hMECs expressing hTERT exhibit the same sets of markers as their parental primary hMECs indicating that stem/progenitor characteristics were maintained through immortalization [20]. Based on these results we speculate that different subtypes of progenitor cells do exist in primary hMEC cultures and a particular subset of cells from this population may give rise to a particular type of breast cancer during transformation process. These results are consistent with microarray expression analyses of various breast cancer specimens [3, 4].

Although we observed markers of stem/progenitor cells, the proof of principle of a stem/progenitor cell is in its ability to self renew and differentiate. Thus, we devised a method to assess ability of mammary progenitor cells to differentiate into myoepithelial and luminal cells *in vitro* [20]. Two types of stem/progenitor cells $K5^+/K19^-$ and $K5^+/K19^+$ cell types when cultured in MEGM medium [14, 20] on 2D plastic substratum, these cells proliferate

as tightly packed epithelial colonies, after one month a proportion of cells near the periphery adopt a spindle shaped morphology. While cells in center (undifferentiated progenitor cells) expressed the basal, luminal and stem cell markers, the spindle-shaped cells forming the peripheral halo were K5-negative and acquired several well known myoepithelial cell markers (α -SMA, CD10, and Thy-1) that were absent on the parental cells as well as in the central compact part of colonies grown in MEGM [20] (Fig. 3). Differential trypsinization and re-plating of central part of cells showed their ability to regenerate similar colonies as well as to generate differentiated myoepithelial cells. In contrast, most of the differentiated myoepithelial cells failed to proliferate, however if myoepithelial cells were transferred to DFCI-1 medium, some cells continued to self renew as myoepithelial cells, suggesting presence of myoepithelial progenitor cells. Furthermore, a subset of cells within the center of the colonies, were MUC1-positive consistent with their differentiation along the luminal cell lineage [13, 20 and references therein] (Fig. 3). Taken together these results support the conclusion that immortalized K5⁺/K19⁻ and K5⁺/K19⁺ cell types retain mammary stem or progenitor properties with capability of self renewal and differentiation along luminal and myoepithelial lineages.

Mammary progenitor/stem cells express markers of well defined stem cell pathways

There is increasing evidence that several pathways, such as Notch, Hedgehog, Wnt and Sox are altered in stem cell differentiation [22-25]. Thus, we performed Affymetrix-based genome-wide mRNA expression profiling of both K5⁺/K19⁻ and K5⁺/K19⁺ cell types and compared these with myoepithelial differentiated cells. These experiments showed significant alteration in known pathways in stem cell differentiation further substantiating our findings that K5⁺/K19⁻ and K5⁺/K19⁺ cell types represent stem/progenitor cells [20].

Mammary progenitor cells are highly susceptible to pre-neoplastic transformation

Over two decades, our studies have defined high susceptibility of mammary progenitor cells K5⁺/K19⁻ to oncogenic transformation. We have shown that introduction of various viral oncogenes, such as urogenital carcinoma-associated human papillomavirus (HPV) oncogenes E6 and E7, exposure to gamma-irradiation, as well as cellular genes (such as mutant p53, activated Rho GTPases and Bmi-1, a member of the polycomb group of transcriptional repressors) induce pre-neoplastic transformation i.e. immortalization of progenitor cells [18, 19, 26-28]. Other study has shown that ZNF217, a zinc finger protein that is overexpressed in breast cancers, can promote the immortalization of post-selection human MECs [29] (Fig. 4).

Cellular targets of E6 and their potential role in hMEC immortalization

Characterization of oncogenesis-related cellular targets of HPV E6 and E7 oncoproteins has been facilitated by their ability to dominantly immortalize primary human cells *in vitro* [18, 19, 30, 31]. The cellular protein that was first shown to interact with high-risk HPV E6 proteins was the tumor suppressor protein p53 via a ubiquitin ligase E6AP [32]. The p53 protein is a key regulator of DNA repair, cell cycle arrest and apoptosis in response to genotoxic stress [31-33]. These responses are essential to prevent the emergence of cells with unstable genomes that are expected to be susceptible to oncogenic transformation. Indeed, mis-sense mutations that inactivate p53, deletion of its gene or loss of its expression, occur in nearly half of all human cancers [31-33 and references there in]. The reproducibility and relatively high efficiency with which E6 (in post-selection hMECs) or E6 and E7 combination can induce the immortalization of hMECs have therefore opened a practical approach to elucidate the biochemical mechanisms of hMEC immortalization. In recent years, using the HPV16 E6 as a bait in yeast two hybrid analyses of a hMEC cDNA library, we have identified several known as well as novel targets of E6 oncogene that represent

