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In Situ Malignant Transformation and Progenitor-Mediated Cell Budding: Two Different Pathways for Breast Ductal and Lobular Tumor Invasion

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Abstract

The human breast lobular and ductal structures and the derived tumors from these structures differ substantial in their morphology, microenvironment, biological presentation, functions, and clinical prognosis. Based on these differences, we have proposed that pre-invasive lobular tumors may progress to invasive lesions through "*in situ malignant transformation*", in which the entire myoepithelial cell layer within a given lobule or lobular clusters undergoes extensive degeneration and disruptions, which allows the entire epithelial cell population associated with these myoepithelial cell layers directly invade the stroma or vascular structures. In contrast, pre-invasive ductal tumors may invade the stroma or vascular structures through "*progenitor-mediated cell budding*", in which focal myoepithelial cell degeneration-induced aberrant leukocyte infiltration causes focal disruptions in the tumor capsules, which selectively favor monoclonal proliferation of the overlying tumor stem cells or a biologically more aggressive cell clone. Our current study attempted to provide more direct morphological and immunohistochemical data that are consistent with our hypotheses.

Key words: Breast cancer; Tumor invasion; Tumor metastasis; Malignant transformation; Tumor cell budding; Myoepithelial cells; Tumor microenvironment; Tumor stem cells; lymphocytes

Introduction

The epithelial component of the human breast consists of lobular (or acinar) cells, which are arranged as grape-like structures responsible primarily for the production of milk, and the ductal system, which are arranged as branching, three-like structures responsible mainly for providing the drainage of the secretions [1-3]. Developmentally, the lobular cells are derived from "budding" cells of the terminal ducts during the early puberty [4-11]. The lobular cells undergo extensive proliferation, differentiation, molecular and biochemical changes during the entire lifespan of the female, more notably during pregnancy and lactation [4-11]. Following menopause, the lobular structures in both nulliparous and parous women start to regress and a majority of these structures are eventually replaced by fibrous tissues [4-11]. The ductal system starts at the terminal ducts, merges into larger ducts, and extends to the nipple orifices. Compared to the lobular (acinar) cells, ductal cells are relatively more stable during the entire lifetime of the

subject [4-11].

Structurally, the lobular and ductal systems differ substantially in the following aspects:

1. The physical distribution and relationship to the myoepithelial cells.

Both the lobular and ductal cells are physically separated from the stroma by a layer of basement membrane and a layer of myoepithelial cells. The basement membrane is structurally similar in both lobular and ductal systems. The myoepithelial cells in the ductal system generally form a continuous sheet that completely encircles all the ducal cells. In contrast, the myoepithelail cell layer in the lobular system is often discontinuous, defined as the lack of direct physical contact or the presence of small gaps (generally smaller than the size of two myoepithelial cells) among the neighboring myoepithelial cells [12,13] (Fig 1a-1d).

2. The expression pattern and frequency of tumor suppressors in the myoepithelial cell layer

The myoepithelial cell layer produces a number of tumor suppressors, including maspin, p63, and Wilms' tumor 1 (WT-1) that exert significant paracrine inhibition on proliferation and invasion of associated tumor cells [13-16]. In the normal ductal system, tumor suppressors are consistently expressed in all or nearly all morphologically distinct myoepithelial cells. In contrast, many morphologically distinct myoepithelial cells in the lobular system are often devoid of expression of these established tumor suppressors [17-20]. In some cases, many lobular clusters or an entire lobule show no or significantly reduced expression of p63 and WT-1 as compared to that of duct-associated [17-20] (Fig 1e-1f).

3. The expression pattern and frequency of cell surface adhesion molecules and c-erbB2

A number of cell surface adhesion molecules, including E-cadherin and ß-catenin, are strongly expressed in a vast majority of the ductal epithelial cells and their malignant derivatives, but are absent in the acinar counterpart, which, however, often harbors isolated lobules with aberrant c-erbB2 expression [21, 22] (Fig 1g-1j).

4. The size and length of the lumen.

The lumen of the lobular units is very small with a single open end that leads to the terminal duct. Due to this structural feature, any substantially elevated lobular cell proliferation may over-stretch the associated basement membrane and myoepithelial cell layer, or even physically disrupt these two structures. In contrast, the lumen of the ducts is substantially larger and intercalated among ducts, which permits a longitudinal expansion of an increased volume of ductal cells and also preserves the physical integrity of the surrounding myoepithelial cell layer and the basement membrane.

Together, these structural features are apparently more favorable for proliferation, invasion, and metastasis of lobular tumor cells. Consistent with this speculation is the fact that invasive lobular cancers (ILC) tend to be significantly larger in size with a significantly higher rate of positive lymph nodes than stage-matched invasive ductal carcinoma (IDC) [23-25]. Although large tumor size and positive lymph node are two well-recognized risk factors for worse prognosis, patients with ILC have a substantially more favorable clinical outcomes compared to patients with IDC [23-27]. These contradictory impacts have been largely attributed to the unique features of ILC, including the lack of E-cadherin expression, higher expression of ER and PR, lower expression of HER-2, p53, EGFR, and lower S-phase fraction [28-31]. The trigger factor for the significant differences in clinical outcomes between stage-matched ILC and IDC, however, has not been identified.

