Review Article

Gene Expression Analysis of *In Vitro* Cocultures to Study Interactions between Breast Epithelium and Stroma

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The interactions between breast epithelium and stroma are fundamental to normal tissue homeostasis and for tumor initiation and progression. Gene expression studies of *in vitro* coculture models demonstrate that *in vitro* models have relevance for tumor progression *in vivo*. For example, stromal gene expression has been shown to vary in association with tumor subtype *in vivo*, and analogous *in vitro* cocultures recapitulate subtype-specific biological interactions. Cocultures can be used to study cancer cell interactions with specific stromal components (e.g., immune cells, fibroblasts, endothelium) and different representative cell lines (e.g., cancer-associated versus normal-associated fibroblasts versus established, immortalized fibroblasts) can help elucidate the role of stromal variation in tumor phenotypes. Gene expression data can also be combined with cell-based assays to identify cellular phenotypes associated with gene expression changes. Coculture systems are manipulable systems that can yield important insights about cell-cell interactions and the cellular phenotypes that occur as tumor and stroma co-evolve.

1. The Tumor Microenvironment: The Value of Studying Heterotypic Interactions in Cancer Biology

While mutations in oncogenes and tumor suppressors cause neoplastic epithelial cells to lose many of their growth constraints, neoplastic cells do not lose their interactions with the surrounding nonmalignant cells or with the extracellular architecture [1]. Instead, the interactions with cells in the microenvironment change during cancer progression and can promote or repress the tumorigenic process [2, 3]. Growth factors, cytokines, and proteolytic enzymes are upregulated and secreted [4, 5], giving a histological appearance of granulation tissue similar to tissue morphology during physiological wound-healing processes. The observation of histological changes in tumor adjacent tissue led Dvorak to propose that tumors are "wounds that do not heal" [6]. More recent experimental and observational studies have expanded on these observations to further suggest that an activated stroma may be dominant in cancer progression.

Some key evidence for the dominance of stroma comes from work identifying windows of susceptibility for breast cancer initiation and progression (e.g., during pregnancy and postlactational involution). Extracellular matrix (ECM) function and composition are remodeled during pregnancy and lactation [7], and these changes along with other changes in tissue cellular composition appear to contribute to increased breast cancer progression [8]. Conversely, progression can be reversed by stromal changes. Tamoxifen, a drug that primarily targets ER-positive epithelium, induces changes in mammary stroma leading to suppression of transformed phenotypes [9], and premalignant breast cancer cells placed on a reconstituted physiological basement membrane undergo cell growth arrest and form polarized alveolar

structures as normal epithelial cells would [10]. These observations illustrate the important role of stromal response in breast cancer.

In recent years, tissue-level wound and stromal responses have been more thoroughly characterized using molecular data [11, 12]. A growing body of gene microarray data support a role for stromal gene expression in breast cancer progression (Table 1). Finak et al. analyzed biopsies of cancer tissue and nonaffected tissue from breast cancer patients. By laser capture microdissection, they separated the tumor compartment from the stromal compartment and performed microarrays to identify a prognostic gene set from tumor stroma that predicted patient survival [13]. Ma et al. compared gene expression of ductal carcinoma *in situ* (DCIS)-associated stroma to stroma from individuals with invasive disease and showed that the majority of stromal alterations occur at the DCIS stage [14]. These authors argued that invasiveness is dependent on the signals the epithelial cells receive from myoepithelial cells, fibroblasts, and myofibroblasts. Allinen et al. isolated pure stromal cell populations from reduction mammoplasties, DCIS, and invasive breast cancer patients. Analysis of gene expression of these purified cell populations revealed widespread molecular changes in all cell types of the breast cancer stroma [15]. We and others have shown an activated wound response in the tumor microenvironment of breast cancer [11, 16].

Signatures of wound response from *in vitro* [16] or *in vivo* [11, 12] predict breast cancer survival and relapse in independent datasets. Finally, Beck et al. have studied both macrophage infiltration-associated gene expression [17] and fibromatosis-associated gene expression [18] as predictors of outcome. These studies cumulatively suggest that tumor progression occurs due to the concerted action of a variety of stromal responses.

The stromal responses to a tumor can be collectively referred to as "the tumor microenvironment." It includes all the structures and cells that support the tumor: extracellular matrix, blood vasculature, inflammatory cells, adipocytes, myoepithelial cell,s and fibroblasts, all of which have been shown to contribute to cancer development [19]. However, it is important to distinguish two types of microenvironment based on location: intratumoral microenvironment and extratumoral microenvironment. Figure 1 shows a schematic depicting the wide variety of cells that make up intra and extratumoral microenvironments. The *intratumoral microenvironment* is what has classically been referred to as "microenvironment of the tumor" or "tumor stroma." It is physically located within the tumor mass or very directly adjacent [20]. The majority of *in vivo* studies of microenvironment have emphasized this intratumoral microenvironment as shown in Table 1. However, some studies have also examined the *extratumoral microenvironment*, which extends further around the perimeter of the tumor (from millimeters to centimeters, depending on the study) and includes all the histologically benign tissue that surrounds the tumor. This extratumoral tissue also provides support for and influences tumor progression, reflecting either a tissue level response to the tumor or the baseline biological behavior of the tissue in which the tumor developed [21]. Both intratumoral

and extratumoral microenvironments are related to the concept of "field cancerization," initially defined as changes to the epithelium which are found in histologically normal tissue near the site of tumorigenesis and that could account for local recurrences [22]. In recent years, the concept of field cancerization has been broadened to include stromal changes. A review of epithelial-specific field effects has been presented elsewhere [23], but, in the current review, we are focusing on intratumoral and extratumoral stromal changes.

