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Stroma in Breast Development and Disease

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Abstract

It is increasingly apparent that normal and malignant breast tissues require complex local and systemic stromal interactions for development and progression. During development, mammary cell fate specification and differentiation require highly regulated contextual signals derived from the stroma. Likewise, during breast carcinoma development, the tissue stroma can provide tumor suppressing and tumor-promoting environments that serve to regulate neoplastic growth of the epithelium. This review focuses on the role of the stroma as a mediator of normal mammary development, as well as a critical regulator of malignant conversion and progression in breast cancer. Recognition of the important role of the stroma during the progression of breast cancers leads to the possibility of new targets for treatment of the initial breast cancer lesion as well as prevention of recurrence.

Keywords

mammary development; breast cancer; tumorigenesis; stroma; cancer-associated fibroblasts

1. Introduction

The mammary gland is a complex tissue comprised of an epithelial parenchyma embedded in an array of stromal cells that regulate its proliferation, differentiation and survival. The mammary gland undergoes dynamic changes over the lifetime of a woman, from the expanded development at puberty, to hormonally-controlled proliferation and apoptosis during the menstrual cycle, to full lobuloalveolar development for lactation. Pioneering mouse mammary epithelial cell transplant work by DeOme and colleagues demonstrated the regenerative plasticity of the mammary epithelium and the dependence on the stroma for its development [1,2]. Moreover, through similar epithelial transplant experiments, non-mammary cells were reprogrammed to perform mammary epithelial cell functions due, in part, to the contribution of paracrine interactions with the host mammary stroma [3,4].

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Breast cancers are also highly complex tissues with carcinoma cells constituting only one of many distinct cell types. Indeed, within many breast tumor masses, the cancer cells may represent only a small proportion (<20%) of the total cell number. The remaining cell types are often grouped together under the collective term of “tumor-associated stroma", which includes fibroblasts, myofibroblasts, macrophages, other immune cells, adipocytes and endothelial cells, among others. The role of this stroma in breast cancer pathogenesis has become an area of intense investigation due to the mounting evidence demonstrating its ability to promote tumorigenesis [5,6]. It has been repeatedly demonstrated that breast cancer development and progression is highly dependent on specialized stroma, as tumors rarely develop in the absence of this microenvironment [7,8].

This narrative focuses on reviewing the parallels between the role of stroma during normal mammary gland development with that of stroma during breast tumor development and progression. The critical function of the stroma during malignant transformation and progression, suggests that targeting it in conjunction with the carcinoma cells may be a synergistic strategy for therapeutic intervention.

2. Normal Mammary Development

2.1 Stromal influence on mammary fate

Mammary gland development in rodents occurs with the thickening of the ectoderm, forming an epidermal “mammary crest.” Between embryonic day 11 (E11)[1] and E12, mammary placodes develop, which give rise to the mammary nipple and the underlying ductal tree [9]. The placode is surrounded by a primary mesenchyme that is indistinguishable from the rest of the dermis, but by E14, the concentric layers of fibroblasts surrounding the placodes exhibit specialized differences in gene expression such as upregulation of steroid receptors and components of the extracellular matrix (ECM) [10,11]. As development proceeds, the placodes elongate and penetrate the secondary mesenchyme, a cluster of preadipocytes in the deeper dermis that will become the mammary fat pad.

During this developmental stage, the mesenchyme is the critical determinant of mammary fate. In elegant tissue recombination studies, non-mammalian chick and duck epidermis recombined with rabbit mammary mesenchyme was able to develop branched glandular tissue [12]. To explore the effect of the mesenchyme on functional mammary differentiation of non-mammary epithelium, dorsal skin epithelium from mouse embryos was combined with syngeneic mammary mesenchyme and grafted under the renal capsule of syngeneic hosts. When grown in hosts implanted with prolactin secreting pituitary isografts, the epithelial cells of the resulting ductal structures expressed the milk proteins casein and alpha-lactalbumin [13]. Similarly, when embryonic mammary epithelium was recombined with salivary mesenchyme and grafted under the renal capsule, the resulting outgrowths were morphologically similar to salivary glands. However, in response to hormonal stimulation, the grafted epithelium was capable of synthesizing milk proteins [14]. These studies suggest that epithelial cell contact with the mesenchyme determines the architecture of the epithelial outgrowth, however, regulation of its biosynthetic function is less clear.