We have hypothesized that the trigger factor for the significant differences in clinical outcomes between lobular and ductal tumors may result from their substantially different growth patterns during invasion. As the lobular cell population undergoes extensive proliferation and differentiation during puberty, pregnancy and lactation [6-11], most adult females may have largely exhausted or "used up" the residual stem cells, which have been suggested as the primary source of invasive and metastatic lesions [32-35]. In addition, the extensive proliferation of the epithelial cell population during these stages may have also caused the exhaustion of the residual stem cells in the myoepithelial cell population, which impairs the normal replenishment process, resulting in an aged myoepithelial cell population. Thus, pre-invasive lobular tumors may progress to invasive lesions through "*in situ malignant transformation*", in which the entire myoepithelial cell layers within a given lobule or lobular clusters become degenerated and disrupted, which allows the entire tumor cell population to directly invade the stroma. In contrast, pre-invasive ductal tumors may invade the stroma or vascular structures through "*progenitor-mediated cell budding*", in which focal myoepithelial cell degeneration-induced aberrant leukocyte infiltration causes focal disruptions of the tumor capsules, which selectively favor monoclonal proliferation of the overlying tumor stem cells or a biologically more aggressive cell

clone. Our current study attempted to provide more morphological and immunohistochemical data supportive of our hypothesis.

Materials and Methods

Ten cases harboring large normal mammary ductal or acinar clusters or lobules with malignant features were selected from our previous studies [12,13,16-20]. All these samples were retrieved from the files of the Armed Forces Institute of Pathology with IRB approved protocols. Consecutive sections at 7-um thickness were cut and placed sequentially on positively charged slides. For each case, 300-500 sections were made. For each set of 10 consecutive sections, the first 3-4 sections were used for hematoxylin and eosin (H & E) staining and immunohistochemistry (IHC). The remaining sections were used for various molecular assays.

To identify cells with malignancy-associated alterations, sections were double immunostained for p53 (clone: D07, Dako, Carpinteria, CA) and smooth muscle actin (SMA; clone: 1A4; Sigma, St. Louis, MO). To differentiate between ductal and acinar cells, and to identify the potential impact of leukocytes on the physical integrity of myoepithelial cell layers, sections were double immunostained for E-cadherin (clone: 36B5; Lab Vision, Fremont, CA), and leukocyte common antigen (LCA, clone: 2B11+PD7/26), which is present in all normal hematopoietic cells and their neoplastic transformations. To identify disseminated or isolated epithelial cells within leukocyte aggregates, sections were double immunostained for LCA and cytokeratin (CK) AE1/3 (clone; AE1/AE3, Dako, Carpinteria, CA), which are expressed in all epithelium-derived cells.

As our previous studies have suggested that aberrant leukocyte infiltration could trigger cell dissemination and malignant transformation in normal appearing lobules [36-40], the possible sign of *"in situ malignant transformation"* was assessed by examining the morphological and immunohistochemical alterations of such lobules with infiltrated leukocyte aggregates in multiple consecutive sections, to determine: (1) whether cells with malignancy-associated changes can originate from normal lobules, (2) whether cells with malignancy-associated changes in the normal appearing lobules are eventually in physical continuity with clear-cut invasive lesions, (3) whether cells with malignancy-associated changes in normal appearing lobules share the same or similar morphological and immunohistochemical profile with their clear-cut malignant counterparts, and (4) whether leukocyte aggregates are exclusively or preferentially located at or near the intersection between these lobules and clear-cut invasive lesions.

To identify signs of "*progenitor-mediated cell budding*", the morphological and immunohistochemical alterations of hyperplastic or *in situ* tumors were examined in cross and longitudinal profile of consecutive sections, to determine: (1) whether morphologically and immunohistochemically different cell types co-exist within the same duct, (2) whether cell "budding" is exclusively seen at focally disrupted myoepithelial cell layers, (3) whether all "budding" cells share the same morphological and immunohistochemical profile, (4) whether "budding" cells are eventually in physical continuity with clear-cut invasive lesions, (5) whether "budding" cells share the same morphological and immunohistochemical profile with their clear-cut malignant and invasive counterparts.

Immunostaining was carried out using our published protocol with monoclonal mouse anti-human antibodies. The secondary antibody, ABC detection kit, and diaminobenzidine (DAB) chromogen kit were obtained from Vector (Burlingame, CA). The AP red-chromogen kit was purchased from Zymad (South San Francisco, CA). To assess the specificity of the immunostaining, different negative controls were used, including (1) the substitution of the primary antibody with the same isotype or pre-immune serum of the antibody; and, (2) omission of the secondary antibody. Immunostaining procedures were repeated at least twice using the same protocol and under the same conditions. Immunostained sections were independently evaluated by two investigators. A given cell was considered immunoreactive if distinct immunoreactivity was consistently seen in its cytoplasm, membrane, or nucleus, while all negative controls lacked distinct immunostaining.

Results

The findings of our current study are in total agreement with our hypothesis. Examinations of the normal appearing lobules with infiltrated leukocyte aggregate consistently revealed that: (1) cells with malignancy associated changes could originate from normal lobules, (2) cells with malignancy associated changes in the normal lobules were eventually in physical continuity with clear-cut invasive lesions, (3) cells with malignancy-associated changes in normal lobules shared the same or similar morphological and immunohistochemical profile with their clear-cut malignant counterparts, and (4) leukocyte aggregates were almost exclusively located at or near the intersection between these lobules and clear-cut invasive lesions (Fig 2).