Studies of intratumoral and extratumoral stroma in patient specimens have identified interesting biological associations, but it is difficult to evaluate the specific contributions of distinct cellular populations in these complex tissues. Wiseman and Werb [5] concluded a review article in 2002 with an important idea: "if our aim is to find cures for diseases that rely on epithelial and stromal crosstalk we must increase our understanding of how these different cell types communicate with each other." *In vitro* cell-cell communication studies can be integrated with studies from human tissue and with animal studies to better understand how heterotypic communication alters disease. The purpose of this review is to summarize some earlier work on cancer-stromal cocultures, focusing on human breast cancer and especially on studies that evaluated gene expression using whole genome approaches. We will then briefly review mouse gene expression studies focused on the microenvironment and discuss how coculture data and mouse model systems can translate to insights on *in vivo* human microenvironments.

2. *In Vitro* Cocultures as Models to Study the Microenvironment

Monoculture studies of breast cancer cells have been the foundation for much of what we understand about molecular mechanisms and molecular signaling in cancer. Early studies showed that basal-like and luminal breast cancers had distinct responses to chemotherapeutics [24], and more recent studies have comprehensively profiled many established breast cancer cell lines to identify genomic models for each breast cancer subtype [25]. Pathway focused studies in monocultures are also common. For example, Hoadley et al. showed that basal-like breast cancer cell lines are more sensitive to the combination of carboplatin and cetuximab *in vitro* when compared to luminal cancer cell lines, and that EGFR-signatures have prognostic value when projected onto tumor datasets [26]. Other studies have identified p53-loss or p53-mutation associated signatures that can predict mutation status and survival *in vivo* [27, 28]. Studies of individual cell lines in monoculture have contributed to the development of new targeted therapies and are proving to have relevance *in vivo*. However, gene expression studies of monoculture experiments are not informative for microenvironment influences on progression. Coculture systems have become important in studying stromal factors.

Drastic changes occur when coculturing epithelium with different cell types. As a clear example, uterine epithelial cells proliferate in response to estrogen only when cocultured with stromal cells, but not when they are in a monoculture [30].

TABLE 1: Whole genome microarray studies to investigate breast cancer microenvironments in human tissues.

Authors [citation]	Type of specimen studied	Processing of specimen	Type of microenvironment	Major findings
Finak et. al. (2008) [13]	Fresh, frozen tissue from primary cancers (53) and adjacent nonaffected tissue (31) from breast cancer patients	Laser capture microdissection of tumor stroma	Intratumoral versus extratumoral	Stromal derived prognostic Predictor (SDPP), a gene set that stratifies patients by disease outcome. Genes are involved in immune response, angiogenesis, and hypoxic response.
Ma et al. (2009) [14]	Fresh frozen biopsies from disease-free tissue, DCIS, and invasive breast cancer (14).	Laser capture microdissection	Intratumoral	Tumor microenvironment participates in tumorigensis before tumor cells invade. Invasiveness is dependent on the signals from myoepithelial cells, fibroblasts, and myfibroblsts.
Allinen et al. (2004) [15]	Snap-frozen biopsies from reduction mammoplasties, DCIS, and invasive breast cancer.	Isolation of pure cell populations by differential centrifugation	Intratumoral	Widespread genome changes in all stromal cell types. Genetic alterations only occur in epithelial cancer cells.
Troester et al. (2009) [11]	Snap-frozen tissue from histologically normal tissue adjacent to breast cancer (47) and reduction mammoplasties (60).	Whole genome profiles of the tissue	Extratumoral	A wound response is activated in the tumor microenvironment. The wound response signature predicts cancer progression.
Chang et al. (2004) [12], Chang et al. (2005) [16]	Isolated fibroblasts from 10 different anatomical sites and tissue from early breast cancer patients (295)	*In vitro* response of the fibroblast populations to serum	Intratumoral normal tissue	Identification of an *in vitro* wound response, enriched in early stage tumors. High expression of this signature correlates with worse overall survival and increased distant metastasis.
Beck et al. (2008) [18]	Desmoid fibromatosis and solitary fibrous tumors.		Intratumoral	DTF core gene set (derived mainly from fibroblasts) is a robust descriptor of stromal response that is associated with improved clinical outcome in public genomic data from breast cancer patients.
Beck et al. (2009) [17]	Tenosynovial giant cell tumors and pigmented villonodular synovitis		Intratumoral	The CSF1 gene expression signature (derived mainly from macrophages) is present in more aggressive cancers.
Luciani et al. (2011) [29]	Tissue from primary breast tumors and reduction mammoplasties	Isolation of epithelial and fibroblast cells.	Intratumoral	A "fibroblast triggered gene expression" gene set generated by coculture of primary breast tumor cell lines and fibroblasts is enriched for inflammatory signaling, cell death, and cell proliferation genes. Predicts survival in independent datasets.

