Do myoepithelial cells hold the key for breast tumor progression?

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ABSTRACT

Mammary myoepithelial cells have been the foster child of breast cancer biology and have been largely ignored since they were considered to be less important for tumorigenesis than luminal epithelial cells from which most of breast carcinomas are thought to arise. In recent years as our knowledge in stem cell biology and the cellular microenvironment has been increasing myoepithelial cells are slowly starting to gain more attention. Emerging data raise the hypothesis if myoepithelial cells play a key role in breast tumor progression by regulating the in situ to invasive carcinoma transition and if myoepithelial cells are part of the mammary stem cell niche. Paracrine interactions between myoepithelial and luminal epithelial cells are known to be important for cell cycle arrest, establishing epithelial cell polarity, and inhibiting migration and invasion. Based on these functions normal mammary myoepithelial cells have been called "natural tumor suppressors". However, during tumor progression myoepithelial cells seem to loose these properties and eventually they themselves diminish as tumors become invasive. Better understanding of myoepithelial cell function and their role in tumor progression may lead to their exploitation for cancer therapeutic and preventative measures.

KEYWORDS: myoepithelium, tumor progression, DCIS (ductal carcinoma in situ), paracrine factor, SAGE (Serial Analysis of Gene Expression)

INTRODUCTION

Breast cancer is the most commonly identified and one of the deadliest neoplasms in women in Western countries. The recent trend toward improvement in breast cancer mortality rate is largely due to increased diagnosis of early stage disease, while our therapeutic options for advanced stage breast carcinomas are still fairly limited. Thus, there is a need to better understand the molecular basis of breast tumor progression and to use this knowledge for the design of targeted, molecular based therapies. Recently developed technologies have enabled us to analyze molecular differences between normal and cancer cells at a genome-wide level in comprehensive and unbiased ways, allowing the molecular based classification of breast cancer and identification gene signatures correlating with metastatic behavior and clinical outcome (1-5). However, since most of these studies were using bulk tissue samples that are composed of multiple cell types, the specific contribution of epithelial, myoepithelial, and various stromal cells to these tumor classifiers and prognostic signatures is unknown.

In the past decades the major focus of cancer research has been the transformed tumor cell itself, while the role of the cellular microenvironment in tumorigenesis has not been widely explored. Epithelial-mesenchymal interactions are known to be important for the normal development of the mammary gland and to play a role in breast tumorigenesis (6-13). Early studies demonstrated that normal mammary microenvironment is capable of "reverting" the neoplastic phenotype of breast cancer cells by inducing cellular differentiation (14, 15), suggesting that cancer cells can thrive only in a distorted environment or have to become independent of extracellular signals. The contribution of genetic host factors to tumor initiation, progression, and angiogenesis also support a role for non-epithelial cells in carcinogenesis (16, 17). This was dramatically illustrated by the results that systemic inactivation of TGF-β type II receptor in stromal fibroblasts led to prostate and gastric epithelial neoplasia (18), while its inactivation in mammary stromal fibroblasts led to abnormal ductal development and promoted the growth of transplanted tumors (19). Similarly a recent finding demonstrating that mammary tumors were only formed in cleared mammary fat pads of rats treated with carcinogens, regardless of whether the epithelial cells were treated

with carcinogens *in vitro*, also emphasizes the importance of stromal alterations in the initiating steps of breast cancer (20). However, this finding could not be confirmed in mouse mammary glands where DMBA treatment of the stroma had no effect on mammary tumorigenesis (21) suggesting differences among species or in experimental design. Numerous *in vitro* and *in vivo* studies using diverse experimental systems have demonstrated that the growth, survival, polarity, and invasive behavior of breast cancer cells can be modulated by myoepithelial and various stromal cells, and several genes have been implicated to play an important role in this process (6-13, 22-26). In addition, certain histo-pathological features of breast tumors, including lymphocytic infiltration, fibrosis, and angio- and lymphangiogenesis, have proven prognostic significance. Despite these convincing data implicating a role for stromal cells in breast tumorigenesis, our understanding of the genes mediating cellular interactions and paracrine regulatory circuits among various cell types in normal and cancerous breast tissue and their role in breast tumorigenesis is limited.

As a consequence of studies focusing almost exclusively on cancer cells, nearly all of the currently used cancer therapeutic agents target the cancer cells that, due to their inherent genomic instability, frequently acquire therapeutic resistance (27). In part due to frequent therapeutic failures during the course of treatment of advanced stage tumors, increasing emphasis has been placed on targeting various stromal cells, particularly endothelial cells, via therapeutic interventions. Since these cells are thought to be normal and genetically stable, they are less likely to develop acquired resistance to cancer therapy. Thus, molecular targeting of the tumor microenvironment may be a novel promising option for cancer intervention and treatment.

Among all the cells types in the breast, myoepithelial cells have been one of the least analyzed, especially compared to luminal epithelial cells (25, 28). In this review we discuss the characteristics and normal function of myoepithelial cells and their putative role in breast tumor progression including the hypothesis that myoepithelial cells are the key regulators of the in situ to invasive carcinoma transition

and may be part the stem cell niche. We mainly focus on the human mammary gland and breast carcinomas, although also reference studies using various model organisms.

THE IDENTITY OF MYOEPITHELIAL CELLS

The mammary gland is composed to multiple cell types including luminal and myoepithelial cells residing within the ducts and alveoli and various other cells located in the stroma. In the ducts myoepithelial cells form a nearly continuous layer of cells that surrounds the luminal epithelial cells and separates them from the basement membrane and the stroma, while in the alveoli myoepithelial cells form a scaffold like structure and some alveolar epithelial cells have direct contact with the basement membrane (Figure 1). Luminal epithelial and myoepithelial cells are differentiated using cell type specific markers, many of which have been only fortuitously identified following immunohistochemical analysis of breast tissue. Myoepithelial cell specific genes include smooth muscle actin (SMA), CD10/CALLA cell surface marker, calponin, cytokeratins 14 and 17 (CTK14 and CTK17), epidermal growth factor receptor (EGFR), and p63 (29-31). In recent years several genome-wide unbiased studies were performed using various cell purification and profiling approaches to better characterize normal luminal epithelial or myoepithelial cells and identify additional genes specific for a particular cell lineage (32-34). One of these studies analyzed established myoepithelial cell lines and xenografts using Affymetrix arrays and compared them to normal and cancerous breast cell lines and primary tumors (32). Using this approach the author identified numerous genes that distinguished myoepithelial cells from other cell types. Interestingly many of these genes encode for extracellular matrix proteins (collagens, laminin A, fibronectin, osteonectin, etc.), angiogenic (thrombospondin-1, plasminogen, etc.) and protease (maspin, PAI-1, etc.) inhibitors, re-confirming the role of myoepithelial cells as tumor suppressors (32). However, the gene expression profile of cell lines and xenografts may not faithfully reflect the *in vivo* patterns, therefore, profiling of uncultured cells is desirable for the discovery of cell lineage specific markers. Along these lines proteomic analysis of uncultured purified normal luminal and myoepithelial cells

identified 170 proteins differentially expressed between the two cell types and 51 of these were annotated by tandem mass spectrometry (33). Many of these corresponded to abundant cytoplasmic proteins, such as cytokeratins, intermediate filaments, and heat shock proteins. Another gene expression profiling study used SAGE (Serial Analysis of Gene Expression) to analyze freshly isolated, uncultured luminal epithelial and myoepithelial cells purified using BerEP4 and CD10 antibody coupled magnetic beads, respectively, from normal breast tissue (34). This analysis revealed 295 genes statistically significantly differentially expressed between the two cell types, and identified 138 that were more abundant in myoepithelial cells. Interestingly a high fraction (43%) of the genes most highly specifically expressed in myoepithelial cells encode secreted or cell surface proteins suggesting that myoepithelial cells are actively involved in autocrine/paracrine interactions (Table 1).