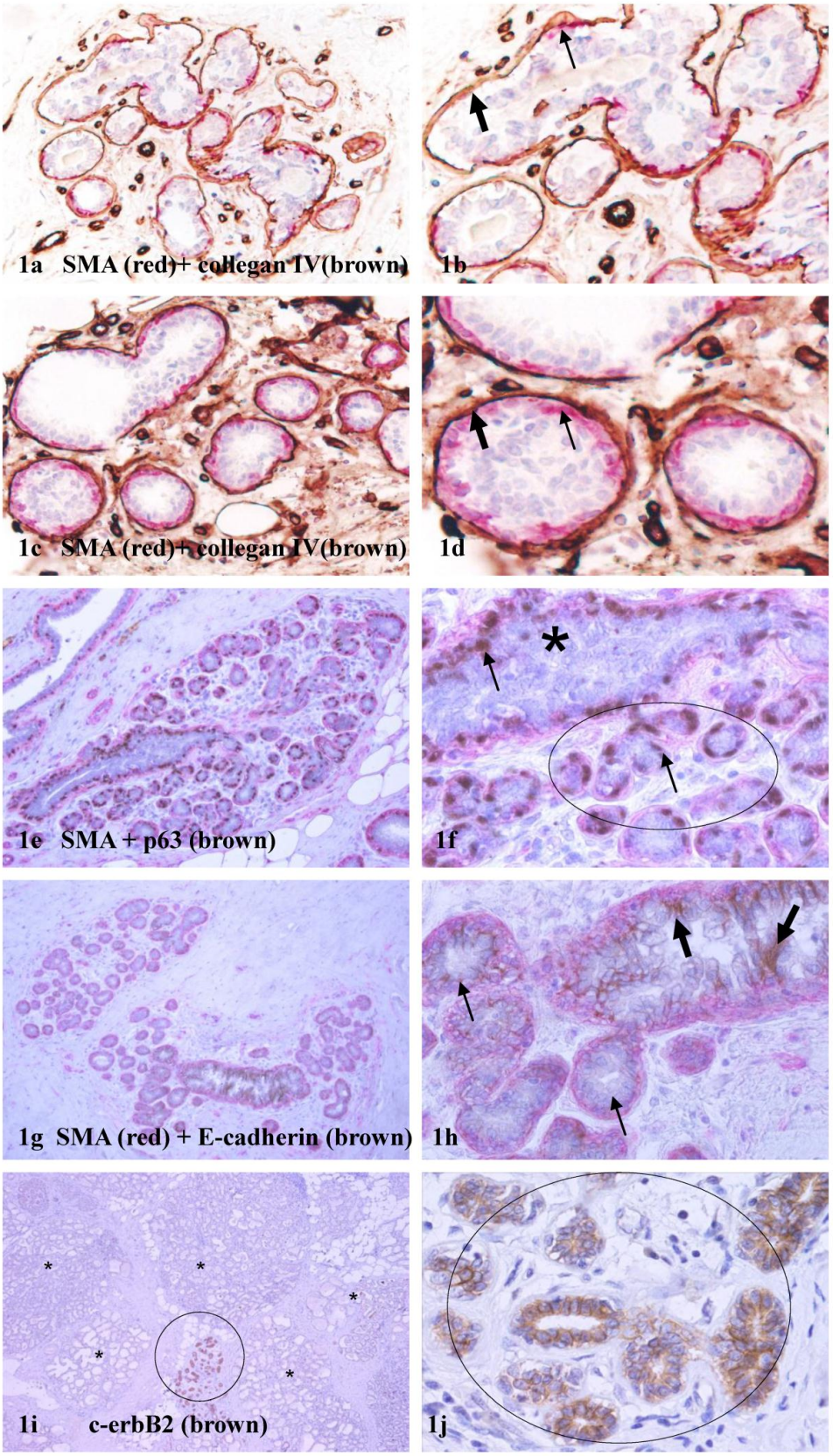


Fig 1.Structure differences between lobules and ducts

In lobular structures (a-b), the basement membrane (thick arrows) is intact, but only a few myoepithelial cells (thin arrows) are seen.

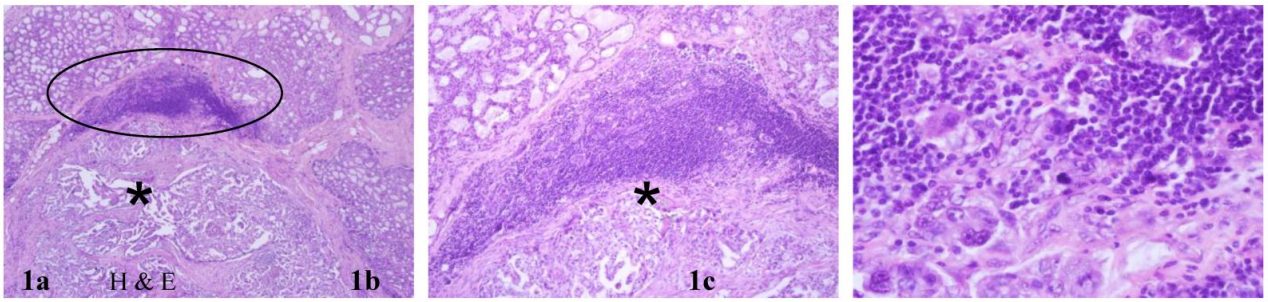
In duct structures (c-d), the basement membrane (thick arrows) is intact, and more myoepithelial cells (thin arrows) are seen.

Most myoepithelial cells (arrows) in the ducts (asterisk) are positive for p63, but only a few p63 positive cells are seen in the lobular structures (circle).