Other studies have demonstrated that breast cancer cell lines in the presence of benign mammary epithelial cells have a more transformed phenotype than when grown in monoculture [3]. Thus, coculture systems can be used to better model key biological behaviors of epithelial and tumor cells advancing the complexity of the system by increments, focusing on one or a small number of particular characteristics of the tissue (e.g., fibroblast-cancer cell interactions or mechanical characteristics).

Cocultures grown in 2-dimensions on plastic are by far the most common type of culture studied by gene expression analysis. For some characteristics, 3D cocultures may be preferable, as they allow cells to organize themselves in space and to mimic tissue structures *in vitro* [31, 32]. The gene expression profiles of 3D and 2D cultures of the same cell lines do show differences [33]. However, 2D cultures are easier to work with and can provide valuable genomic information. A large number of gene expression studies on 2D cocultures (Table 2) have been reported, demonstrating that these cocultures can generate important insights. They can also preserve important physical characteristics. For example, fibroblast-to-myofibroblast transdifferentiation can be

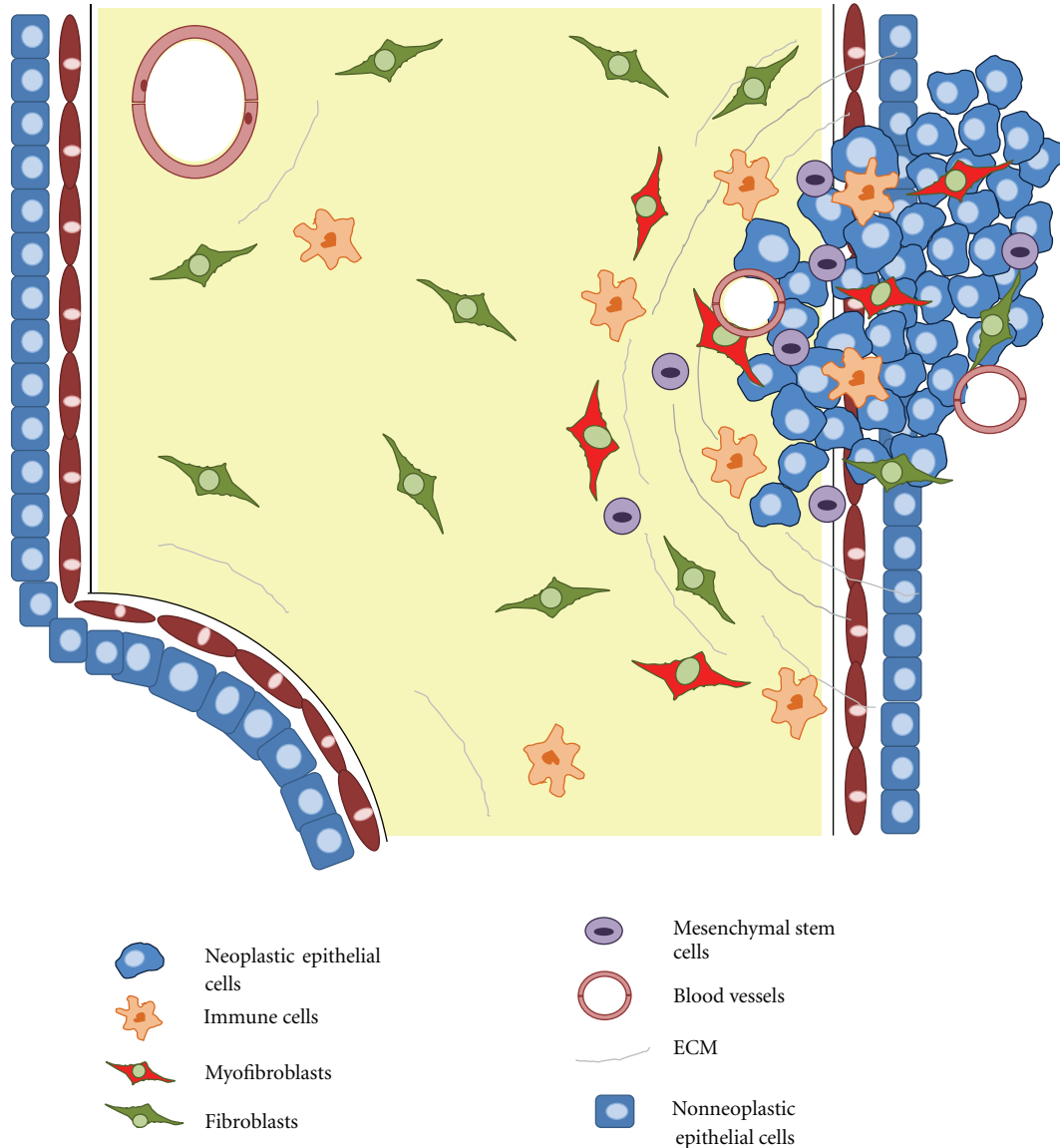| | Neoplastic epithelial cells | | Mesenchymal stem cells |
	Immune cells		Blood vessels
	Myofibroblasts		ECM
	Fibroblasts		Nonneoplastic epithelial cells

FIGURE 1: Intra- and extratumoral microenvironments and cellular components of these compartments. Cell types present in the extratumoral and intratumoral microenvironment are similar and include fibroblasts, immune cells, endothelial cells, and mesenchymal stem cells. Abundance and signaling of these cells vary widely between and within individuals with cancer.

more easily studied on plastic (2D) than in Matrigel due to physical properties of the culture surface [34]. In both 2D and 3D cultures, there are a number of variables that play a role in determining what phenotypes are observed, including the ratios of different cell types (using a convenient prespecified ratio such as 1 : 1 versus identifying multiple different biologically relevant ratios) [35], the number of cell types (e.g., choosing to coculture epithelial cells only with one stromal cell type or combining multiple cell types), mechanical factors (culture of cells with certain matrices or polymers to stimulate stiffness or other biophysical properties) [36], or the degree of cell contact (growing cells in direct physical contact or separating cell types on transwell cultures).