All these studies assumed that there is only one type of myoepithelial cell within the normal breast, but this is unlikely to be true, since not all myoepithelial cells express all myoepithelial markers, and myoepithelial cells localized in the ducts and alveoli may also be different. Therefore, additional analyses are necessary to further define the molecular portrait of the various normal myoepithelial cells.

THE ORIGIN OF MYOEPITHELIAL CELLS

The cell or origin of normal myoepithelial cells is not well defined. Understanding the normal development of the mammary gland, clarifying the relationships among stem cells and their differentiated progeny, and characterizing factors regulating these processes are important not only for furthering our knowledge of basic mammary gland biology, but also for improving our understanding of breast tumorigenesis. Human epidemiologic data indicate that exposure to various hormones, radiation and other environmental agents during intrauterine, infantile, or pubertal mammary gland development influence the subsequent incidence of breast cancer in adulthood (35-38). The development of the mammary gland is a complex process and contrary to that of most other organs it is completed only in adulthood and some aspects of cellular differentiation even require the completion of a full-term pregnancy, lactation, and

involution cycle. The mammary gland is also unique with respect to being continuously remodeled following puberty due to the cyclical influence of reproductive hormones. Most of our data on mammary gland development have been obtained in mice and interpreted for humans despite the well-known differences between human and mouse mammary gland development and function. Studies analyzing the development of the human mammary gland have been limited to structural and immunohistochemical analyses of a limited number of samples collected at different stages of fetal, infantile, childhood, and pubertal development (39-42). In the human embryo the breast bud arises as a result of proliferation of basal cells of the epidermis, and some markers of these basal keratinocytes are maintained (CTK19), while others are extinguished (CTK14) in the mammary epithelial cells potentially due to factors secreted by the mesenchymal cells present in the breast bud (40). Myoepithelial cell differentiation, as determined by the expression of myoepithelial cell specific markers (SMA, CD10, p63, etc.) and the lack of expression of luminal cell markers (CTK19 etc.) seems to occur at 21-28 weeks of gestational age (41, 42). However, not all markers characteristic for myoepithelial cells are expressed in the SMA positive basal cells of the fetal mammary gland, suggesting that the terminal differentiation of myoepithelial cells is a multi-step process and there may be several intermediary cells with varying stem cell potential and lineage commitment. This seems to the case even in breast tissue of adult women based on a recent immunohistochemical analysis of myoepithelial cell using multiple lineage specific markers (43). Specifically, a subset of morphologically myoepithelial appearing cells lack the expression of the nine cell lineage specific markers analyzed. However, it is possible that these cells are not truly differentiated myoepithelial cells, but bipotential or myoepithelial progenitor cells. Characterization of mammary stem cells and identification of stem cells specific markers would be necessary to conclusively answer this question.

Almost nothing is known about the identity of factors that regulate myoepithelial cell differentiation, although recent data indicate the involvement of the Notch pathway (44, 45). Specifically, activation of the Notch pathway not only appears to enhance the proliferation of the putative mammary

stem cells, but also increases the number of cells committed to the myoepithelial lineage by promoting the proliferation of bipotential or myoepithelial progenitor cells and enhancing myoepithelial differentiation. Further studies are needed to identify and characterize regulators of mammary stem cell proliferation and differentiation and determine how abnormal activity of these pathways may contribute to breast tumorigenesis.

THE FUNCTION OF MYOEPITHELIAL CELLS IN BREAST TUMOR PROGRESSION

Breast tumors evolve via sequential progression through defined clinical and pathologic stages starting with ductal epithelial hyperproliferation, progressing into *in situ* then invasive, and metastatic carcinomas. DCIS is believed to be the true precursor of invasive ductal carcinoma based on molecular-based clonality studies, its increased incidence in women with high risk of invasive breast cancer, its frequent coexistence with invasive lesions, and on its high rate of recurrence as an invasive tumor at its original site (46-52). Until 1980, DCIS was diagnosed very rarely and represented less than 1% of all breast cancer cases. Due to the increased use of mammograms DCIS became the most rapidly increasing subset of breast cancers and currently it accounts for 15-25% of newly diagnosed breast cancer cases in the United States (53, 54). In contrast to the dramatic improvement in our ability to detect DCIS, our understanding of the pathophysiology of this disease and factors involved in its progression to invasive carcinoma are still poorly defined.

The major diagnostic criteria that pathologists use to differentiate in situ from invasive carcinomas is the presence or absence of an intact myoepithelial cell layer, which is usually confirmed by performing immunohistochemical analyses against myoepithelial cell specific genes such as smooth muscle actin, p63, or CD10 (55). However, it is unknown what leads to the disappearance of the myoepithelial cells in invasive tumors (selective elimination by apoptosis or lack of proper myoepithelial cells differentiation from stem cells) and how this contributes to tumor progression. Exposure of myoepithelial cells in culture to even low concentrations of carrageenans, naturally occurring sulfated polysaccharides used in

commercial food preparation, leads to cell death (56). However, it is unknown if destruction of myoepithelial cells could occur by these compounds or by other environmental agents in human breast cancer patients.

The tumor suppressor function of myoepithelial cells

Myoepithelial cells have been called natural tumor suppressors due to their negative effect on various neoplastic phenotypes including tumor cell growth, invasion, and angiogenesis (23-25, 28). Myoepithelial cells also synthesize the basement membrane of the ducts and alveoli and form a structural barrier between the luminal epithelial cells and the surrounding stroma, thus, physically preventing tumor cell invasion. The tumor suppressor phenotype was determined based on the ability of myoepithelial cells to inhibit the growth and invasion of breast cancer cells in co-culture assays *in vitro* and inhibit tumor growth in xenograft assays (23, 24, 57, 58). These effects have been largely attributed to paracrine factors secreted by myoepithelial cells that exert their effects on the tumor epithelial cells. Some of these factors include ECM proteins, protease inhibitors, various growth factors, and some are still unidentified. Most of these studies were performed using myoepithelial cell lines derived from benign or low-grade human myoepitheliomas of the breast, salivary gland, and bronchi, thus, these cells may no completely reflect the function of normal mammary epithelial cells (59). However, the myoepithelial cell lines used in these experiments are genetically fairly normal, have maintained the expression of all myoepithelial markers analyzed (SMA, S100A2, CTK14, etc.) even after prolonged passage *in vitro*, and the expression of all anti-tumorigenic genes was confirmed in primary human breast tissue by immunohistochemistry. In addition, the results were reproduced using freshly isolated primary normal mammary myoepithelial cells (60). In these experiments co-culturing normal breast myoepithelial cells (purified using anti-CD10/CALLA antibody) using transwell insert with various human breast cancer cell lines led to decreased expression of MMPs (Matrix Metallo-proteases) in the cancer cells. This effect of the

myoepithelial cells was observed even in the presence of co-cultured fibroblasts, known promoters of tumor cell invasion, thus, demonstrating the dominance of myoepithelial cells.