While the primary fibroblastic mammary mesenchyme defines the cellular fate of the mammary gland, the secondary preadipocyte mesenchyme is critical for the characteristic shaping of

[1]Abbreviations used: E, embryonic day; ECM, extracellular matrix; FGF, fibroblast growth factor; PTHrP, parathyroid hormone related peptide; TEB, terminal end bud; ER, estrogen receptor; EGFR, epidermal growth factor receptor; GH, growth hormone; IGF, insulin-like growth factor; TGFβ, transforming growth factor beta; MMTV, mouse mammary tumor virus; αSMA, alpha smooth muscle actin; FAP, fibroblast activated protein; HIM, human-in-mouse; RMFs, reduction mammary fibroblasts; HGF, hepatocyte growth factor; DCIS, ductal carcinoma *in situ*; CAF, cancer-associated fibroblast; VEGF, vascular endothelial growth factor; PDGF, platelet derived growth factor; MSC, mesenchymal stem cell; EMT, epithelial-mesenchymal transition; MD, mammographic density.

ductal branching structures. Recombination of embryonic or adult mammary epithelial cells with the fibroblastic mesenchyme led to atypical ductal branching and hyperplasia, whereas grafting with preadipocytes led to normal ductal elongation [15], possibly due to differences in the composition of the basement membrane [16]. It is not clear if the preadipocytes play a similar role in human mammary development. While the mature murine mammary fat pad consists primarily of adipocytes, the developing mammary epithelium in humans remains encased in fibroblastic stroma, eventually resulting in the development of specialized interlobular and intralobular stroma in the mature tissue; further, it is thought that adipose rich tissue inhibits the growth of the human mammary epithelium [17].

Complex signaling through multiple families of ligands and their cognate receptors appear to function through temporally restricted and highly localized expression in the epidermis and mesenchyme to control development during the embryonic period. The most characterized of these families include Wnt, fibroblast growth factor (FGF), parathyroid hormone related peptide (PTHrP), and Hedgehog; their signaling patterns at specific times during embryonic development have been recently reviewed [9,18–20]. Gene knockout studies in mice have demonstrated non-redundant roles for specific genes. For example, failure to express FGF10 or its receptor FGFR2b during placode development results in the inability to form mammary buds 1, 2, 3, and 5, and maintain bud 4 [21]. Although expressed during similar points in embryonic development, FGF family members appear to act in parallel with the Wnt family, as inhibition of Wnt pathways do not alter expression of FGF10 or FGFR1 [11,22]. However, these families appear to influence each other indirectly through induced transcription factors [23], such as Tbx3 [22]. While these interactions are starting to be elucidated in the mouse, little is known about the roles these families play during development in the human gland.

2.2 Stroma and growth of the ductal tree

Unlike the embryonic phase of growth, full development and differentiation of the mouse mammary gland relies on coordinated communication between circulating hormones and localized growth factors. Terminal end buds (TEBs) form at the tips of the ducts and begin to grow allometrically into the mammary fat pad [24]. At puberty, elevated circulating estrogen acts through its receptors, estrogen receptor alpha (ERα) and beta (ERβ). Transplants of $ERα^{-/-}$ epithelium into wild type glands developed only a rudimentary ductal structure limited to the nipple region [25,26], demonstrating that this receptor is critical for estrogen-induced growth of the ductal tree. Early studies suggested that ERα expression in the stroma was critical during puberty for ductal elongation, and expression within both the epithelial cells and stroma were necessary for function in the adult [27]. However, these studies were confounded by incomplete removal of ERα activity, and further investigation with a complete functional knock out revealed that epithelial ERα expression was critical at both points [26]. Epithelial cells expressing ERα do not proliferate [28–30], suggesting a paracrine interaction for growth. Interestingly, $ERβ^{-/-}$ mice do not show any overt mammary abnormalities and lactate normally [31].