potential mediators of E6-mediated hMEC immortalization [34]. These include ADA3 (alteration/deficiency in activation 3), a novel coactivator of p53 and steroid receptors (estrogen receptor and retinoic acid receptor) that enhances transcription. shRNA-mediated knockdown of ADA3 dramatically inhibits function of p53, ER and RXR proteins [35-37]; E6 targeted protein 1 (E6TP1), a novel GTPase activating for Rap small G protein; and protein kinase N (PKN), an effector for Rho small G protein [34, 38, 27]. Furthermore, MamL1, a human homologue of *Drosophila* mastermind gene and a known coactivator for Notch also interacts with E6 [39, 40]. These studies have implicated the p53, Notch, ER, Rho and Rap signaling pathways in early transformation of hMECs. Consistent with these analyses we have shown that expression of mutant p53 [26] or activated Rho [27] directly induces the immortalization of hMECs. Recently, we identified a human orthologue of *drosophila Ecdysoneless* (Ecd) protein as a E6 binding protein that also associates with p53 and induces p53-mediated cellular senescence in fibroblasts but not in epithelial cells [41]. In order to study the cellular function of Ecd in mammalian cells, we generated *Ecd^{lox/lox}* mouse embryonic fibroblast cells from *Ecd* floxed mouse embryo. Cre-mediated deletion of Ecd in *Ecd^{lox/lox}* MEFs led to a proliferative block due to a delay in G₁-S cell cycle progression; this defect was reversed by the introduction of human Ecd. Loss of Ecd led to marked downregulation of E2F target gene expression. Furthermore, Ecd directly binds to Rb at the pocket domain, and competes with E2F for binding to hypophosphorylated Rb. These results demonstrate that mammalian Ecd plays a role in cell cycle progression via Rb-E2F pathway [42, 43]. Recent studies from our laboratory show Ecd is overexpressed in several carcinomas, including breast cancers and overexpression of Ecd in mammary cells enhances cellular entry to S-phase of synchronized cells upon growth factor stimulation. Importantly, overexpression of Ecd promotes oncogenic characteristics in human mammary epithelial cells (Bele et al., unpublished data). Taken together, these studies underscore importance of identification and characterization of E6 cellular targets in breast cells.

Conclusions

Our ability to culture and immortalize mammary stem/progenitor cells *in vitro* has provided the first reliable model of immortal mammary stem/progenitor cells and a wealth of knowledge about the behavior of mammary cells and the genes involved in normal cell growth and oncogenesis. Characterization of these cells has provided potential novel markers for early diagnosis and prognosis of breast cancers and given knowledge about potential precursor cells for breast cancers. *In vitro* transformation analyses have also proven important to understand the multistep nature of breast cancer. Lastly, using gene profiling we have begun to appreciate that breast cancers do not originate only from luminal cells but also from basal (and probably from myoepithelial progenitor) cells and that there are subtypes of breast cancers possibly originating from distinct normal precursors that have distinct clinical outcomes and may require different treatment strategies.

However, many critical questions remain unanswered-How do different subtypes of breast cancer originate? Are breast cancer stem cells originate from normal mammary stem cells? How to define cancer stem cell specific therapeutic strategy? In conclusion, experimental immortalization of breast progenitor cells has led to substantial progress in our understanding of the biology of breast cancer and future studies in these model systems should go a long way towards helping elucidate the nature of breast cancer heterogeneity and thus facilitate the development of more individualized stem cell based therapy for breast cancer patients.

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Fig. 1. Immunofluorescence analyses of hTERT immortalized stem/progenitor cells from human mammary tissue. Please note two types of stem/progenitor cells $K5^{+}/K19^{-}$ and $K5^{+}/K19^{+}$ express uniformly basal (K5), stem cell (CD49f), luminal (K19), epithelial (E-cadherin) and mesenchymal (vimentin) markers in same cells. CD49f, K5, K19, Vimentin, E-cadherin (shown in red) or DAPI (shown in blue)

Fig. 2.

Immunofluorescence staining of human mammary tissue specimen shows presence of stem/progenitor cells. Staining was done using antibodies against K5 (green), K19 (red) double staining and co-staining with myoepithelial marker α -SMA (red) or CD10 (red).

Fig. 3. In vitro self-renewal and differentiation of K5⁺/K19⁻ cell type in MEGM medium. (A). Phase contrast microscopy showing morphology of cells before and after differentiation. (B). Immunofluorescence staining of cells after differentiation with antibodies against K5 (green), luminal cell marker MUC1 (red), and myoepithelial cell marker CD10 (red).

Fig. 4.

Human mammary stem/progenitor cells are highly susceptible to pre-neoplastic transformation. Several viral oncogenes, such as HPV E6 and SV40, irradiation and cellular mutant/overexpressed genes (ZNF217, mutant p53, RhoA and Bmi-1) induce immortalization/preneoplastic transformation. Genomic instability induced by additional oncogenes or by exposure to DNA damage further leads to full transformation of hMECs.