The anti-invasive and anti-proliferative effects of myoepithelial cells can be further increased following treatment with tamoxifen due to the increased secretion of maspin and production of inducible nitric oxide synthase (iNOS) and these effects are mediated by the estrogen receptor (61). Although myoepithelial cells do not express estrogen receptor-α (ERα) they express ERβ both *in vitro* and *in vivo* (61, 62) and the induction of maspin and iNOS appears to be mediated by ERβ dependent activation of AP1. It remains to be determined if this effect of tamoxifen is also observed in breast cancer patients and if it contributes to the cancer preventative effects of anti-estrogens.

In addition to being responsive to tamoxifen mammary epithelial cells may also influence the response of luminal epithelial cells to estrogenic hormones, since they express high levels of steroid sulfatase (STS), a lysosomal hydrolyse that converts estrone 3-sulfate (E1S) and dehydroepiandrosterone-sulfate (DHEA-S) into their active, unsulfated forms (63). The expression of STS in the myoepithelial cells may lead to increased local concentration of these hormones making them available for the ERα luminal epithelial cells. However, this result has not been confirmed in vivo, thus, the potential role of myoepithelial cells as regulators of the local concentration of estrogenic hormones remains to be determined.

Myoepithelial cells also influence the differentiation and polarity of the adjacent luminal epithelial cells. Luminal epithelial cells are polarized as determined by the expression of sialomucin, epithelial specific antigen (ESA), and occludin on the luminal membrane and integrin β4 on the basolateral membrane. This polarity is observed *in vitro* when luminal epithelial cells are cultured in reconstituted basement membrane (matrigel), but lost when the cells are grown in collagen I (22). However, mixing the luminal epithelial cells with normal myoepithelial cells was able to restore epithelial cell polarity even in collagen cultures, and this effect was shown to be mediated by laminin-1. Interestingly myoepithelial cells

isolated from invasive breast tumors were unable to exert this effect (22). These tumor myoepithelial cells were isolated from primary breast carcinomas using a Thy-1 antibody column and they were considered myoepithelial cells due to their emergence from luminal epithelial cells, expression of cytokeratins, vimentin, and SMA, and inability to form tumors in nude mice, but they were not analyzed for genetic changes and clonal relationship to the epithelial cells from the same tumor. Invasive breast tumors by definition devoid of myoepithelial cells, but a subset of tumor cells, including the presumable 'cancer stem cells", express high levels of Thy-1, vimentin, and cytokeratins. Similarly, stromal myofibroblasts are highly Thy-1 and vimentin positive, although lack cytokeratins. Thus, the identity of these tumor myoepithelial cells is somewhat of a mystery and would require further studies, although it is clear that they are not able to recapitulate the function of normal myoepithelial cells presumably due to their lack of laminin-1 expression.

Alterations in myoepithelial and stromal cells during breast tumor progression

In the past few years the role of the cellular microenvironment in tumorigenesis has become an intense area of research. This is in part due to studies demonstrating that genetic abnormalities, such as loss of heterozygosity (LOH), occur not only in cancer cells, but in myoepithelial or stromal cells, or even normal appearing epithelial cells surrounding the tumor and in benign stromal and epithelial hyperproliferative diseases (64-72). In several cases the tumor epithelial and stromal cells had discordant genetic changes suggesting a clonal co-evolution for these two cell types. Due to the low probability of two adjacent cells simultaneously acquiring different genetic changes, the authors suggested that in some breast tumors cancer epithelial and stromal cells may be derived from a common stem cell, but subsequently undergo divergent genetic selection processes. One study demonstrated discordant mutations in TP53 and PTEN in tumor epithelial and stromal cells, and low frequency of WFDC1 mutations exclusively in the stromal cells (67). However, no mechanistic insight was provided explaining the clonal selection for mutation in the same gene in the two different cellular compartments. Thus, the

biological relevance of these mutations and LOH events in stromal cells and their role in breast tumorigenesis is still largely unknown. One potential problem with these studies is that the cellular identity of the stromal cells was not conclusively confirmed (e.g. by cell type specific markers), and since they were isolated using LCM (laser capture microdissection) from formalin fixed and paraffin embedded or frozen breast tumors, the possibility of contaminating tumor cells is difficult to rule out. Furthermore, due to the methods used relatively few stromal cells were analyzed and from a relatively small area of the tumor. Thus, it is uncertain if these LOH events reflect true clonally selected genetic events that are observed in the majority of the tumor stroma or they are just random LOH that occur at low frequency in every dividing cell. Similarly the report describing LOH in normal luminal and myoepithelial cells obtained from reduction mammoplasty specimens analyzed clones of cells expanded in *in vitro* cultures (73). Thus, the possibility that the clone of a single cell expanded *in vitro* was analyzed or that the LOH occurred in vitro cannot e excluded.

Immunohistochemical analyses of normal breast tissue and in situ and invasive breast carcinomas aiming to identify markers of tumor progression using a candidate gene approach have identified several genes that are differentially expressed between normal and DCIS myoepithelial cells. Among others lysyl oxidase, an enzyme involved in collagen and elastin crosslinking, was most highly expressed in myoepithelial cells and myofibroblasts in DCIS tumors (74). Similarly the expression of neuropilin-1 was found to be up-regulated in DCIS myoepithelial and vascular smooth muscle cells compared to normal breast tissue and invasive carcinomas (75). To identify molecular changes in the cellular microenvironment in an unbiased way, Hu et al. purified and analyzed all cell types (epithelial, myoepithelial, and endothelial cells, infiltrating leukocytes, fibroblasts and myofibroblasts) from normal breast tissue, and in situ and invasive carcinomas, and concluded that gene expression changes occur in all cell types during breast tumor progression, but clonally selected genetic changes are only observed in tumor epithelial cells (76). In this study each cell type was purified using cell type specific cell surface markers and the purity of the isolated cell population was confirmed using RT-PCR and cell type specific

genes. The comprehensive gene expression profile of each purified cell population was then analyzed by SAGE (Serial Analysis of Gene Expression), while genetic changes were analyzed by cDNA array CGH (Comprehensive Genomic Hybridization) and SNP (Single Nucleotide Polymorphism) arrays. Interestingly the comparison of myoepithelial cells from normal breast tissue and DCIS yielded the highest number of consistently differentially expressed genes, and a significant fraction of these encoded for secreted and cell surface proteins including several chemokines (Tables 1 and 2). Many of the genes specific for normal myoepithelial cells (CTK14, CTK17, OXTR, EGFR, etc.) were absent or dramatically downregulated in DCIS myoepithelial cells suggesting that myoepithelial cells in DCIS are not really "normal" since they appear to be less differentiated and likely have lost some of the functions of normal myoepithelial cells (Tables 1 and 2). However, demonstrating functional differences between normal and DCIS myoepithelial cells and demonstrating that these changes play a role in breast tumor progression require further studies.