Although expression of ERα in the epithelium is critical for development, stromal ERα expression appears to have a role in modulating the expression of the growth hormone receptors and their ligands that are necessary for development. Through transplant studies, roles for stromal epidermal growth factor receptor (EGFR) and growth hormone (GH) receptor in ductal elongation have been uncovered. Although embryonic lethal, $EGFR^{-/-}$ females showed normal mammary ductal development before birth, however, transplant studies demonstrated impaired ductal outgrowth at puberty, which was dependent upon stromal EGFR expression [32,33]. Exogenous EGFR ligands can rescue ductal development in both ovariectomized [34] and $ERα^{-/-}$mice [35], and exogenous estradiol elicits EGFR activation in ovariectomized mice, demonstrating crosstalk between these pathways [32]. Although EGFR has multiple ligands,

during ductal elongation, paracrine interactions between amphiregulin expressed in the epithelium and EGFR in the stroma are essential for normal development [36]. Similarly, GH expression in the stroma is necessary for normal ductal elongation [37], mediated at least in part by upregulation of insulin-like growth factor I (IGF-I). Locally produced IGF-I is critical, suggested by the observation that mammary growth proceeds normally in mice with a liver-specific deletion of IGF-I that causes a 75% reduction in circulating IGF-I [38]. GH signaling induces both IGF-I and ERα expression in mammary fat pads cleared of endogenous epithelium, the induction of IGF-I by GH is enhanced by estradiol, and only GH treated glands express stromal ERα (for review, [39]). These observations support the idea that both the epithelium and stroma are critical for integrating the signaling effects of ovarian estrogen for ductal elongation.

Besides its proliferative effects during ductal elongation, estrogen may also exert control over this growth through localized activity of transforming growth factor beta (TGFβ). The TGFβ superfamily is a large family of secreted multifunctional peptides involved in regulating almost every aspect of cellular behavior [40,41]. The most characterized of this family is TGFβ1, which is expressed in both the epithelium and stroma [42,43]. Localized TGFβ, either under control of the mouse mammary tumor virus (MMTV) promoter or from mammary implants has demonstrated an integral role for TGFβ in inhibition of ductal elongation during puberty [44,45]. The main effect of TGFβ on mammary epithelium appears to be growth inhibitory [46,47] and is regulated by ovarian hormones [42,47]. In contrast, TGFβ increases proliferation in fibroblasts in culture [48], and estrogen significantly enhances TGFβ levels in dermal fibroblasts [49,50], suggesting estrogen may have opposing effects on proliferation in the epithelial and stromal compartment mediated through TGFβ. This differential effect on proliferation may define the specific patterns of ductal branching demonstrated during development in the mammary gland.

3. Malignant Breast Development

3.1 Differences between normal breast stroma and tumor associated stroma

It is well established that stroma associated with normal mammary gland development is strikingly different from that associated with carcinomas [8]. When compared to normal tissues, the stroma accompanying breast tumors contains an increased number of fibroblasts and immune cell infiltrates, enhanced capillary density, increased collagen I and fibrin deposition, all which collectively alter the structure and stiffness of the ECM and induce changes in signaling within the adjacent epithelium [8,51]. Compared to normal mammary gland stroma, tumor-associated stroma shows elevated expression of alpha smooth muscle actin (αSMA), collagen IV, prolyl-4-hydroxylase, fibroblast activated protein (FAP), tenascin, desmin, calponin, caldesmon and others [52–54].

Several reports have used transcriptome-wide analyses to report the changes in stromal gene expression associated with tumor development [55–58]. The genes prominently upregulated include components of the ECM and matrix metalloproteases responsible for stromal remodeling [59] as well as secreted and cell surface proteins [55]. In fact, based on SAGE and SNP analyses, the most dramatic and consistent modifications in gene expression occurred within the fibroblast and myoepithelial fractions sorted from primary human breast tumors [55]. Whether these stromal changes in gene expression are the result of genetic alterations remains controversial [60,61], however, it is generally accepted that epigenetic alterations are at least in part responsible [62].