In addition to these variables that can be explicitly controlled, there are some experimental variables that are less easily manipulated but important to consider in designing a study. For example, every cell line is unique and shows individual characteristics. To make experiments generalizable, it may be necessary to use multiple cell lines (at least 3 or more) to establish reproducible trends for a given cell type (e.g., three cell lines or more may show consistency in luminal breast cancer behaviors, whereas one cell line alone cannot establish behavior of the class of luminal cell lines). Also, changes in the stromal cells over time should be considered. If using primary rather than established cell lines for stromal populations, it is important to consider that primary cells, such as fibroblasts, ultimately undergo senescence. Senescent fibroblasts create very different signaling milieus [43], so intraindividual variation in a given fibroblast line in culture (e.g., due to *in vitro* aging or passage number) should be

TABLE 2: Whole genome microarray studies to investigate breast cancer tumor microenvironment *in vitro*.

Authors (citation)	Cancer cell lines used	Stromal cell lines used	Type of coculture	Special separation techniques	Linked to human *in vivo* data	Major findings
Rozenchan et al. (2009) [37]	MCF10A, MDA-MB-231	Primary CAFs and NAFs	Transwell	No	No	Epithelial cell lines upregulate different pathways when cocultured with the two types of fibroblasts. MDA-MB-231-CAF cocultures (CAFs) upregulate β-catenin/TCF pathway genes; MDA-MB-231-NAF cocultures downregulate glycolipid and fatty acid biosynthesis. MCF10A-CAF cocultures upregulate stress response genes, while MCF10A-NAF cocultures downregulate growth control and adhesion genes.
Santos et al. (2011) [38]	MDA-MB-231, MDA-MB-435, MCF7	Primary fibroblasts from positive and negative LN	Transwell	No	No	Gene expression changes induced by coculture with fibroblasts from positive and negative nodes are distinct and intrinsic to each tumor subtype.
Camp et al. (2010) [35]	MCF7, T47D, ZR75, Sum102, Sum149, HCC1537	Immortalized reduction mammary fibroblasts	Direct physical contact and transwell	Yes	Computational deconvolution	The response to fibroblast coculture differs between basal-like and luminal cancer cell lines. The genes that distinguish basal-like versus luminal cultures also distinguishes human tumors. Basal-likes upregulate interleukins and chemokines (IL-6, IL-8, CXCL1, CXCL3, TGF-β) and TWIST and SOD1. Luminal cells increase stress response genes.
Buess et al. (2009) [39]	Hs578T, BT549, MDA-MB-436, MDA-MB-231, HMEC, SKBR-3, MCF7, T47D, HMECs	Stromal fibroblasts: human dermal fibroblasts, embryonic lung fibroblasts, and breasts fibroblasts	Direct physical contact & transwell	Yes	Computational deconvolution	Interaction between some breast cancer cells and stromal fibroblasts induced interferon response. The presence of this response is associated with higher risk of tumor progression.
Buess et al. (2009) [40]	HMECs, MCF7, T47D, MDA-MB-231, SKBR-3, Hs578T, BT549	HuVECs and human dermal microvascular endothelial cells	Direct physical contact & transwell	Yes	Computational deconvolution	Induction of an "M-phase cell cycle genes" in breast cancer cell lines but not in normal epithelium. Tumors with this gene signature have increased metastasis and worse overall survival. Endothelial cells induce proliferation in CD44+/CD24− cancer cells.
Liu et al. (2011) [41]	Sum159, Sum149, MCF7	Human bone marrow-derived mesenchymal cells	Direct physical contact and transwell	No	No	MSCs regulate cancer cell behavior through their effects on cancer stem cells. Networks of cytokines (IL-6, IL-8, CXCL1, CXCL5, and CXCL6 are associated with migration of cancer cells).
Wadlow et al. (2009) [42]	Many commercially available cancer cell lines	Many commercially available normal skin and lung fibroblasts	Direct physical contact	No	No	Cancer cell proliferation is modulated both by the cancer cell and the fibroblasts. Two functionally distinct pathways associated with altered proliferation were identified, one of which showed features of activated mesenchyme.

considered when interpreting results. If a primary cell line will be used, variation in patient characteristics should be considered (i.e., due to collection of cells from different patients with different tumor subtypes, ages, and genetic or environmental exposure history). It is known that cell lines can persistently harbor changes due to the exposure history of their donors [44]. Some studies have used hTERT immortalized cells to create a renewable source of isogenic cell lines for coculture studies [35, 45], and this has some advantages for reproducibility. On the other hand, variation may be of interest itself, such as variation that suggest differences between African-American and Caucasian fibroblast lines [46]. Aligning the strengths and weaknesses of a given model system with the research question is more important than perfectly recapitulating the complexities of the tissue.