The dramatic gene expression changes and lack of genetic alterations in the myoepithelial and stromal cells suggested underlying epigenetic changes, since cells isolated from normal and tumor tissue are known to maintain their differences even after prolonged cell culture *in vitro* (8, 13, 77, 78). Indeed, a follow up study by the same group using a newly developed method (MSDK-Methylation Specific Digital Karyotyping) for the analysis of genome-wide methylation profiles identified alterations in DNA methylation patterns not only in tumor epithelial cells, but in stromal fibroblasts and DCIS myoepithelial cells as well (79). Consistent with prior results, increased DNA methylation in the promoter region of the genes negatively correlated with gene expression, while hypermethylation of introns and 3' exons positively influenced gene expression suggesting the presence of silencer elements that are regulated by DNA methylation (79, 80). The best characterized example for this is the imprinting of the H19/IGF2 genes that is regulated by a silencer containing a binding site for CTCF (81, 82). The imprinting of *IGF2* is dependent on *CTCF* binding to this enhancer-blocking element and its methylation inhibits *CTCF* binding and leads to loss of imprinting(81, 82). Interestingly based on their sequence two of the

differentially methylated genes identified by Hu et al. appear to have a *CTCF* binding site in their hypermethylated region, but demonstrating their silencer function requires follow up studies.

The relationship between myoepithelial cells and myofibroblasts

Close "relatives" of myoepithelial cells of myoepithelial cells are the myofibroblasts, since they share the expression of many genes including SMA, Thy-1, vimentin, CD10/CALLA, and several proteases and protease inhibitors (76). However, myoepithelial cells express cytokeratins and are located within the breast ducts, while myofibroblasts are mesenchymal cells located in the stroma. The cell of origin of myofibroblasts is still subject to debate with two main hypotheses dominating. According to one model myofibroblasts are fibroblasts "transformed" by TGFβ and PDGF potentially secreted by the tumor cells or infiltrating leukocytes, since treatment of fibroblasts in *in vitro* cultures with these growth factors leads to the expression of SMA and other myofibroblast specific genes (83, 84). However, in vivo studies performed both in mice and human provide strong evidence that myofibroblasts are derived from circulating mesenchymal stem cells recruited to the tumors either by the tumor cells themselves or by the inflammatory reaction initiated by infiltrating leukocytes (85, 86). Despite the similarity of gene expression patterns between myoepithelial cells and myofibroblasts, it is unlikely that the two cell types are clonally related. Correlating with this comprehensive analysis of their gene expression profiles identified many genes differentially expressed between the two cell types (Table 2). A significant fraction (49%) of these genes encode secreted and cell surface proteins including chemokines, ECM molecules, proteases and protease inhibitors, implicating both cells in autocrine/paracrine interactions and ECM remodeling. The hypothesis that myoepithelial cells may become myofibroblasts during the in situ to invasive carcinoma transition is unlikely, but deserves further investigation.

Models of breast tumor progression

Our fairly limited understanding of in situ to invasive carcinoma transition is in part due to the fact that there are no good experimental models for DCIS that would faithfully reproduce the human disease. Carcinogen induced mammary gland tumors in rats reproduce certain aspects of human DCIS, such as ovarian hormone dependence and gradual progression to invasive disease(87). However, the carcinogen used for the initiation of these tumors may have caused numerous genetic changes that are not easy to identify making this model unattractive for molecular studies addressing the role of specific genes in mammary tumorigenesis. The same limitation applies to the use of DCIS xenografts formed by subcutaneous injection of pieces of human DCIS tumors into nude mice(88).

Although no model is ideal, they allow the functional testing of genes implicated in breast cancer and the evaluation of novel cancer preventative and therapeutic interventions. A good model of DCIS would have to resemble the histology of high-risk human pre-malignant breast lesion that with time progress to invasive carcinomas. The MCF10AT human breast cell line is one of the most well characterized human models of breast tumor progression that fulfills these criteria(89, 90). These cells were derived from the immortalized MCF-10A cells via transformation with T24 mutant c-Ha-ras (89, 90). Interestingly, the MCF10AT cells appear to contain multi-potent (or bipotential) breast stem cells, since both luminal epithelial and myoepithelial cells can be derived from these cells *in vivo*(91). Recently a derivative of the MCF10AT premalignant human cell line model was established MCF10DCIS.com that reproducibly forms comedo DCIS like lesions that spontaneously progress to invasive tumors (89, 90). However, just because a tumor looks like a DCIS it does not mean that it is "really" a DCIS and cells expressing myoepithelial markers may not always be "real" myoepithelial cells. This is illustrated by the detection of "revertant" DCIS in lymph nodes of some breast cancer patients with metastatic breast tumors (92). Specifically, a subset of invasive or metastatic breast tumors demonstrate a DCIS-like growth pattern as determined by cells growing in tight clusters surrounded by basement membrane, but these structures lack a myoepithelial cell layer emphasizing the importance of staining for myoepithelial cell specific markers in cases of questionable diagnosis. Similarly, in invasive breast tumors with basal phenotype the tumor

cells themselves express several genes normally detected in myoepithelial cells including p63 and cytokeratin 14, presumably because these tumors originate from bipotential progenitor cells and show some degree of myoepithelial differentiation, but they never express smooth muscle actin (93). Thus, to conclusively prove that a cell is a normal terminally differentiated myoepithelial cell the examination of multiple markers is necessary.

The "escape" or "release" model of in situ to invasive carcinoma transition

The prevailing view of breast tumor progression is tumor epithelial cell driven, since tumor epithelial cells have acquired genetic changes and demonstrate genomic instability, and thus, the most aggressive invasive cells can be selected out due to clonal selection. However, this model has recently been challenged by multiple independent studies. First, demonstrating genetic changes in tumor stroma, although still controversial, raises the possibility that clonal selection occurs in non-epithelial cells as well, thus, the microenvironment may play an active role in driving tumor progression. Second, demonstrating global gene expression and epigenetic alterations in all cell types during breast tumor progression and the finding that the genetic background of the host influences metastatic behavior suggest that tumorigenesis is a "teamwork" and although genetic changes may only occur in tumor epithelial cells, changes in the cellular microenvironment are likely to play a role in tumor progression. Studies demonstrating increased cancer risk in patients with chronic inflammatory disease and decreased risk in users of anti-inflammatory drugs, and experiments performed in model systems addressing the role of genetically modified stroma in tumorigenesis, even raise the possibility that alterations in the microenvironment play a role in tumor initiation.

Based on these data we propose two alternative models of the in situ to invasive carcinoma transition (Figure 3). One hypothesis emphasizes the role of tumor epithelial cells and suggests that genetic changes in these cells lead to the selection of a clone with invasive properties that will "escape" from the duct, spread into the stroma, and subsequently expand. The second model gives more credit to

the non-epithelial cells and implies that phenotypic changes in DCIS myoepithelial cells, accumulation of inflammatory cells and myofibroblasts work together and lead to the break down of the ducts and results in the "release" of tumor epithelial cells. Recently published studies describing focal myoepithelial cell layer disruption in DCIS breast tumors at sites of leukocytic infiltration gives support to the "release" model (94). Similarly experiments performed using the MCF10DCIS.com model system suggest that myoepithelial cells play a key role in the regulation of in situ to invasive carcinoma transition (Hu et al. unpublished data). However, the two models are not mutually exclusive. For example, it is possible that factors secreted by DCIS myoepithelial cells, infiltrating leukocytes and myofibroblasts may influence the clonal evolution of the tumor epithelial cells. Supporting this combined view is the finding that tumor epithelial cells overlaying sites of focally disrupted myoepithelial cell layers are phenotypically and genetically different from their adjacent counterparts (94). Since presumably these are the cells responsible for the subsequent progression of the tumors, thus, presumably are the putative "cancer stem cells", this raises the question if paracrine factors secreted by myoepithelial and stromal cells may influence the cancer stem cell phenotype. Thus, in a way cells of the tumor microenvironment may constitute the stem cell niche, a hypothesis worth investigating in the future. Of course it is difficult to know what comes first, the development of a genetically distinct tumor epithelial cell clone or the myoepithelial cell layer disruption. Clarifying this "chicken or egg" dilemma will likely to keep investigators busy for years to come.