3.2 The role of stromal activation in promoting tumor formation

Studies in mice have attempted to address the direct involvement of activated stromal cells in breast tumor formation. Irradiation of the mouse mammary stroma promotes an activated mesenchymal response with the release of active TGFβ, resulting in tumor formation after injection of COMMA-D cells, non-tumorigenic murine epithelial cells that harbor a mutation in p53 [5]. These results suggest that molecular or epigenetic activation of the stroma promotes tumor formation, but raises the question as to whether or not the tumor cells themselves must initially contain genetic alterations in order to be susceptible to activated stromal influences. To address this question using dissociated normal human mammary epithelial cells (organoids), Kuperwasser *et al.* established a humanized mouse model of normal and malignant breast growth (human-in-mouse, HIM model) [63,64]. In this model, ad-mixed irradiated and unirradiated immortalized mammary fibroblasts (RMFs) were introduced into cleared mouse mammary fat pads to create a fibroblast-enriched microenvironment that more closely mimics human breast tissue and allows for normal human mammary epithelial outgrowths. The irradiated fibroblasts enabled the unirradiated fibroblasts to survive and colonize the mammary gland by remodeling the ECM proteins of the adipose stroma [63,64]. To create a microenvironment that shares some of the features of tumor-associated stroma, RMFs overexpressing hepatocyte growth factor (HGF) or TGFβ, alone or together, were used to humanize cleared fat pads prior to the introduction of breast organoids [63]. Unlike non-immortalized, normal primary human mammary fibroblasts, which allowed for only normal outgrowths, the growth factor enriched RMFs allowed for the rare (1/10 patient samples) promotion of ductal carcinoma *in situ* (DCIS)-like lesions, adenomas and poorly differentiated tumors from ostensibly normal organoids [63].

To further explore the influence of stromal fibroblasts on the development of human breast cancer, the HIM model was recently combined with lentiviral gene transduction of human breast organoids and used for tissue reconstitution [65]. Tumors were efficiently generated from tissue recombinants when genetically modified organoids were co-mixed with immortalized fibroblasts with or without expression of HGF. However, tumor development was rarely observed when organoids were implanted either alone or co-mixed with normal primary fibroblasts further demonstrating that human breast cancer formation, even in the presence of oncogene-driving mutations, requires activated stoma [65]. These results further underscore the notion that even in the presence of robust oncogene signaling, activation of the stromal environment is an important component for malignant transformation of human breast epithelium *in vivo*.

The HIM model represents a unique *in vivo* platform to investigate how particular signaling molecules, such as those expressed by cancer-associated fibroblasts (CAFs) and other cell types that constitute the tumor-associated stroma contribute to tumor progression. Significantly, unlike many cell line based xenograft models of human breast cancer, human breast cancers generated by lentivirally-transformed organoids and single cell suspensions in the HIM model demonstrate a robust recruitment of several components of tumor-associated stroma seen in human patients, namely, angiogenic capillaries, αSMA-positive myofibroblasts, macrophages and other immune cells (Fig 1), indicating that this is a useful model to investigate the influence of the stromal microenvironment on tumor development and progression.

3.3 Tumor fibrosis and progression

Both tumors and wounds elicit stromal reactions that are characterized by ECM remodeling, growth factor secretion, cell migration, and angiogenesis. During normal wound healing, this stromal response is initiated by bone marrow-derived hematopoietic cells and is accompanied by a marked increase in vascular permeability, plasma extravasation, fibrin deposition, platelet activation and inflammatory cell infiltration, which together result in the release of numerous

of cytokines and growth factors [66]. This response leads to the generation of granulation tissue, which is characterized by angiogenesis, activation of fibroblasts into αSMA positive myofibroblasts, and matrix remodeling.

Myofibroblasts within the stroma of wounded tissues are distinguished from αSMA positive fibroblasts within the stroma of tumors (CAFs) based on the latter's co-evolvement with tumor cells and the ability to support tumor growth in mice [52,67]. However, both cell types express similar markers and their appearance within the stroma coincides with the disruption of basement membrane and features of fibrosis. Notably TGFβ is a major instigator of fibrotic reactions as it can promote the assembly of stress fibers and fibronectin-containing fibrils which generate the contractile forces characteristic of the myofibroblast [7]. However, whether TGFβ can promote the conversion of resident tissue fibroblasts into tumor-promoting CAFs rather than myofibroblasts remains unknown, given the lack of molecular distinctions between these cell types.

