4

Does Inter-Individual Heterogeneity in the Normal Breast Corrupt Cancer Stem Cell and/or Cancer-Specific Signaling Characterization?

Harikrishna Nakshatri

Departments of Surgery, Biochemistry and Molecular Biology,
Indiana University Simon Cancer Center,
Indiana University School of Medicine,
Indianapolis, Indiana, USA

Abstract

Several studies have described cell surface markers that phenotypically define stem-progenitor-mature cell hierarchy in the normal breast. The same markers have been used to identify subpopulation of cancer cells with enhanced tumor initiating capacity. These subpopulations of cells, also called cancer stem cells (CSCs), have been the focus of intense research for the last few years. Identifying and characterizing cancer-specific differences in CSCs from their normal counter part is not trivial due to non-availability of replenishable source of primary normal and CSCs to perform functional assays. Moreover, recent discovery of widespread genetic variation in humans leading to functional transcriptome diversity makes the task of defining "global normal" very difficult. Thus, "normal" breast epithelial hierarchy and corresponding gene expression profiles have to be defined at individual patient level for comparison with cancer. Recent advances in human mammary epithelial cell reprogramming growth conditions and single cell genome analyses should overcome these limitations and enable characterization of "normal" and "tumor" at individual levels. By propagating cells from core breast biopsies of healthy donors, tumors and adjacent normal followed by flow cytometry analysis, we have recently observed remarkable inter-individual phenotypic heterogeneity

in normal breast stem, luminal progenitor, and mature cell numbers and possibly epithelial cell plasticity. Comparison of adjacent normal and tumor from the same patient showed distinct differences in differentiation status between normal and tumor. This observation has important implications as cancer-specific defect in differentiation alone could account for the majority of gene expression differences observed between cancer and normal cells. In addition, most of the differentially expressed genes including genes with highest expression difference, which are often considered for functional studies or as biomarkers of cancer cell behavior, are not causally linked to cancer. Collectively, inter-individual heterogeneity in the normal breast, the differences in the differentiation status between normal and tumor of the same patient, and differences in epithelial cellularity between normal and tumors used for gene expression studies may be the reasons for discrepancy in the literature with respect to gene expression based prognostic signatures, cancer-specific signaling pathway alterations, and CSC characterization. As a way forward, we propose that the magnitude of tumor heterogeneity and CSC phenotype is the product of individual's epithelial cell plasticity and cancer-specific mutation. In addition, we need to characterize normal and tumor on an individual basis for clear understanding of pathobiology of tumors.

Keywords: Normal breast, Epithelial hierarchy, Heterogeneity, Breast cancer, Cancer stem cells.

4.1 Introduction

Recent advances in genomics have shown profound inter-individual functional diversity in transcriptome due to genetic regulatory variations [1]. In a study involving non-transformed fibroblasts from 62 unrelated individuals, Wagner et al. found significant inter-individual differences in the expression levels of 9,493 out of 16,952 genes with strongest differences in the expression levels of a subset of developmentally regulated Hox gene cluster [2]. This breakthrough should force us to redefine "normal" and reassess cancer-specific variation from normal variation in gene expression. The integrative cluster classification of breast cancer study addressed this issue partially, where the impact of inherited copy number variations and single nucleotide variations were taken into consideration to derive cancer-specific transcriptome [3]. However, the problem still persists when one defines "normal" without any consideration to ethnic differences in the normal tissues used as controls. In normal breast,

menstrual cycle at the time of tissue collection adds another variability to transcriptome [4]. Along this line, lack of reproducibility of gene expression signatures or biomarkers with prognostic significance may in part be due to genetic regulatory variation in "normal". In addition, methods to develop targeted therapies need to be reevaluated because some of signaling network considered to be active based on comparison of cancer transcriptome with "global normal" breast transcriptome may be misleading. Indeed, analysis using newer computational tools suggest that driver mutations required for oncogenesis are relatively small suggesting that few of the previously described cancer-specific aberrations in gene expression are not causally linked to cancer [5]. Instead, most of the documented differences in gene expression between normal and cancer are likely due to inter-individual heterogeneity in normal breast transcriptome.