CONCLUSIONS

Due to decades of neglect the function of mammary myoepithelial cells and their role in breast tumorigenesis is still largely unknown. However, recent and emerging studies implicate them as potential regulators of in situ to invasive carcinoma transition and stem cell function. In light of the importance of these issues, myoepithelial cells are now the focus of intense investigations and via understanding their function we are likely to gain new, valuable targets for breast cancer prevention and treatment.

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FIGURE LEGENDS

Figure 1. Location of myoepithelial cells in normal breast ducts and lobules. A. Schematic depiction of a normal duct and lobule. Myoepithelial cells are indicated in black, ductal luminal epithelial cells in pink, while alveolar epithelial cells in beige. Black line denotes the basement membrane. In the ducts myoepithelial cells form a nearly complete layer around the luminal epithelial cells, while in the alveoli the myoepithelial layer is more fenestrated and some luminal epithelial cells are in direct contact with the basement membrane. B. Immunohistochemical analysis of the expression of myoepithelial cell specific genes in normal human breast tissue. Top panel: expression of smooth muscle actin (SMA) is indicated by brown color, while the nuclei are counterstained with hematoxylin. Bottom panel: dual staining for p63 (brown nuclear signal) and pan-cytokeratin (pink cytoplasmic staining) expression. All cells in the basal (myoepithelial) cell layer appear to be positive for SMA, but not for p63.

Figure 2. Hypothetical model depicting breast tumor progression with the different cell types and cell-cell interactions indicated. A. Schematic view of a normal duct, and in situ and invasive carcinomas. In normal breast ducts luminal epithelial cells (pink) lay on top of a layer of myoepithelial cells (black) that are in direct contact with the basement membrane (black line). Stromal cell types include various leukocytes (blue), fibroblasts (pink spindle shape cells), myofibroblasts (black spindle shape cells), adipocytes (yellow round shape cells), and endothelial cells (red rod like cells). Cellular interactions among the different cell types mediated by autocrine and paracrine factors are indicated with arrows. epithelial cells and stromal cells, including fibroblasts (pink spindle-shape cells), adipocytes (round yellow), macrophages (white irregular shape), eosinophil granulocytes (red), lymphocytes (blue circles), and endothelial cells (red rods). In *in situ* carcinomas, potentially due to the loss of expression of certain chemokines and cytokines and up-regulation of some others, luminal epithelial cells loose their ability to maintain a single epithelial layer. At the same time, the number of myoepithelial cells decreases and the number of stromal fibroblasts, lymphocytes and endothelial cells increases. In invasive

carcinomas myoepithelial cells and the basement membrane are absent and tumor cells are dispersed in the stroma. B. Immunohistochemical analysis of myoepithelial cell specific gene expression in human normal and cancerous tissue samples. Smooth muscle actin (SMA) is expressed in myoepithelial cells and in myofibroblasts, while p63 is expressed in myoepithelial cells in normal and DCIS tissue, but in a subset of epithelial cells in invasive tumor indicative of basaloid/squamous cell differentiation.

Figure 3. Hypothetical model depicting two views of in situ to invasive carcinoma transition. Cells are depicted as in Figures 1 and 2. In the "escape" model the tumor epithelial cells disrupt the myoepithelial cell layer, degrade the basement membrane, and migrate into the stroma. While in the "release" model the myoepithelial cells disappear and the basement membrane is disrupted at sites coinciding with areas of leukocytic infiltration and accumulation of myofibroblasts.

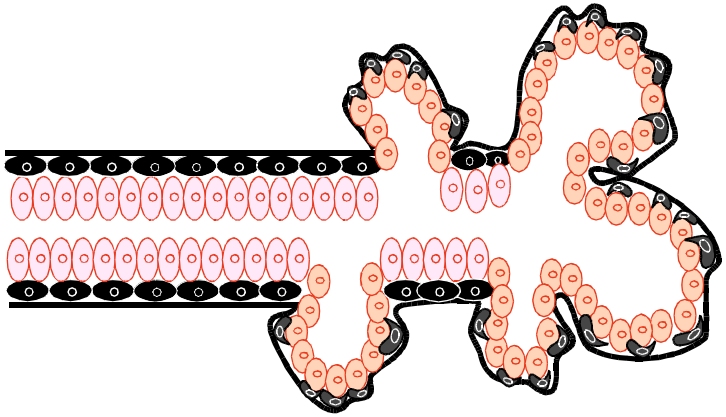
Table 1. List of genes specific for normal myoepithelial cells and abnormally expressed in DCIS myoepithelial cells. SAGE tag sequence (Tag), normalized tag numbers in normal epithelial (NE), myoepithelial (NM), and DCIS myoepithelial (DM) cells, their ratios, and the subcellular localization of the encoded protein are listed. Genes were selected based on pair-wise comparison of SAGE libraries generated from normal epithelial and myoepithelial, and DCIS myoepithelial cells, and identifying the statistically significantly ($p<0.02$) differentially expressed tags demonstrating at least 10-fold difference.