Likewise, platelet derived growth factor isoform BB (PDGF-BB) has also been shown to promote a desmoplastic and fibrotic response within tumors. Stable transfection of PDGF-B cDNA into human WM9 melanoma cells induced formation of vascularized tumors within nests of connective tissue septa compared to control cells which lack a stromal response [68]. Similarly, enforced expression of PDGF-B in immortalized, nontumorigenic human keratinocytes also enhanced mesenchymal cell proliferation, angiogenesis and epithelial cell proliferation *in vivo* [6]. Using 3D co-culture systems, it was shown that tumor cells are sufficient to induce a myofibroblast phenotype in cultured resident tissue fibroblasts; however only a fraction of the fibroblasts, those in closest contact with the tumor cells, responded in this fashion [54]. These and other studies collectively suggest that the CAFs can be generated by stromal-epithelial cell crosstalk [6], with PDGF and TGFβ as possible signals capable of inducing the CAF phenotype in breast tumors.

While it is clear that CAFs promote tumor growth [52,67], their origins remain largely unknown. CAFs and myofibroblasts can be derived from circulating fibrocytes, [69] cells that express hematopoietic stem cell markers as well as monocyte lineage and fibroblast markers. Fibrocytes are known to differentiate into myofibroblasts and have been identified within invasive ductal carcinomas and DCIS lesions of the breast [70,71]. In addition, bone marrow derived-mesenchymal stem cells (MSCs) have also been shown to differentiate into αSMA positive cells with CAF-like characteristics [72,73]. The transdifferentiation of a variety of cell types has also been proposed to be a source of CAFs. For example, the endothelial mesenchymal transition has been shown to produce myofibroblast-like cells upon exposure to TGFβ [74]. Tumors formed from endothelial cell-specific LacZ reporter mice contain LacZ-positive fibroblasts [74], suggesting that endothelial transdifferentiation can contribute to the CAF content of the microenvironment. The epithelial-mesenchymal transition (EMT) has long been regarded as a necessary step in the progression to invasive tumors. Interestingly, there is evidence to suggest that tumor cells undergoing an EMT may transdifferentiate into myofibroblasts. In a mouse model of pulmonary fibrosis, the fate of lung epithelial cells was tracked through labeling with β-galactosidase, demonstrating that vimentin-positive cells accumulating within the injured lung were of epithelial origin [75]. A mesenchymal-like cell line derived from a metaplastic human breast carcinoma retains genetic linkage to the epithelial tumor of origin, yet resembles the myofibroblast phenotype *in vivo* and promotes MCF7 breast cancer cell tumor growth in nude mice, similar to that promoted by CAFs [76].

4. Clinical Perspectives and Therapeutic Targeting

4.1 Stroma as a prognostic factor

An active area of research for breast cancer involves the identification of prognostic and predictive factors that will help to guide the best course treatment for both early-stage and established breast cancers. The stroma that surrounds pre-cancerous mammary tissue, DCIS lesions and established tumors provides a rich source of potential biomarkers and prognostic information.

Mammographic density (MD) refers to the relative abundance of low-density adipose tissue to high-density glandular and fibroblastic stromal tissue within the breast. Since the concept was first described in the 1970's, it has become clear that MD is an important risk factor for the development of breast cancer; involvement of 60% or more of the breast with mammographically dense tissue confers an 3–5 fold increased relative risk for breast cancer [77,78]. Numerous studies have been undertaken to look for genetic polymorphisms and other biomarkers that might correlate with MD and evidence exists for the involvement of both the IGF-1 and hormone signaling cascades in promoting MD [79,80]. Circulating IGF-1 levels and IGF-1 expression in breast tissue has been positively correlated with increased MD in pre-menopausal women [81,82]. In general, breast density decreases after menopause but studies have shown that post-menopausal hormone replacement therapy (estrogen and progesterone) is associated with measurable increases in MD, which may account for part of the increased risk of breast cancers seen with this intervention [83,84]. Conversely, treatment with the ER inhibitor tamoxifen has been shown to decrease MD [85,86]. Mammographically dense tissues are also associated with increased collagen-1 deposition in the tissue [82,87]. A recently described mouse model indicates directly that higher collagen levels in the mammary gland increase tumor formation and invasive behavior [88], suggesting a manner in which areas of dense tissue may be tumor promoting.