Most ductal cells (thick arrows) show E-cadherin expression, whereas all lobular cells (thin arrows) are devoid of E-cadherin expression.

Nearly all cells in a small normal appearing lobule (circles) show c-erbB2 expression, whereas all cells in the adjacent lobules (asterisks) lack c-erbB2 expression.

Figure 1. Structure differences between lobules and ducts.



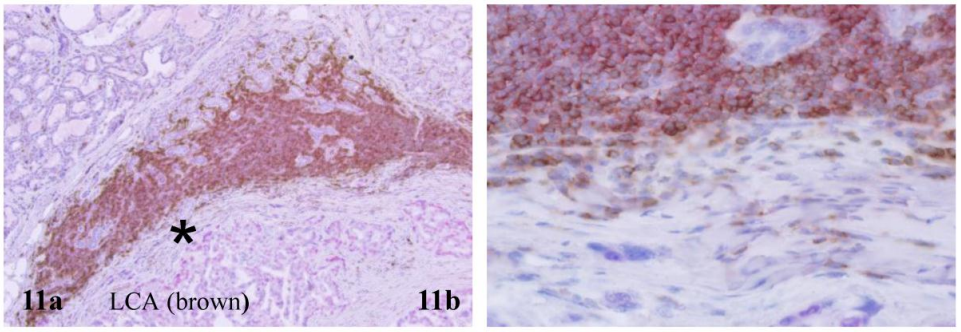
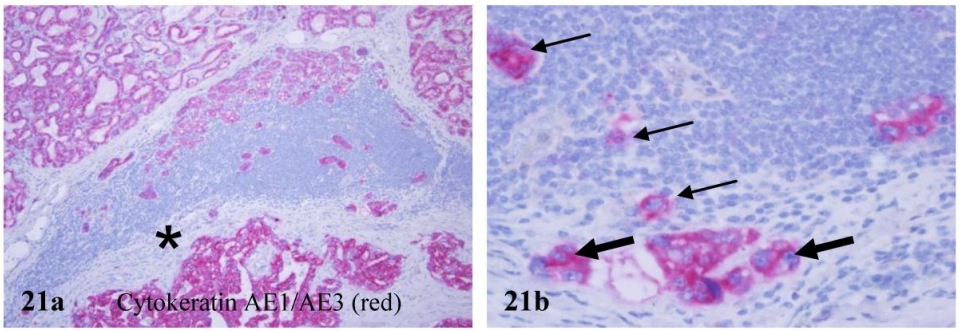
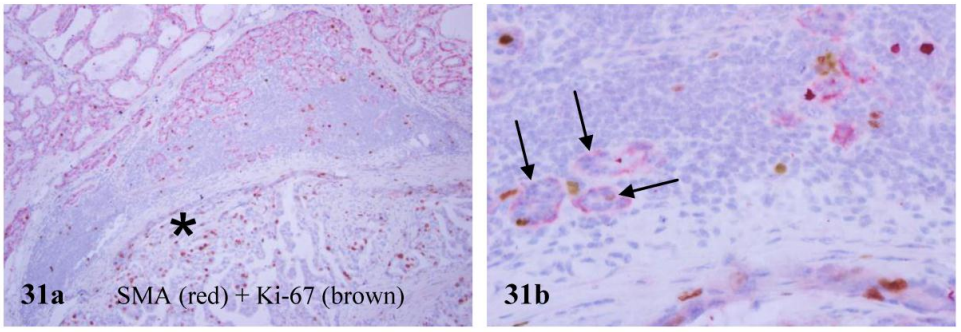


Fig 2. *In situ* malignant transformation of lobules

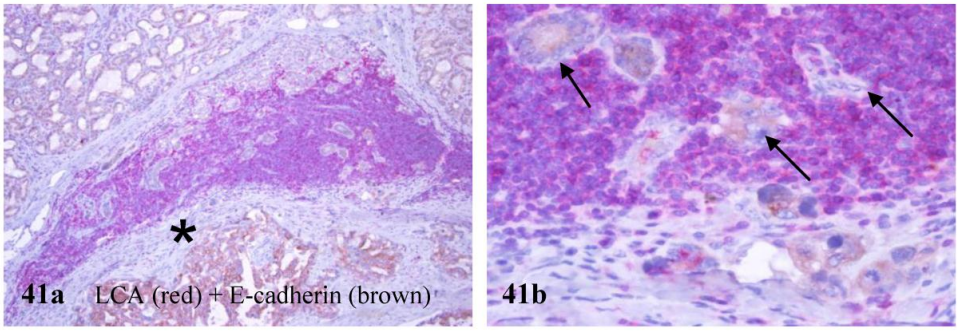
On H&E (1a-c) and immuno-stained sections (11a-b), a normal lobule (circle) adjacent to invasive cancer (asterisks) shows extensive leukocyte infiltration.



On sections immunostained for cytokeratin AE1/AE3, some isolated epithelial cells or cell clusters (thin arrows) within the leukocyte aggregate share the same morphological and immune-profiles with adjacent invasive cancer cells (thick arrows).



The myoepithelial cell layer (arrows) of these isolated epithelial structures is either totally absent or focally disrupted. These epithelial structures also show elevated cell proliferation.