Tag	NE	NM	DM	Ratio NM/NE	Ratio DM/NM	UniGene	Gene Sym	Description	Location
TACTTTATAA	0	287	2	287	-170	Hs.534115	ADAMTS1	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	secreted
GGGAAGGGAC	0	178	3	178	-53	Hs.269128	PPP2R1B	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	
ACCTTGGTGC	0	140	2	140	-83	Hs.355214	KRT14	Keratin 14	
GTAAATATGG	2	280	5	120	-55	Hs.485616	DST	Dystonin	secreted
TGCTTGTGTA	0	115	0	115	-115	Hs.121520	AMIGO2	Adhesion molecule with Ig-like domain 2	cell membrane
AGGTCTTCAA	2	192	22	83	-9	Hs.164226	THBS1	Thrombospondin 1	secreted
TTTTGTATTT	0	80	2	80	-48	Hs.434914	C14orf31	FERM domain containing 6	
TCCTGTAAAG	0	73	7	73	-11	Hs.74034	CAV1	Caveolin 1, caveolae protein, 22kDa	cell membrane
ATGGTGTATG	0	70	2	70	-42	Hs.128548	WDR1	WD repeat domain 1	
TGTAGGTCAT	0	66	5	66	-13	Hs.58561	GPR87	G protein-coupled receptor 87	
TTTCCTCTCA	5	297	15	64	-20	Hs.523718	SFN	Stratifin	cytosol/secreted
GATATGTTAT	0	63	0	63	-63	Hs.117938	COL17A1	Collagen, type XVII, alpha 1	secreted
CTTCCTTGCC	44	2698	118	61	-23	Hs.2785	KRT17	Keratin 17	
TTCTTTTCAT	0	59	0	59	-59	Hs.522590	EIF1AX	Eukaryotic translation initiation factor 1A, X-linked	
AAAGTCATTG	2	133	3	57	-39	Hs.21145	ODAG	GATA zinc finger domain containing 1	
ACTACTAAGG	0	52	0	52	-52	Hs.2820	OXTR	Oxytocin receptor	cell membrane
GCTGTAGCCA	0	52	0	52	-52	Hs.483816	PPARGC1B	Peroxisome proliferative activated receptor, gamma, coactivator 1, beta	
GGAAAGATGT	0	49	0	49	-49	Hs.488293	EGFR	Epidermal growth factor receptor	cell membrane
CTTTCTTTGA	2	77	8	33	-9	Hs.292156	DKK3	Dickkopf homolog 3 (Xenopus laevis)	secreted
TCATCATCAG	2	77	0	33	-77	Hs.303870	KCNC1	Potassium voltage-gated channel, Shaw-related subfamily, member 1	cell membrane
AAGCTGTATA	2	73	8	32	-9	Hs.143250	TNC	Tenascin C (hexabrachion)	secreted
TGATTGGTGG	5	122	3	26	-36	Hs.74615	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	cell membrane
GTTTCATCTC	5	84	3	18	-25	Hs.408767	CRYAB	Crystallin, alpha B	
TCACTGCACT	9	154	10	17	-15	Hs.491322	PTK2B	PTK2B protein tyrosine kinase 2 beta	
TTTGTAGATG	5	70	3	15	-21	Hs.184233	HSPA9B	Heat shock 70kDa protein 9B	
TATCTGTCTA	12	136	2	12	-81	Hs.436687	SET	SET translocation	
TCTCTACTAA	9	108	13	12	-8	Hs.466088	TPM4	Tropomyosin 4	
ACTGAGGAAA	9	105	19	11	-6	Hs.450230	IGFBP3	Insulin-like growth factor binding protein 3	secreted
TTCTATTTCA	7	73	2	11	-44	Hs.87752	MSN	Moesin	
TAGCTCTATG	9	98	8	11	-12	Hs.371889	ATP1A1	Hypothetical protein MGC16179	cell membrane
GTCCTTTCTG	12	119	5	10	-24	Hs.799	HBEGF	Heparin-binding EGF-like growth factor	secreted
TACCAGTGTA	9	94	2	10	-56	Hs.113684	HSPD1	Cadherin 12, type 2	cell membrane
ACACTTGGAG	9	91	3	10	-27	Hs.221889	CSDA	Cold shock domain protein A	
GTGATGGTGT	9	84	3	9	-25	Hs.292493	G22P1	X-ray repair complementing defective repair in Chinese hamster cells 6	
CTTAAGGATT	12	101	5	9	-20	Hs.530412	PAI-RBP1	PAI-1 mRNA binding protein	
TGCTGTGCAT	19	157	25	8	-6	Hs.380774	DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	
AGTGTCTGTG	37	304	32	8	-10	Hs.8867	CYR61	Cysteine-rich, angiogenic inducer, 61	secreted
TAAAACAAGA	12	94	12	8	-8	Hs.527653	DAF	Decay accelerating factor for complement	secreted
TTCATTATAA	23	171	5	7	-34	Hs.459927	PTMA	Prothymosin, alpha (gene sequence 28)	
GTGTAATAAG	26	175	17	7	-10	Hs.487774	HNRPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	
AGTATGAGGA	26	147	30	6	-5	Hs.211600	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	
TACTAGTCCT	19	105	7	6	-16	Hs.525600	HSPCA	Heat shock 90kDa protein 1, alpha	
TTAAAGATTT	33	175	15	5	-12	Hs.133892	TPM1	Tropomyosin 1 (alpha)	
TTCTTGTTTT	26	126	13	5	-9	Hs.472010	PRNP	Prion protein (p27-30)	
TACCATCAAT	107	444	64	4	-7	Hs.544577	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	
TTCATACACC	240	793	64	3	-12	Hs.527213	DCOHM	6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha	
TAATAAAGGT	137	409	64	3	-6	Hs.512675	RPS8	Ribosomal protein S8	
AGAAAGATGT	184	521	79	3	-7	Hs.494173	ANXA1	Annexin A1	cell membrane
TTGAAGCTTT	396	1007	249	3	-4	Hs.75765	CXCL2	Chemokine (C-X-C motif) ligand 2	secreted

Table 2. List of genes differentially expressed between normal and DCIS myoepithelial cells, and myofibroblasts. SAGE tag sequence (Tag), normalized tag numbers in normal (NM) and DCIS (DM) myoepithelial cells, and in myofibroblasts (MF), their ratios, and the subcellular localization of the encoded protein are listed. Genes were selected as described above (Table 1).