2.1. Cellular Phenotypes of Epithelial Cells in Coculture: Changes in Gene Expression. Epithelial gene expression has been examined in relation to exposure to different cell types such as fibroblasts, immune cells, and even adipocytes (Table 2). Fibroblasts are abundant in the extratumoral and intratumoral microenvironment and play an essential role in the maintenance of normal tissue. The activation of fibroblasts to myofibroblasts creates a sustained fibrosis and wound-healing response leading to the desmoplastic reaction in advanced breast carcinomas [21, 47]. Fibroblasts also deposit the ECM necessary for cells to adhere, and their activation changes ECM and signaling to alter tumor initiation and progression [19].

Rozenchan et al. exposed MCF10A, a benign mammary epithelial cell line and the transformed MDA-MB-231 cell line to cancer-associated fibroblasts (CAFs) and normal tissue-associated fibroblasts (NAFs) from the same patient. Through this indirect coculture, they found many changes in the gene expression; MDA-MB-231 cells upregulated genes involved in the β-catenin/TCF pathway probably related to regulating cell polarity (DDX21 and DICER) while MCF10A cells induced stress response (S100A9, HSP90B1, and SPRR3), prosurvival genes when cultured with CAFs. Meanwhile, in culture with NAFs, MDA-MB-231 responded by downregulating genes associated with glycolipid and fatty acid biosynthesis (ACSL5 and AGTPAT4), potentially affecting membrane biogenesis, and MCF10A downregulated genes critical for growth control and adhesion (DDIT4, CTNND1, and PCDH1) [37]. The influence of the fibroblast on breast cancer cell gene expression has also been observed in cocultures comparing fibroblasts from negative and positive lymph nodes [38] and in comparing fibroblasts from different anatomical sites and patients [42]. Each of these studies showed that fibroblasts from different sites and patients had distinct effects on the cancer cells with which they were cultured in both cell based assays and gene expression. The fibroblasts from different anatomical sites (skin and lung) induce distinct proliferation effects on breast cancer cell lines. These effects can be used to segregate these cell lines on the basis of their tissue of origin [42]. The transcriptional changes induced in breast cancer cell lines when cocultured with fibroblasts from positive and negative lymph

nodes had some common features. However, the fibroblast responses were distinct for each breast cancer cell line, suggesting a stromal response intrinsic to breast cancer subtype [38]. Likewise, in a different model system, soluble interactions between basal and luminal cancer cells had distinct effects on fibroblast gene expression. When in a transwell coculture system, basal-like breast cancer cells induced the upregulation of genes such as IL-6, IL-8, CXCL3, TWIST, and SOD2 in fibroblasts, while luminal breast cancers did not [35]. These studies echo one another in demonstrating that both the fibroblasts and the cancer cells influence the character of the interaction.

The studies discussed above were conducted using transwells where cells were not in direct contact, but several gene expression studies have incorporated direct cell-cell contact with some additional technical or analytical steps. Direct cell-cell contact can create gene expression profiles that are distinct from those produced through soluble factors alone. However, methods for separating the cells may aid interpretation of the resulting gene expression profiles. For example, cells can be transfected with a GFP reporter and grown in coculture with fibroblasts. The GFP-producing cells can then be isolated using flow cytometry and subsequently analyzed [48]. Similarly, magnetic beads have been used to separate cells and demonstrate that tumor fibroblasts support neoplastic progression by altering the epigenome of mammary epithelial cells [49], specifically increasing hypermethylation of the CST6 gene. The authors of that study speculated that the direct cell-to-cell contact is involved in the epigenetic cascade that produces long-term silencing of this gene. Others have performed a variety of cell-sorting methods, ranging from the use of surface markers to labeling of cells with short-lived cell tracking dyes [35]. These cell sorting methods are proving to be an important tool for deconvoluting cocultures.

Cocultures can also be deconvoluted using computational methods rather than physical cell sorting. Buess et al. described a deconvolution method that computationally controls for cellular composition of cocultures. Using this approach, it was demonstrated that the interaction between some breast cancer cells and stromal fibroblasts induces an interferon-response signature which is correlated with survival [39]. Using the same deconvolution method, Camp et al. [35] have recently showen that luminal and basal-like breast cancer cells respond differently to the coculture with fibroblasts, but both show substantially altered expression relative to the monocultures. Furthermore, the direct coculture of basal-like breast cancer cells and fibroblasts induced the expression of interleukins and chemokines such as IL-6, IL-8, CXCL3, TGF-β also TWIST, and SOD1, while luminal breast cancer cell line cocultures with fibroblasts upregulated genes involved in stress response such as the S100AB, S100A9 genes as well as certain transcription factors (FOXP1 and FOXA2). These cocultures studies raised the hypothesis that heterotypic interactions are intrinsic to breast cancer subtypes, and better understanding of cell-cell interactions will yield important insights relative to treating and clinical course of these cancer subtypes.