All or nearly all cells within these isolated epithelial structures surrounded by leukocytes show no distinct E-cadherin expression (arrows)

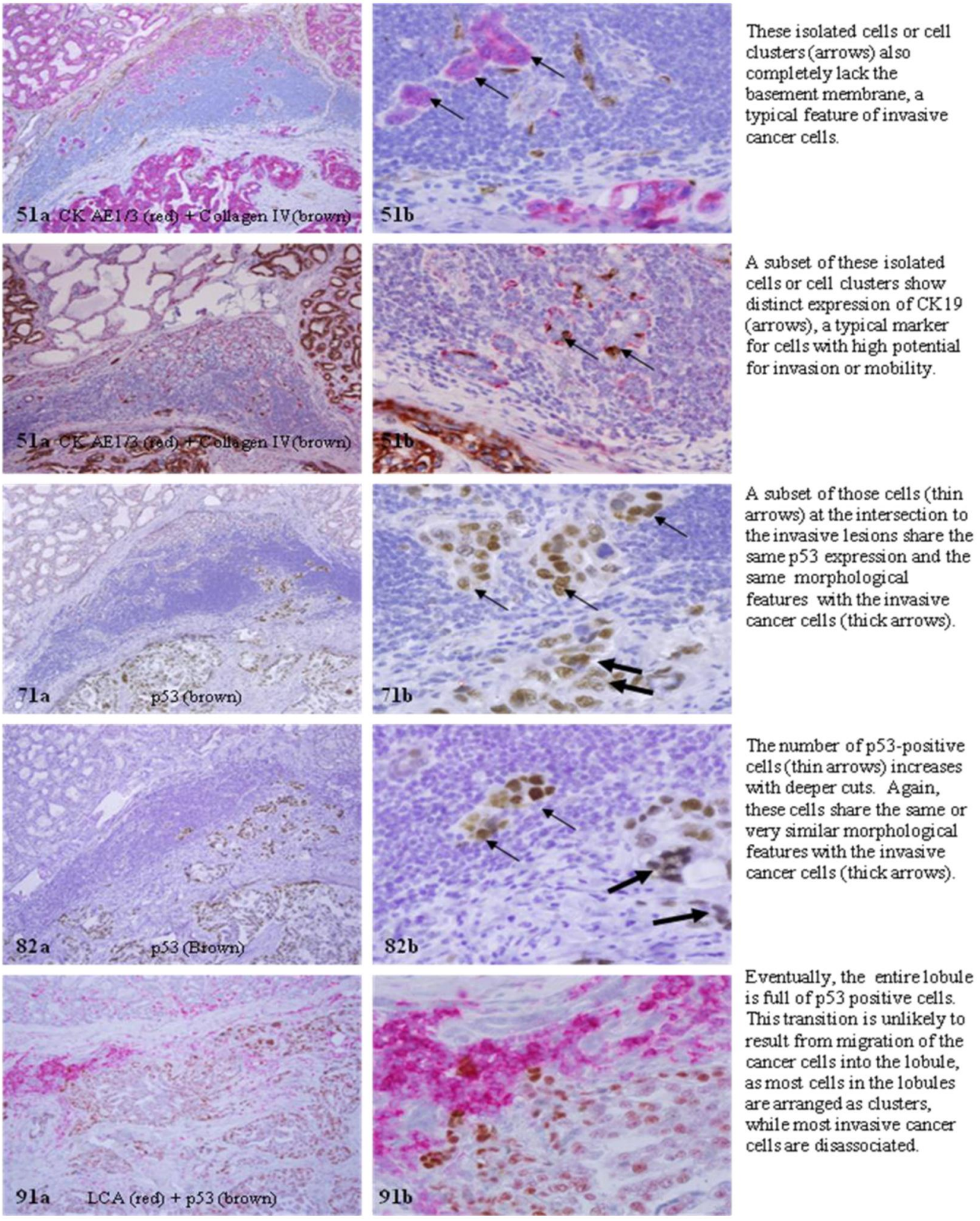


Figure 2. *In situ* malignant transformation of lobules.