4.2 Defining Normal Breast Hierarchy

Although it is ideal to document inter-individual heterogeneity of the normal breast at genomic levels, it is not is easily achievable. A simplest assay would be to characterize normal cells for cell surface markers that have previously been used for stem, progenitor and mature cell identification. Several recent reviews by others and us provide a list of such markers [6–8]. For example, CD49f+/EpCAM−, CD49f+/EpCAM+, and CD49f−/EpCAM+ cells correspond to stem, luminal progenitor, and mature/differentiated cells of breast, respectively [8]. EpCAM+/CD49f+CD10+ are bipotent cells, EpCAM+/CD49f+/MUC1+ cells are luminal-restricted colony forming cells, EpCAM+/CD49f−/MUC1+ cells are mature luminal cells and EpCAM+/CD49f−/MUC1−/CD10+ cells are differentiated myoepithelial cells of the human breast [9]. Basal cells of the breast express CD271 [10]. Basal cells of the normal breast also demonstrate CD44+/CD24− phenotype [11]. CD73+/CD90− cells correspond to rare cells in the breast that exhibit extensive lineage plasticity [12]. Limited gene expression studies using purified subpopulation of cells from normal breast have demonstrated significant gene expression differences between these populations. For example, ∼2000 genes are differentially expressed between bipotent luminal and mature luminal cells [9]. Similar differences in gene expression between CD44+/CD24− and CD44−/CD24+ cells have been observed [11]. Using non-transformed MCF-10A breast epithelial cell line, we had demonstrated differential expression of >2000 genes between CD44+/CD24− and CD44−/CD24+ epithelial cells [13]. These subpopulation-specific

differences also extend to microRNAs; basal, luminal progenitor and mature cells of normal breast express different set of microRNAs [14].

Although the above-mentioned studies documented the presence of different populations of cells in the normal breast, this knowledge has not been utilized extensively to characterize tumor-specific gene expression. The following limitations may have had a negative impact on cancer-specific transcriptome analyses. 1) Most of the "normal" tissues used for comparative gene expression studies were derived from either reduction mammoplasty or contiguous with the tumor; 2) There are no replenishable primary cells to determine the function of genes uniquely expressed in a subpopulation of cells; 3) Since gene expression analyses were done using flow sorted cells directly [9, 11], in which gene expression could still be under the influence of microenvironment, it is difficult to determine intrinsic gene expression pattern in subpopulation of cells; 4) Because of the nature of normal tissues utilized, inter-individual heterogeneity in number of phenotypically defined subpopulations in the normal breast due to age, body mass index, ethnicity, parity, breast feeding, use of birth control pills, menstrual cycle at the time of tissue collection, menopausal status, or age at menarche corrupts the definition of normal gene expression pattern in breast. One would expect expression changes in $>$2000 genes simply due to differences in progenitor to mature cell ratio between healthy individuals. Attempting to address these issues is not easy but achievable as explained below.

4.3 Need for *In Vitro* Assays to Document Inter-Individual Heterogeneity in the Normal Breast

Over the years, a series of breast epithelial cell lines have been generated to study the role of specific oncogenes, transcription factors, epithelial to mesenchymal transition (EMT), growth factors and cytokines in breast cancer progression. However, most of these cell lines display basal cell features [15]. Using different media composition, Weinberg's group was able to generate two distinct subtypes of normal cells (with basal and luminal features) but neither contained estrogen receptor alpha (ERα)-positive cells [16]. Thus the currently available model systems cannot document or study inter-individual heterogeneity. Lack of the model systems has also prevented any mechanistic studies on tumor initiating events responsible for specific subtypes of breast cancer. For example, based on microarray analysis, breast cancer is classified into five intrinsic subtypes; luminal A, luminal B, normal-like/claudin-low, Her2+ and basal type [17]. Luminal A and luminal B express ERα.

ERα-positive breast cancer represents ∼70% of breast cancer cases and is a major clinical problem. Although ERα-positive breast cancers are generally thought to be less aggressive, luminal B ERα-positive breast cancers show poor outcome, almost similar to basal-type breast cancers [3, 17]. Similarly, integrative cluster analysis has also identified three ERα-positive integrative clusters with differing outcomes [3]. Mechanisms responsible for differential outcome in these ERα-positive subtypes can be revealed only when we know the ERα-signaling network in non-transformed breast epithelial cells.

In the normal mammary gland of human, rats, mice and cows, ERα-positive cells are heterogeneously located in the luminal compartment of the duct and rarely co-localize with proliferating cells [18]. ERα-positive tumor cells, in contrast, proliferate in response to estradiol (E2) treatment. In mouse models, autocrine activity of transforming growth factor beta (TGFβ) prevents ERα-positive cells from responding to E2 and impairment of TGFβ signaling is essential for ERα-positive cells to acquire E2-dependent proliferation [19]. However, similar studies in human breast epithelial cells have not been conducted due to the failure of currently available culture systems to support growth of non-transformed ERα-positive cells. Since the number of normal ERα-positive cells vary between individuals (5−20%), understanding inter-individual heterogeneity in E2-dependent autocrine and paracrine gene expression changes in the normal breast is essential for defining transcriptome in the normal breast.