Other stromal cell types (beyond fibroblasts) have been less well studied but certainly play a critical role in tumor microenvironment. The most widely studied are endothelial, inflammatory, and mesenchymal stem cells. For example, Buess et al. have documented that endothelial cells cocultured with epithelial cells induce M-phase genes in the CD44+/CD24− epithelial cell population. This "M-phase cell cycle gene set" consists of 70 genes such as HMGN2, CDC2, CDKN3, DICER, and so forth and can predict metastasis *in vivo*. But perhaps more importantly, endothelial cocultures mirrored results with fibroblasts; gene expression studies showed complex patterns reflecting substantial variation in the abilities of normal and malignant cells to send and respond to extrinsic signals [40].

Macrophages have been evaluated for their role in tumor progression using coculture models. For example, Hagemann et al. showed that coculture with macrophages increased tumor cell invasiveness through TNF-α dependant upregulation of matrix metalloproteinases (MMP-2,-3,-7,-9) [50]. Hou et al. have recently demonstrated that macrophages induce COX-2 expression in breast cancer cells through IL-1β signaling [51]. These observations gain greater importance when they are designed to confirm *in vivo*, biology, such as work following on recent studies [52] showing that tumor-associated macrophages may enhance metastasis through the activation of epidermal growth factor receptor signaling in neoplastic mammary epithelial cells. Continued work in cocultures with macrophages can elucidate whether these macrophage-cancer cell associations are subtype specific, as many of the markers induced in cancer cells (e.g., EGFR and COX2) are strongly associated with breast cancer subtype.

A common theme across stromal cell-breast cancer cocultures has been an increase in cytokines and inflammatory cells. These results have been observed for a variety of mesenchymal cell types; therefore, it may not be surprising that similar responses have been observed in cocultures with mesenchymal stem cells (MSCs). MSCs are important players in the tumor microenvironment [1], as they migrate and engraft into the primary tumor site. This was compellingly demonstrated in a humanized mouse model; tibial injections of human MSCs induced increased proliferation and progression of tumor xenografts [41]. These results also demonstrate that species differences are important because the mouse mesenchymal cells in the control animals (no tibial injection) were not capable of promoting progression as strongly. Complementary *in vitro* cocultures used in this study clearly demonstrated a role of CXCL7 and IL-6 signaling in the aggressive, invasive phenotypes induced by MSCs. Other recent results also have supported the role of MSCs in promoting a more aggressive phenotype, showing that, after direct coculture of MDA-MB-231, T47D, and SK-Br3 with MSCs, the cancer cells upregulate genes such as SNAIL, TWIST, vimentin, N-cadherin, and so forth, [53]. Similar observations were detected in transwell assays with SUM149 and HMEC cells [54], suggesting that many of these signals may be communicated via soluble factors, potentially including those factors identified by Liu et al. [41].

An emerging area that will require additional investigation is how microRNAs modify the stromal-epithelial gene expression patterns. In a recent study, neoplastic epithelial cells have been directly cocultured with bone marrow stromal cells, and microRNAs were shown to be transported via gap junctions between cancer cells and MSCs. These microRNAs led to reduced CXCL12 expression and a decreased proliferation [55]. Thus, future studies of gene expression changes in cocultured cells may find that microRNAs play an important role in controlling some of the observed gene expression profiles. The direct cell-cell transport of a critical mRNA regulators suggests that the complexity of cell-cell interactions far exceeds what we have begun to understand. However, a growing database of gene expression data from coculture studies will help to advance our understanding of the unique cell-cell interactions that influence cancer progression.

While *in vitro* cocultures allow controlled investigation of signaling pathways and help to reconstruct step by step the complexity of cancer biology, human tumors *in vivo* are the ultimate system of interest. Thus, most studies have tested their signatures in coculture by linking the gene expression patterns with published microarray findings in patients. For example, the aforementioned "M-phase cell cycle gene set," obtained through the coculture of endothelial cells with the stem cell portion of cancer cell lines, was projected onto tumor data to demonstrate that this gene set can predict metastasis *in vivo* and patient survival [40]. Luciani et al. also used their *in vitro* signature of seven independent primary tumor cell line cocultures with primary fibroblasts to define two groups of patients with distinct overall survival rates [29]. Other approaches include showing that coculture-derived signatures recapitulate established gene expression classes. For example, a subtype-specific fibroblast-coculture signature predicts breast cancer subtype in tumors, demonstrating that the *in vitro* signature is relevant *in vivo* [35]. These *in vivo* comparisons can also be combined with experimental data that demonstrate function, either in cell-based assays *in vitro* or in mouse models.

A next step with important *in vivo* implications is extension of cocultures to study responsiveness to cytotoxic chemotherapeutics. If signatures associated with increased toxicity were able to predict pathologic response *in vivo*, cocultures could help identify pathways that are promising as biomarkers or targets in neoadjuvant therapies. Given the wealth of public data from tumors and their adjacent microenvironment that has accrued in recent decades, it is becoming increasingly possible to test the relevance of *in vitro* gene expression results in patient populations at little additional cost. These types of analyses could help define candidate pathways involved in interactions to those that are most likely to play an important role in disease progression.