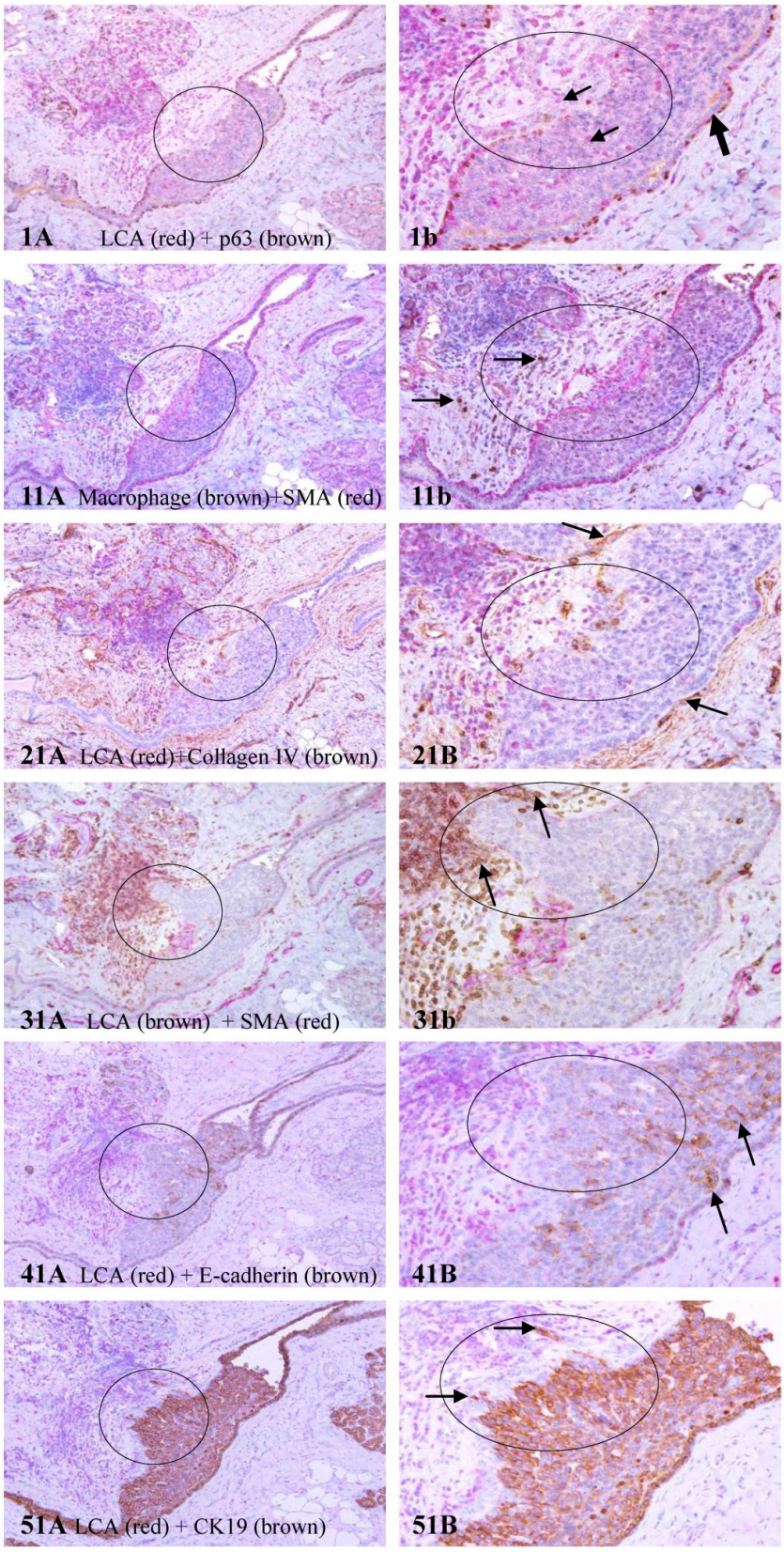


Fig 3. Progenitor-mediated cell budding in mammary ducts

The longitudinal section of a duct shows a focal disruption (circles) in the surrounding myoepithelial cell layer. Many leukocytes are seen at or near the disruption (thin arrows), but no leukocytes are seen at or near intact myoepithelial cell layer (thick arrow).

The focal disruption was present in adjacent sections, but macrophages (thin arrows) appear to be distant from the disruption, suggesting that myoepithelial cell degeneration may not be associated with non-specific immunoreactions.

The basement membrane (arrows) is also absent at the focal disruption, suggesting that absence of these two structures may be a correlated event.

A finger-like cell projection is seen to protrude into the stroma from the focal disruption, and the tip of the cell projection is surrounded by leukocytes (arrows)

Nearly all cells at the tip of the cell projection are devoid of E-cadherin expression, while most cells distant from the disruption show distinct expression of this cellular surface adhesion molecule (arrows).

A subset of those cells (arrows) at the tip of the cell projection are disassociated from the tumor core, and invade into the stroma.