Tag	MF	DM	NM	Ratio DM/NM	Ratio DM/MF	UniGene	Gene Sym	Description	Location
AACTTTTGGC	9	81	15	12	9	Hs.195471	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	
TTCATACACC	377	64	1648	-12	-6	Hs.527213	DCOHM	6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha	
TAGCTGGAAA	31	395	0	235	13	Hs.475125	ATXN10	Ataxin 10	
GCCTGTCCCT	203	56	0	33	-4	Hs.821	BGN	Biglycan	secreted
GCGAAACCCA	11	69	0	41	6	Hs.17569	CHST11	Carbohydrate (chondroitin 4) sulfotransferase 11	
CGGGGTGGCC	41	173	0	103	4	Hs.1584	COMP	Cartilage oligomeric matrix protein	secreted
TCCAGGAAAC	31	109	0	65	4	Hs.11590	CTSF	Cathepsin F	cytosolic/secreted
GCTGAACGCG	71	336	65	11	5	Hs.517106	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	
AGGCTCCTGG	18	200	15	29	11	Hs.483444	CXCL14	Chemokine (C-X-C motif) ligand 14	secreted
GAATTCACAA	20	113	0	67	6	Hs.482562	F2R	Coagulation factor II (thrombin) receptor	cell membrane
GGGCCAACCC	14	87	0	52	6	Hs.501309	CIRBP	Cold inducible RNA binding protein	
CTGTCAGCGT	213	69	0	41	-3	Hs.405614	CTHRC1	Collagen triple helix repeat containing 1	secreted
TTTGGTTTTC	760	252	0	150	-3	Hs.489142	COL1A2	Collagen, type I, alpha 2	secreted
GGTGAGCCGG	10	69	0	41	7	Hs.420269	COL6A2	Collagen, type VI, alpha 2	secreted
CAGATAAGTT	23	207	0	123	9	Hs.409662	COL14A1	Collagen, type XIV, alpha 1	secreted
TTGGGATGGG	13	116	0	69	9	Hs.154224	CFHL1	Complement factor H-related 1 pseudogene	secreted
ACCGGCGCCC	2	77	0	46	39	Hs.476092	TNA	C-type lectin domain family 3, member B	secreted
AGTGTCTGTG	120	32	632	-10	-4	Hs.8867	CYR61	Cysteine-rich, angiogenic inducer, 61	secreted
GCGTCGGTGC	9	89	0	53	10	Hs.155597	DF	D component of complement (adipsin)	secreted
GAGGAGGAGA	8	64	0	38	8	Hs.127092	DHX38	DEAH (Asp-Glu-Ala-His) box polypeptide 38	
ACTTATTATG	448	121	7	35	-4	Hs.156316	DCN	Decorin	secreted
GGCAGGCGGG	27	138	29	10	5	Hs.440332	ERF	Ets2 repressor factor	
GGGTAGGGGG	80	458	15	65	6	Hs.75678	FOSB	FBJ murine osteosarcoma viral oncogene homolog B	
CCGTGACTCT	55	187	58	7	3	Hs.269512	FSTL1	Follistatin-like 1	secreted
TCACCGGTCA	72	352	7	101	5	Hs.522373	GSN	Gelsolin	secreted
TAGCTGGAAC	5	59	0	35	12	Hs.508364	GPC5	Glypican 5	cell membrane
AACTCCCAGT	73	839	15	120	12	Hs.110571	GADD45B	Growth arrest and DNA-damage-inducible, beta	
TACTAGTCCT	90	7	218	-16	-13	Hs.525600	HSPCA	Heat shock 90kDa protein 1, alpha	
TGTAATCAAT	59	7	152	-11	-9	Hs.546261	HNRPA1	Heterogeneous nuclear ribonucleoprotein A1	
GGGCAGGCGT	89	320	44	15	4	Hs.501629	IER2	Immediate early response 2	
CTAACGGGGC	11	150	0	89	14	Hs.513022	ISLR	Immunoglobulin superfamily containing leucine-rich repeat	
GGGGGGGGGT	10	136	0	81	14	Hs.523414	IGF2	Insulin-like growth factor 2	secreted
GGCCCCTCAC	8	86	0	51	11	Hs.274313	IGFBP6	Insulin-like growth factor binding protein 6	secreted
CATATCATTA	499	160	0	95	-3	Hs.479808	IGFBP7	Insulin-like growth factor binding protein 7	secreted
GGAAAAATTC	21	82	7	24	4	Hs.429052	ITGB1	Integrin, beta 1	cell membrane
GGCACCTCAG	16	124	7	36	8	Hs.512234	IL6	Interleukin 6	secreted
ACCCCCCCGC	119	1112	22	106	9	Hs.2780	JUND	Jun D proto-oncogene	
GGAGGGGGCT	37	128	44	6	3	Hs.491359	LMNA	Lamin A/C	
TTATGTTTAA	655	77	7	22	-8	Hs.406475	LUM	Lumican	secreted
AGAACCTTCC	49	190	44	9	4	Hs.181244	HLA-A	Major histocompatibility complex, class I, A	cell membrane
GGAAATGTCA	227	562	87	13	2	Hs.513617	MMP2	Matrix metalloproteinase 2	secreted
AGGGAGCAGA	28	172	0	102	6	Hs.296049	MFAP4	Microfibrillar-associated protein 4	secreted
GACGGCTGCA	21	93	7	26	4	Hs.380906	MYADM	Myeloid-associated differentiation marker	
GGTGCCCAGT	14	84	7	24	6	Hs.519909	MARCKS	Myristoylated alanine-rich protein kinase C substrate	
GAAAAATTTA	33	136	22	13	4	Hs.448588	NGFRAP1	Nerve growth factor receptor (TNFRSF16) associated protein 1	
TAGTTGGAAA	23	338	44	16	15	Hs.524430	NR4A1	Nuclear receptor subfamily 4, group A, member 1	
TGCGCTCTCC	22	91	0	54	4	Hs.25391	PI16	Protease inhibitor 16	secreted
GAGATCCGCA	15	76	0	45	5	Hs.75348	PSME1	Proteasome activator subunit 1	
TTCATTATAA	157	5	356	-34	-31	Hs.459927	PTMA	Prothymosin, alpha	
CACACAGTTT	51	192	29	14	4	Hs.502876	RHOB	Ras homolog gene family, member B	
GACACGAACA	19	89	7	26	5	Hs.25829	RASD1	RAS, dexamethasone-induced 1	
ATTTCTTCAA	307	81	0	48	-4	Hs.481022	SFRP2	Secreted frizzled-related protein 2	secreted
TCAACTTCTG	20	133	0	79	7	Hs.419240	SLC2A3	Solute carrier family 2, member 3	cell membrane
AGCCCTACAA	128	498	312	3	4	Hs.469331	STARD7	START domain containing 7	
GCTTGCAAAA	30	219	109	4	7	Hs.487046	SOD2	Superoxide dismutase 2, mitochondrial	
CCTCCCAGCT	28	141	7	40	5	Hs.527973	SOCS3	Suppressor of cytokine signaling 3	
GTCAAAATTT	48	150	0	89	3	Hs.371147	THBS2	Thrombospondin 2	secreted
GGGGAAATCG	272	681	160	9	3	Hs.446574	TMSB10	Thymosin, beta 10	
TACATAATTA	39	3	218	-31	-12	Hs.523789	TncRNA	Trophoblast-derived noncoding RNA	
TCTCTACTAA	79	13	225	-8	-6	Hs.466088	TPM4	Tropomyosin 4	
TGGAAAGTGA	351	1176	65	37	3	Hs.25647	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	
ATGGTGGGGG	143	427	240	4	3	Hs.534052	ZFP36	Zinc finger protein 36, C3H type, homolog	
CTGCCAAGTT	51	187	65	6	4	Hs.490415	ZYX	Zyxin	cell membrane

A



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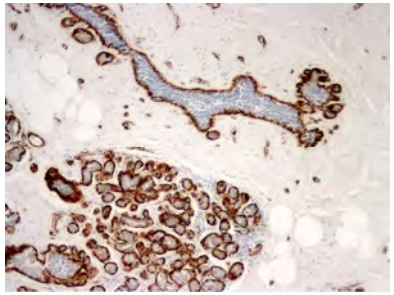

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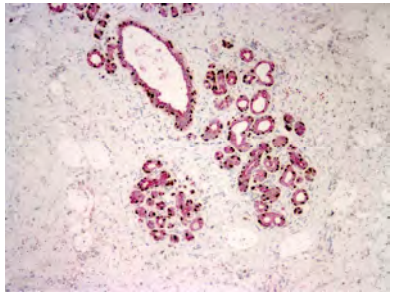
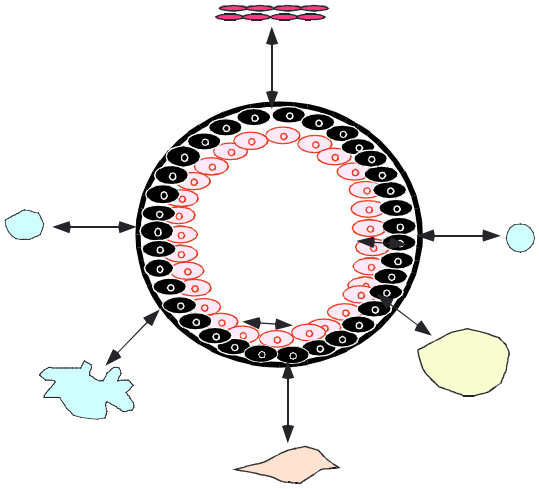