2.2. Confirming Changes in Cellular Phenotypes: Using Cell-Based Assays to Corroborate Gene Expression Data with Cocultures. Because much of the research on cocultures has focused on how the stroma modulates invasive potential, cell-based assays demonstrating changes in migration and anchorage-independent growth can help to establish biologic plausibility. Epithelial cells, in normal physiological conditions, are immobile, attached to a basement membrane, and

bound to neighboring cells through several types of cell-junctions. They present apical versus basal polarity- and these characteristics are essential for them to carry out their function *in vivo* [56]. One of the most drastic and visible changes that epithelial cells can acquire during carcinogenesis is the capacity to migrate, a hallmark of cancer [47]. Thus, migration assays *in vitro* can help assess how gene expression changes alter the capacity to migrate and invade. Common migration assays are transwell/Boyden chamber assays and scratch/wound-healing assay. The *Boyden chamber assay* allows for paracrine and autocrine communication because it uses a transwell coculture system in which the cells share the same medium but cannot physically interact. The chemotactic cells are placed on the bottom well, and the migrating cells are placed on the top insert. This insert has pores big enough (usually $8.0\,\mu$m) to allow cells to migrate through. The transwell migration assays may or may not include an extracellular matrix (ECM) layer. If this ECM layer is present, the assay models the capacity of cells to break down ECM and invade, whereas in the absence of ECM, the migratory capacity alone is investigated. In either case, the cells migrating to the opposite side of the transwell insert are fixed, stained, and counted. The *wound/scratch assay* allows for paracrine, autocrine, and cell-to-cell communication. Cells are seeded on the same surface in direct contact, a scratch is made when cells are nearly confluent, and cells migrating into the scratch are measured overtime. By labeling one cell type with a fluorescent label, it is possible to identify which of the two cell types are closing the wound.

Many of the stromal cocultures discussed above have been evaluated for their effects on migration of cancer cells. For example, focusing on fibroblasts, Potter et al. showed that tumor stromal cells (compared to normal stromal cells) caused greater chemotaxis of MDA-MB-231 and that this effect could be blocked by the addition of a monoclonal antibody to CCL2 [57]. MCF7s also become more migratory when cocultured with fibroblasts [58]. Fibroblast populations isolated from different distances relative to a breast tumor had distinct effects on the migratory capacity of MCF7 cells in scratch assays [59]. Similar findings have been observed for MSCs [60] and macrophages [61]. Breast adipocytes are abundant, comprising a major percentage of the extratumoral microenvironment, and have also been cocultured with breast cancer cells. Adipocytes are challenging to culture and coculture because they terminally differentiate and cannot be propagated to achieve a reproducible culture system; however, they are proving to have important implications for cancer progression. Dirat et al. showed that the estrogen-receptor positive breast cancer cell line ZR75.1, and the estrogen-receptor negative line SUM159PT, both increased their invasive capacity after 72 hours in coculture with mature primary adipocytes [62].

As an epithelial cell becomes more aggressive, it becomes less dependent on ECM and basement membrane interactions for survival. After coculture with certain types of cells, benign or malignant epithelial cells can acquire or enhance their anchorage-independent growth properties. There are two main types of assays to address anchorage independent growth, mammosphere, and soft-agar colony formation

assays. In both cases, coculture studies can be designed to evaluate paracrine, autocrine, and/or cell-to-cell contacts. In the *mammosphere formation assay,* cells are cultured in suspension in a defined growth media [62]. Colonies are allowed to grow for 7–10 days and then analyzed. Only cells with anchorage-independent growth capacity will grow, so the number and size of colonies reflect acquisition of this phenotype. In *soft-agar colony formation assays,* cells are grown in a gel-like matrix that provides more structure than a suspension culture, but the same phenotypes (colony number and size) are assessed after a period of growth, typically at least two weeks. These assays have been used to confirm anchorage-independent growth changes in coculture. For example, breast cancer fibroblasts decreased time required for MCF7s to form mammospheres and increased the overall number of spheres relative to cocultures with normal fibroblasts. Additionally, when MDA-MB-468, a basal-like breast cancer cell line, was cocultured with CAFs, the number of soft-agar colonies were higher than when cocultured with NAFs [63]. MSCs have also been shown to induce mammosphere formation in human mammary epithelial cells (HMECs), and SUM149 but not in primary inflammatory breast cancer cells (MDA-IBC-3). These effects occurred though paracrine factors, as conditioned media from the MSCs had the same effects [54].

By combining the expression data suggesting a certain phenotypic trait and with cell-based assays, new treatment-relevant advances are possible. Genome expression data along with cell-based assays can be used to test targeted perturbations (e.g., blocking cytokines or treating with growth factors such as in [41]) to study how these phenotypes are regulated. Given that these studies can be done with human cells, and with careful control of cell ratio, cell physical environment, polarity, and so forth, these systems can provide interesting and important insights about how cancers become more invasive and aggressive through interactions with their environments.

3. Mouse Models for Comparative Biology of Tumor Microenvironment

Given identification of novel hypotheses from *in vitro* cocultures and confirmation of the cellular phenotypes *in vitro*, a complete picture of stromal-epithelial interactions requires linkages with studies *in vivo*. As described above, public genomic data can be useful for this purpose, but mouse models have contributed to our fundamental understanding of the reciprocal signaling between stroma and epithelial compartments. Noël et al. performed the first inoculation of cocultured fibroblasts and breast cancer cell lines with matrigel in an athymic mice model. The inoculation of these cocultures decreased the latency time and enhanced tumor growth. Both tumor growth and latency time were dependent on the number of inoculated fibroblasts in the coculture [64]. In another classic example, it was demonstrated that when nontumorigenic cell lines are introduced into irradiated cleared fat pads, they form tumors. Conversely, when introduced into cleared fat pads that have not been irradiated

the same tumorigenic cell lines do not form tumors. This indicates that radiation induces changes in the stromal microenvironment that contribute to neoplastic progression *in vivo* [65]. More recently, Hu et al. have shown that myoepithelial cells suppress, while fibroblasts enhance, tumor progression from DCIS to invasive cancer in a mouse xenograf model [66]. Novel models for combining and humanizing the microenvironment have also been proposed, including a humanized mouse xenograft model into cleared fat pads [67], and intraductal xenografts, where human cell lines can be injected alone or with stromal components [68]. An advantage of these models is that some of the innate immune responses are preserved, as is the systemic circulation and the three-dimensional structure of the tissue.