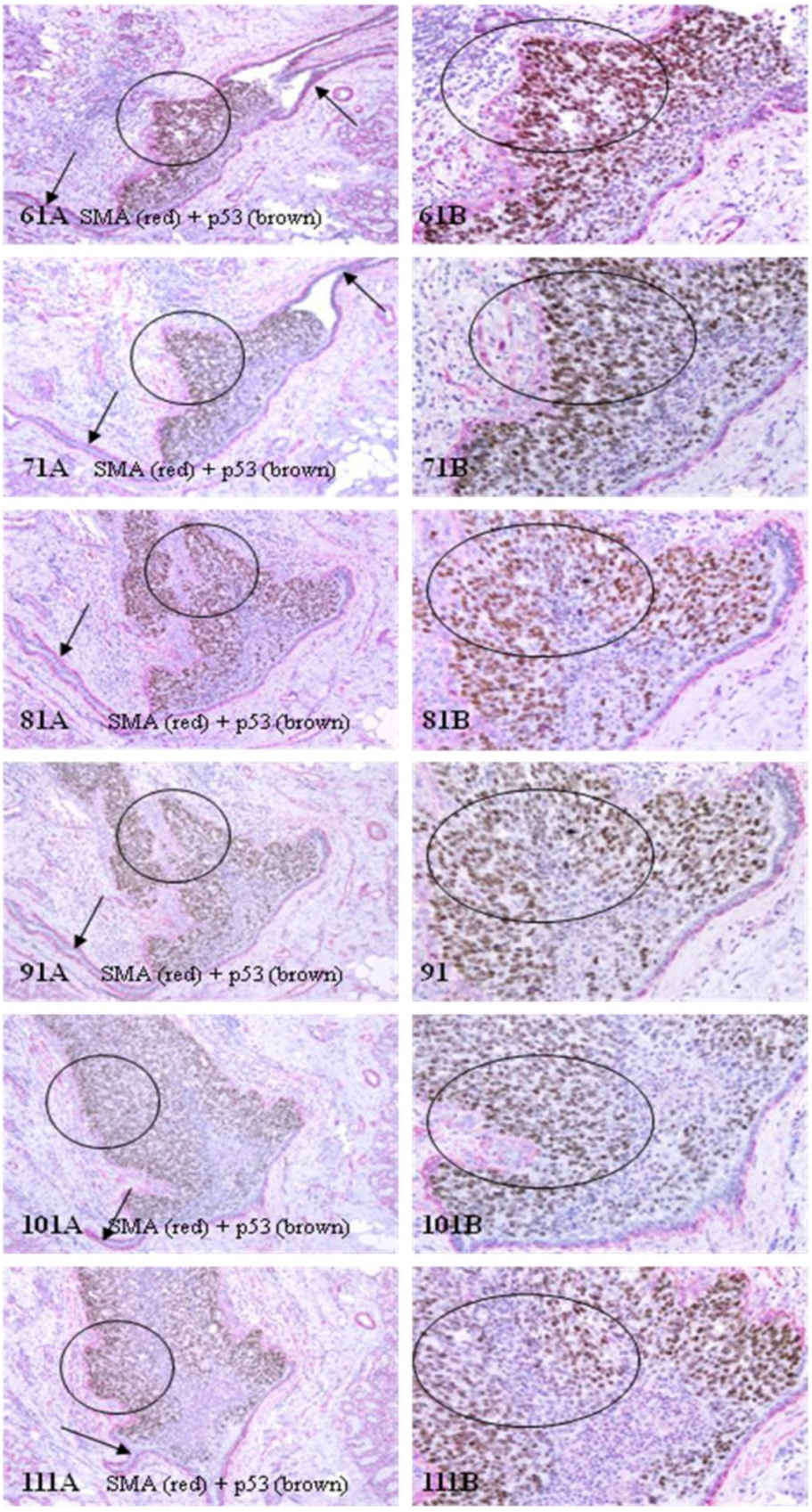


Figure 3. Progenitor-mediated cell budding in mammary ducts.

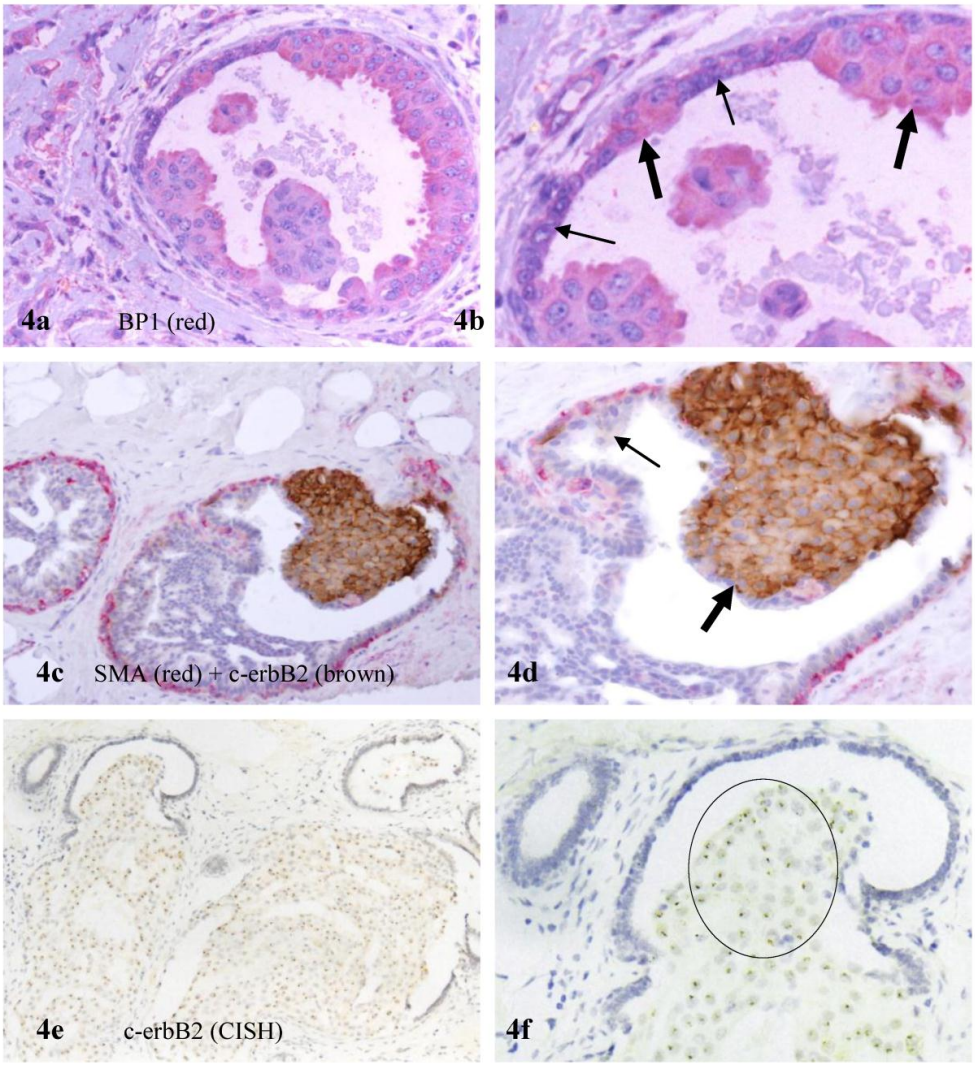


Fig 4. Multiple cell types within mammary ducts

At the cross section of a duct, multiple cell types with different morphological and biochemical profiles co-exist. As shown in a-b, some cells (thick arrows) are positive for BP1, a oncoprotein, but residual normal cells (thin arrows) are devoid of BP1.
A cluster of cells within a duct are strongly positive for oncoprotein c-erbB2 (thick arrows), but their adjacent counterparts are devoid of c-erbB2 (thin arrows)
The adjacent section of c-d are used for in situ hybridization with a probe for c-erbB2. High levels of c-erbB2 amplification is seen exclusively in the tumor cells (circles), but not in the normal residual cells within the same duct.