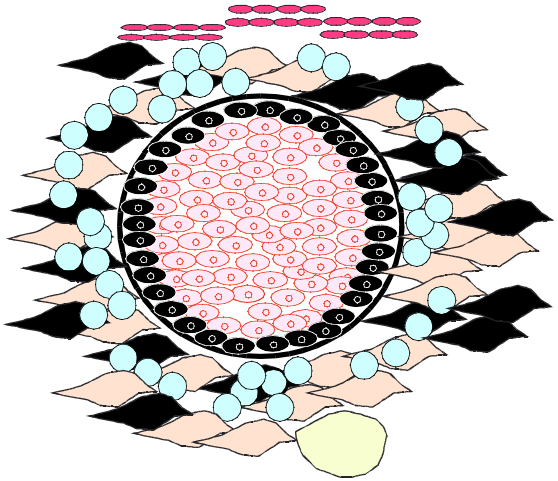
p63+CTK

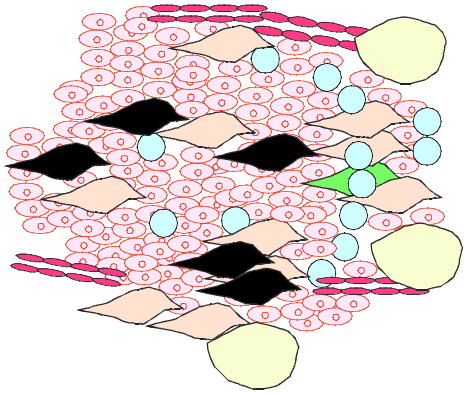
NORMAL

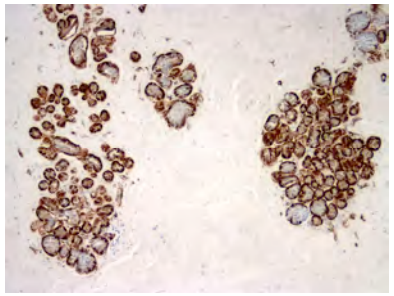
IN SITU CARCINOMA

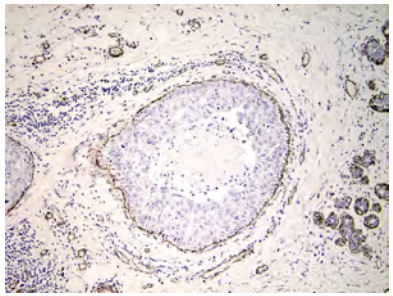
INVASIVE CARCINOMA

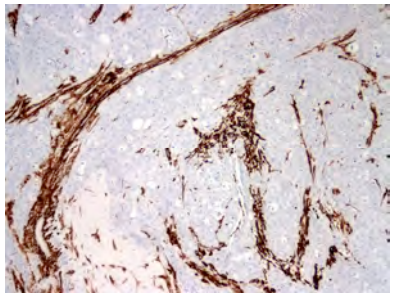












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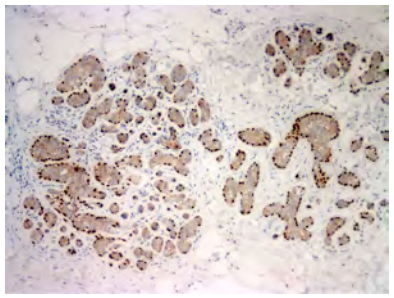

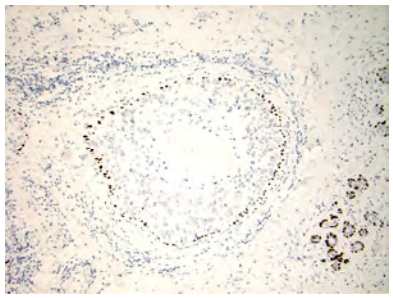

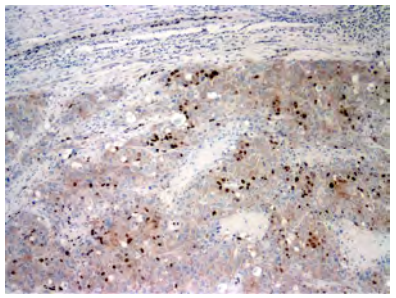

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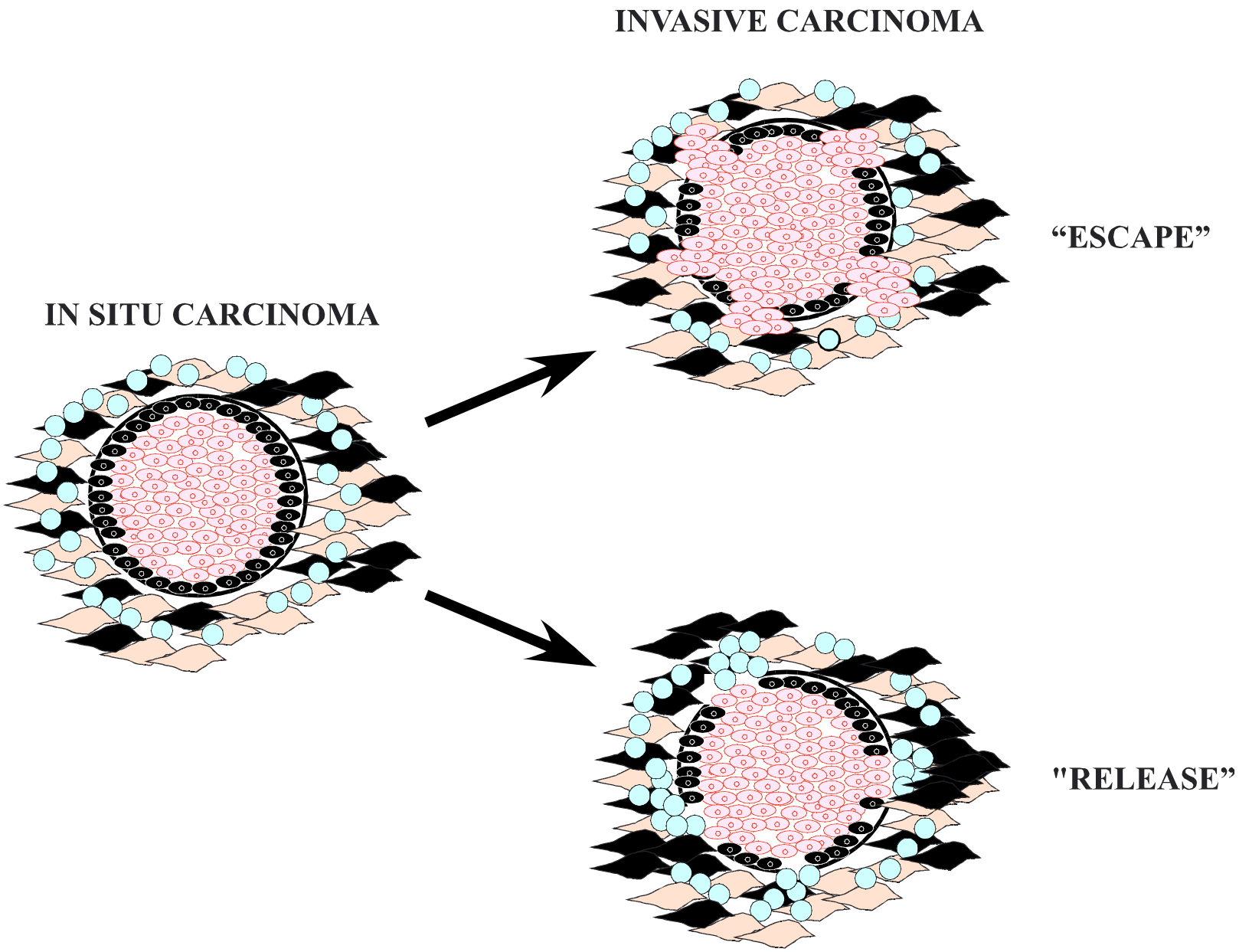
INVASIVE CARCINOMA
"ESCAPE"
IN SITU CARCINOMA
"RELEASE"