Recently, it has also been established that different mouse models can be used to represent the heterogeneity of human breast cancers [69]. For example, the C3Tag mice overexpress the SV40Tag transgene in distal mammary ductal epithelium and terminal ductal lobular units. This overexpression allows for a targeted inactivation of two tumor suppressor genes: p53 and Rb, giving rise to a very predictable onset of tumors [70]. They most commonly develop tumors with features of basal-like breast cancer. Thus, these models may be useful for studying basal-like microenvironments. Future studies should examine how microenvironment characteristics, such as obesity or immune cell ablation, influence the progression of tumors in some of these model systems, to gain a perspective on the role of microenvironment in different breast cancer subtypes. These models, when combined with coculture-based mechanistic studies, can be a powerful combination.

4. Limitations and Future Directions of the Coculture Models of Gene Expression

In vitro cocultures have led to significant advances in our understanding of heterotypic interactions among different cell types, complementing mouse studies and human *in vivo* studies. They provide an easy and controllable technique to study heterotypic interactions among different cell types. Because stromal interactions have been proven to be key in the carcinogenesis process, cocultures are important tools. However, making inferences about the relevance of coculture findings to human patients *in vivo* requires some assumptions. An important limitation of cocultures is that the unit of study is often a single pathway or a small number of pathways in isolation, and typically limited to cancer cells and one other stromal cell type. Each coculture system recapitulates some aspect of the whole tissue and the interactions that are occurring *in vivo*, but given the complexity of the whole tissue, cocultures cannot fully recapitulate all dynamics and dimensions of the tissue. Assays and bioinformatics methods are available for studying interactions between two or three cell types, but incorporating more cell types has not yet been accomplished. In any case, models with increasing complexity will be needed to advance our understanding of heterotypic interactions in breast cancer. Studies focusing on ECM in heterotypic contexts [71], the role of mechanical forces and the overall 3D architecture

of the tissue [72] will add new biological insights. Some of these factors can be addressed one by one in monoculture and coculture experiments, but it may be that the whole tissue is more than the sum of its parts. Consider for example, a model that incorporates a variety of cell types but does not account properly for biophysical characteristics of the tissue. Biomechanical features are of established importance in cancer progression, and nanotechnology tools for dynamically altering physical environments may help address this [5, 71]. However, our ability to design cocultures depend on the knowledge we have about which factors matter. Limitations of the cocultures, and areas where they fail to explain *in vivo* phenomena will also potentially be informative.

There is still much to be learned about tumor-stroma interactions and cocultures will play an essential role in further understanding key processes in carcinogenesis. Future studies using these coculture system should address several issues, including how the microenvironment of a tumor responds to hypoxic conditions and how this affects disease progression. It is established that hypoxia occurs during tumor progression but the stromal-epithelial interactions affected by these conditions are unknown. These cocultures also afford the possibility of investigating controversial hypotheses, such as the Warburg effect, which is not easily studied in solid tumors. As metabolism has gained recognition as an important driver of cancer progression [47], novel methods for studying metabolic microenvironments are needed. Other studies should explore how cells of different origins, epithelial versus mesenchymal, affect tumor initiation and progression. While a variety of mesenchymal cells appear to induce similar responses in cancer cells, it remains unknown whether similar responses can also be induced by dedifferentiating epithelial cells. For example, the concept of epithelial-to mesenchymal transition (EMT) is gaining strength in the cancer field, and if epithelial cells take on more mesenchymal phenotypes, the influences on cancer progression will be important to study. These transient phenotypes are challenging or impossible to study *in vivo* but could be more readily manipulated in coculture systems. Finally, while inflammatory responses and cytokine milieus emerge as important biological determinants of basal-like versus luminal cancer microenvironments, it will be interesting to investigate the effect of different inflammatory environments on epithelial cells in the context of a coculture. Cancer cell responses to inflammatory signals have been studied in monoculture, but adding other cells to the culture system will improve the *in vivo* relevance of these findings.

5. Conclusions

The tissue stroma is crucial for normal organ homeostasis as well as for tumor initiation and progression. Additionally, both intra- and extratumoral microenvironments play essential roles in tumor biology. Thus, improved understanding of the interactions that take place between epithelial cells and stromal compartments is critical to advancing our knowledge of human cancer. *In vitro* coculture systems are controllable systems that can be used to study gene expression

changes and corresponding cellular phenotypes that occur as tumor and stroma co-evolve. These systems can be used to define critical factors mediating the communication between the cell types. Although cocultures have limitations, the growing body of gene expression coculture data demonstrates that these models are generating important insights in the biology of breast cancer.

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