Figure 4. Multiple cell types within mammary ducts.

Examinations of the cross and longitudinal section profiles of hyperplastic and *in situ* breast tumors showed that: (1) morphologically and immunohistochemically different cell types co-existed within the same duct, (2) cell budding was exclusively seen at focally disrupted myoepithelial cell layers, (3) all budding cells shared the same morphological and immunohistochemical profile, (4) budding cells were eventually in physical continuity with clear-cut invasive lesions, and (5) budding cells shared a very similar profile with their clear-cut malignant counterparts (Figs 3-4).

Discussion

Based on our hypothesis, although the lobular cell population has a less suppressive microenvironment and growth advantage, it may retain fewer residual stem or progenitor cells, compared to its ductal counterpart. Therefore, lobular tumors may at a greater risk for invasion or metastasis, whereas the invasive or metastatic lobular tumor cells may have lower potential to form new tumor nests in new tissue sites. Consequently, lobular tumors may have substantially more favorable prognosis than their stage-matched ductal counterpart. Our speculation is in total agreement with a case control study of 37,692 ductal carcinomas *in situ (DCIS)* and 4,490 lobular carcinoma *in situ (LCIS)*, which showed that patients with LCIS were 5.3-fold more likely than patients with DCIS to develop invasive lobular lesions [41]. Our speculation is also consistent with the pooled data of a number of epidemiological studies, which have shown that although invasive lobular tumors tends to be significantly larger in size with a significantly

higher rate of positive lymph nodes than its stage-matched ductal counterpart, patients with invasive lobular tumors have a substantially more favorable clinical outcome [23-27]. Together, these findings suggest the exhaustion or "use-up" the stem population with a normal full term pregnancy or multiple pregnancies may represent an effective mean to reduce breast cancer risk [32-35].

In sharp contrast, as the epithelial component is normally devoid of blood vessels and lymphatic ducts and totally depends on the stroma for its metabolic needs and even survival, a focal myoepithelial cell layer disruption in a given duct could have a number of consequences, including: (a) a localized loss or reduction of tumor suppressors and the paracrine inhibitory functions, which allow the associated tumor cells to undergo elevated proliferation [42]; (b) focal alterations in the permeability for oxygen, which selectively triggers the exit of stem or progenitor cells from quiescence [43,44]; (c) a localized increase of leukocyte infiltration, which directly export growth factors to the associated epithelial cells through direct physical contact [45-47]; (d) the direct epithelial-stromal cell contact, which augments the expression of stromal MMP or represses the normal production and distribution of E-cadherin, and other cell adhesion molecules, facilitating epithelial-mesenchymal transition and cell motility [48-50]; (e) the direct exposure of the epithelial cells to different cytokines, which stimulate an aberrant expression of c-erbB2, which facilitates vasculogenic mimicry and tumor angiogenesis [51,52]; and, (f) the direct physical contact between newly formed cell clusters and stromal cells stimulates the production of tenascin and other invasion-associated molecules that facilitate the stromal tissue remodeling and angiogenesis, providing a favorable micro-environment for epithelial cell proliferation and migration [53,54]. Together, these alterations could selectively favor monoclonal proliferation of the overlying tumor progenitors or a biologically more aggressive cell clone. Thus, the invasive and metastatic cells derived from the duct system may have greater potential to form tumor nests in the new tissue sites, and consequently lead to worse prognosis.

If confirmed, our hypothesis would have a number of clinical implications. *First*, the application of double immunohistochemistry to identify normal appearing lobular clusters with malignancy-associated alterations and focal myoepithelial cell layer disruptions with "budding" tumor cells in clinical biopsies would significantly facilitate early detection of individuals at greater risk to develop invasive cancer or pending invasive lesions. *Second*, as if two independent mechanisms or pathways are responsible for lobular and ductal cancer invasion, the precursors of invasive lesions for these tumors are very likely to differ substantially in their morphological, molecular, and/or biochemical profiles. Consequently, micro-dissection of these potential precursors of invasive lesions for gene expression profiling may lead to identification of more specific molecules for differentiation and intervention of invasive lobular and ductal cancer. *Third*, as it has been well documented that invasive cancer cells derived from lobular cancer tend to be more ER (+), PR (+), and HER-2 (-), compared to their stage-matched ductal counterparts [1-6], invasive and metastatic lesions derived from these tumors may have different responses to the same therapeutic regimen. Therefore, the development of more specific reagents or detection methods to differentiate lobular and ductal cells and their malignant derivatives may have significant therapeutic value. More importantly, as leukocyte aggregates have been consistently seen at the junction between normal lobules harboring cells with malignancy-associated changes and invasive lesions, and also at or near focally disrupted myoepithelial cell layers with budding tumor cells, anti-inflammatory therapy may have significant clinical value for lobular cancers.

Acknowledgments

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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