Researchers have attempted to address the above issue by reintroducing ERα to ERα-negative cells. Paradoxically, introduced ERα inhibited growth instead of supporting E2-dependent proliferation [20]. Furthermore, there is evidence in the literature that ERα target genes are methylated in the absence of ERα [21]. Only way to activate endogenous ERα in these ERα-negative cells is to treat cells with DNA methyltransferase and histone deacetylase inhibitors, which have additional effects on the genome [22]. Therefore, resources need to be applied to develop a system that allows culturing of non-transformed ERα-positive cells.

Recently developed epithelial cell reprogramming assay will likely change the landscape of breast cancer research and will enable us to address several of these unmet needs stated above [23]. Indiana University houses Susan G Komen for the Cure normal breast tissue bank to which healthy donors donate breast core biopsies. This resource should eliminate the major concern regarding "normal" breast tissues that are being used as healthy controls in various gene expression studies including The Cancer Genome Atlas (TCGA) [24]. We have begun to address these issues by culturing core

biopsies of healthy donors, high risk patients, adjacent normal and tumor cells from the same patient for s short duration and subjected these cells to phenotypic analysis using various cell surface markers and flow cytometry. We observed remarkable phenotypic heterogeneity in the normal breast among healthy donors irrespective of their age, menstrual cycle, body mass index, and parity [25]. The number of CD49f+/EpCAM−, CD49f+/EpCAM+, and CD49f−/EpCAM+ cells varied from individual to individual.

Tumor and adjacent normal cells from the same patient were phenotypically different indicating differences in differentiation status, which we expect to have an impact on gene expression pattern [25]. Furthermore, we did not find significant differences in the levels of CD44+/CD24− cells between adjacent normal and tumor cells of the same patient. Therefore, without characterizing normal breast of the same patient, it is difficult to conclude enrichment of CD44+/CD24− CSCs in a tumor of a patient. In contrast, we found enrichment of ALDEFLUOR-positive CSCs in tumors compared with adjacent normal depending on the cancer type. Since luminal progenitor or committed luminal cells are ALDEFLUOR-positive [26], the above observation indicates that cancers that originate from luminal cells and/or maintain luminal features are ALDEFLUOR-positive.

The ability to culture primary cells cultured under reprogramming condition may allow us to address another longstanding question related to ERα function in non-transformed cells. We expect a subpopulation of these cells to express ERα and consequently respond to E2, which will likely be different from ERα-positive breast cancer cells.

Epidemiologic studies have shown pregnancy protects against breast cancer [27]. A recent study has shown that CD44+p27+ positive cells with progenitor properties confer susceptibility to breast cancer and the number of CD44+p27+ cells in the breast decline with pregnancy [28]. As noted above, there can also be inter-individual heterogeneity in the number of p27+ stem cells and rate at which they decline after each pregnancy may help to assess breast cancer risk. Primary breast epithelial cells grown from nulliparous and parous women may allow risk assessment as well as to conduct mechanistic studies related to susceptibility of CD44+/p27+ cells to tumorigenesis.

4.4 Future Directions

Recent advances in next-generation sequencing, primary cell culturing, availability of truly normal breast tissues, and 3D culture techniques should enable major strides against breast cancer. There have been lots of efforts to understand tumor heterogeneity. However, heterogeneity in normal breast

has not been the forefront of research efforts. Tumor heterogeneity in some cases could be a reflection of inherent properties of breast epithelial cells of the individual rater than due to cancer-specific genomic aberration. Distinguishing inherent heterogeneity from mutation-induced heterogeneity will have an impact on how tumors are characterized, cancer-specific signaling networks are identified, and treatment decisions are made.

List of Abbreviations:
CSC, Cancer Stem Cells
E2, estradiol
ERα, Estrogen Receptor alpha
TCGA, The Cancer Genome Atlas
TGFβ, Transforming Growth Factor Beta

Acknowledgements

The Susan G. Komen for the Cure supports research in Nakshatri's laboratory.

References

[1] Lappalainen T, Sammeth M, Friedlander MR, *et al.* Transcriptome and genome sequencing uncovers functional variation in humans. Nature 2013;501(7468):506–11.
[2] Wagner JR, Busche S, Ge B, *et al.* The relationship between DNA methylation, genetic and expression inter-individual variation in untransformed human fibroblasts. Genome Biol 2014;15(2):R37.
[3] Curtis C, Shah SP, Chin SF, *et al.* The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012;486(7403):346–52.
[4] Pardo I, Lillemoe HA, Blosser RJ, *et al.* Next-generation transcriptome sequencing of the premenopausal breast epithelium using specimens from a normal human breast tissue bank. Breast Cancer Res 2014;16(2):R26.
[5] Kandoth C, McLellan MD, Vandin F, *et al.* Mutational landscape and significance across 12 major cancer types. Nature 2013;502(7471): 333–9.
[6] Nakshatri H, Srour EF, Badve S. Breast cancer stem cells and intrinsic subtypes: controversies rage on. Curr Stem Cell Res Ther 2009;4(1): 50–60.

[7] Badve S, Nakshatri H. Breast-cancer stem cells-beyond semantics. Lancet Oncol 2012;13(1):e43–8.
[8] Visvader JE, Stingl J. Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes Dev 2014;28(11): 1143–1158.
[9] Raouf A, Zhao Y, To K, *et al.* Transcriptome analysis of the normal human mammary cell commitment and differentiation process. Cell Stem Cell 2008;3(1):109–18.
[10] Kim J, Villadsen R, Sorlie T, *et al.* Tumor initiating but differentiated luminal-like breast cancer cells are highly invasive in the absence of basal-like activity. Proc Natl Acad Sci USA 2012;109(16):6124–9.
[11] Shipitsin M, Campbell LL, Argani P, *et al.* Molecular definition of breast tumor heterogeneity. Cancer Cell 2007;11(3):259–73.
[12] Roy S, Gascard P, Dumont N, *et al.* Rare somatic cells from human breast tissue exhibit extensive lineage plasticity. Proc Natl Acad Sci USA 2013;110(12):4598–603.
[13] Bhat-Nakshatri P, Appaiah H, Ballas C, *et al.* SLUG/SNAI2 and tumor necrosis factor generate breast cells with CD44+/CD24− phenotype. BMC Cancer 2010;10:411.
[14] Pal B, Chen Y, Bert A, *et al.* Integration of microRNA signatures of distinct mammary epithelial cell types with their gene expression and epigenetic portraits. Breast Cancer Res 2015;17:85.
[15] Stampfer MR, Yaswen P. Culture models of human mammary epithelial cell transformation. J Mammary Gland Biol Neoplasia 2000;5(4): 365–78.
[16] Ince TA, Richardson AL, Bell GW, *et al.* Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. Cancer Cell 2007;12(2):160–70.
[17] Sorlie T, Perou CM, Tibshirani R, *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001;98(19):10869–74.
[18] Grimm SL, Rosen JM. Stop! In the name of transforming growth factor-beta: keeping estrogen receptor-alpha-positive mammary epithelial cells from proliferating. Breast Cancer Res 2006;8(4):106.
[19] Ewan KB, Oketch-Rabah HA, Ravani SA, *et al.* Proliferation of estrogen receptor-alpha-positive mammary epithelial cells is restrained by transforming growth factor-beta1 in adult mice. Am J Pathol 2005;167(2):409–17.

[20] Jeng MH, Jiang SY, Jordan VC. Paradoxical regulation of estrogen-dependent growth factor gene expression in estrogen receptor (ER)-negative human breast cancer cells stably expressing ER. Cancer Lett 1994;82(2):123–8.

[21] Leu YW, Yan PS, Fan M, *et al.* Loss of estrogen receptor signaling triggers epigenetic silencing of downstream targets in breast cancer. Cancer Res 2004;64(22):8184–92

[22] Sharma D, Blum J, Yang X, *et al.* Release of methyl CpG binding proteins and histone deacetylase 1 from the Estrogen receptor alpha (ER) promoter upon reactivation in ER-negative human breast cancer cells. Mol. Endocrinol 2005;19(7):1740–51.

[23] Liu X, Ory V, Chapman S, *et al.* ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. Am J Pathol 2012;180(2):599–607.

[24] Koboldt DC, Fulton RS, McLellan MD, *et al.* Comprehensive molecular portraits of human breast tumours. Nature 2012;490:61–70.

[25] Nakshatri H, Anjanappa M, Bhat-Nakshatri P. Ethnicity-Dependent and –Independent Heterogeneity in Healthy Normal Breast Hierarchy Impacts Tumor Characterization. Sci Rep 2015;5:13526.

[26] Lim E, Wu D, Pal B, *et al.* Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. Breast Cancer Res 2010;12(2):R21.

[27] Colditz GA, Rosner BA, Chen WY, *et al.* Risk factors for breast cancer according to estrogen and progesterone receptor status. J Natl Cancer Inst 2004;96(3):218–28.

[28] Choudhury S, Almendro V, Merino VF, *et al.* Molecular profiling of human mammary gland links breast cancer risk to a p27(+) cell population with progenitor characteristics. Cell Stem Cell 2013;13(1):117–30.