Expression profiling has done much to illuminate the heterogeneous nature of human breast tumors and has also been used to identify stromal signatures that have predictive value for breast cancers. A 'wound-healing' gene signature, originally derived from microarray analysis of the response of cultured fibroblasts to serum [89] has shown to have the ability to predict survival in breast cancer patients [90]. Recently, by using laser-capture microdissection to isolate tumor-associated stroma, a 26-gene stroma-derived prognostic predictor was generated that was predictive of relapse-free survival [57]. Genes associated with poor survival were involved in hypoxic and angiogenic responses within the tumor as well as a tumor-associated macrophage immune response. Conversely, genes indicating a tumor-inhibitory immune response were associated with good prognosis for the patient [57]. Another recent microarray study of the tumor-stroma showed an association of a reactive stromal gene signature (suggestive of large stromal content within the tumor) with resistance to neo-adjuvant chemotherapy [91]. These studies suggest that much can be learned about the potential tumor course and its responsiveness to treatment by screening the stroma associated with breast cancers.

4.2 Targeting tumor-associated stroma as a clinical strategy

The tumor microenvironment has become an attractive clinical drug target as it has become recognized that there is dysfunction in not only tumor epithelial cells but also tumor-associated stromal cells [92]. It is increasingly clear that cells within the tumor stroma are communicating with other components of the tumor microenvironment as well as with the tumor epithelial cells, thus, drug targets that can disrupt the tumor 'ecosystem' are highly sought after. The most active avenues for drug development have been in targeting tumor-promoting inflammatory processes and tumor-associated angiogenesis [92,93]. The most clinically

advanced of these are VEGF inhibitors that target tumor endothelial cells, such as anti-VEGF monoclonal antibodies bevacizimab and tyrosine kinase inhibitors such as sorafenib and sunitinib with clinical efficacy seen in metastatic breast and colon cancers in combination with chemotherapy [93]. Drugs already in wide clinical use for breast cancer such as tamoxifen and letrozole, while aimed at inhibiting the estrogen activity in breast tumor epithelial cells, have the dual benefit of acting on tumor-associated stroma as well. Tamoxifen can act on the tumor-associated ECM, leading to less aggressive behavior of breast cancer cells *in vitro* and *in vivo* [94]. The aromatase inhibitor letrozole can also be thought of as a stomal targeting drug as much of the local estrogen activity is derived from aromatase action in adipose tissue of post-menopausal women. Additionally, letrozole was shown to block the tumor promoting effects of estrogen on the tumor-associated stroma in a mouse xenograft model of ER-negative breast tumors [95].

Recent efforts have been undertaken to identify and exploit potential drug targets associated specifically with CAFs and tumor-stroma paracrine signaling networks. Several approaches have been used to directly target CAFs due to the overexpression of FAP, which is widely expressed on the stromal cells of epithelial tumors. Anti-FAP antibodies have been engineered to deliver drugs to the tumor site, the serine-protease activity of FAP has been exploited to activate pro-toxins in the vicinity of the tumor, and vaccines have been developed to generate an immune reaction to the FAP antigen [96–98]. In mouse models of cervical and colon cancer, disruption of the paracrine signaling loop between tumor cell-derived PDGF ligands and stromal PDGF receptor with the tyrosine kinase inhibitor imatinib was effective in reducing both tumor growth and tumor vessel formation [99,100]. Similar tumor-stromal signaling crosstalk exists with the expression of hedgehog ligands by tumor cells and the signaling effector Gli-1 in the tumor-associated stroma [101,102]. Depletion of tumor-associated stroma through inhibition of hedgehog signaling in a mouse model of pancreatic cancer allowed for increased tumor perfusion by gemcitabine and decreased tumor growth [103]. These studies (as well as others) demonstrate novel ways in which the tumor stroma can be targeted to facilitate effective treatment of the tumor.

5. Conclusions

Malignant breast tumors are composed of heterogeneous cell types, including aberrantly regulated epithelial cells surrounded by extracellular matrix, cancer associated fibroblasts, inflammatory cells, and blood vessels. Current therapies target primarily the carcinoma cells, however, many women develop recurrent disease and/or distant metastases following treatment. Given the supportive and instructive role of the stroma in cancer progression, therapeutics tailored to both the stroma and epithelium may have more clinical efficacy for prevention of local recurrence and metastases. Examining signaling interactions among the mammary epithelial cells and its associated stroma during normal development and tumorigenesis may provide critical insight to additional chemotherapeutic targets for future therapeutics.

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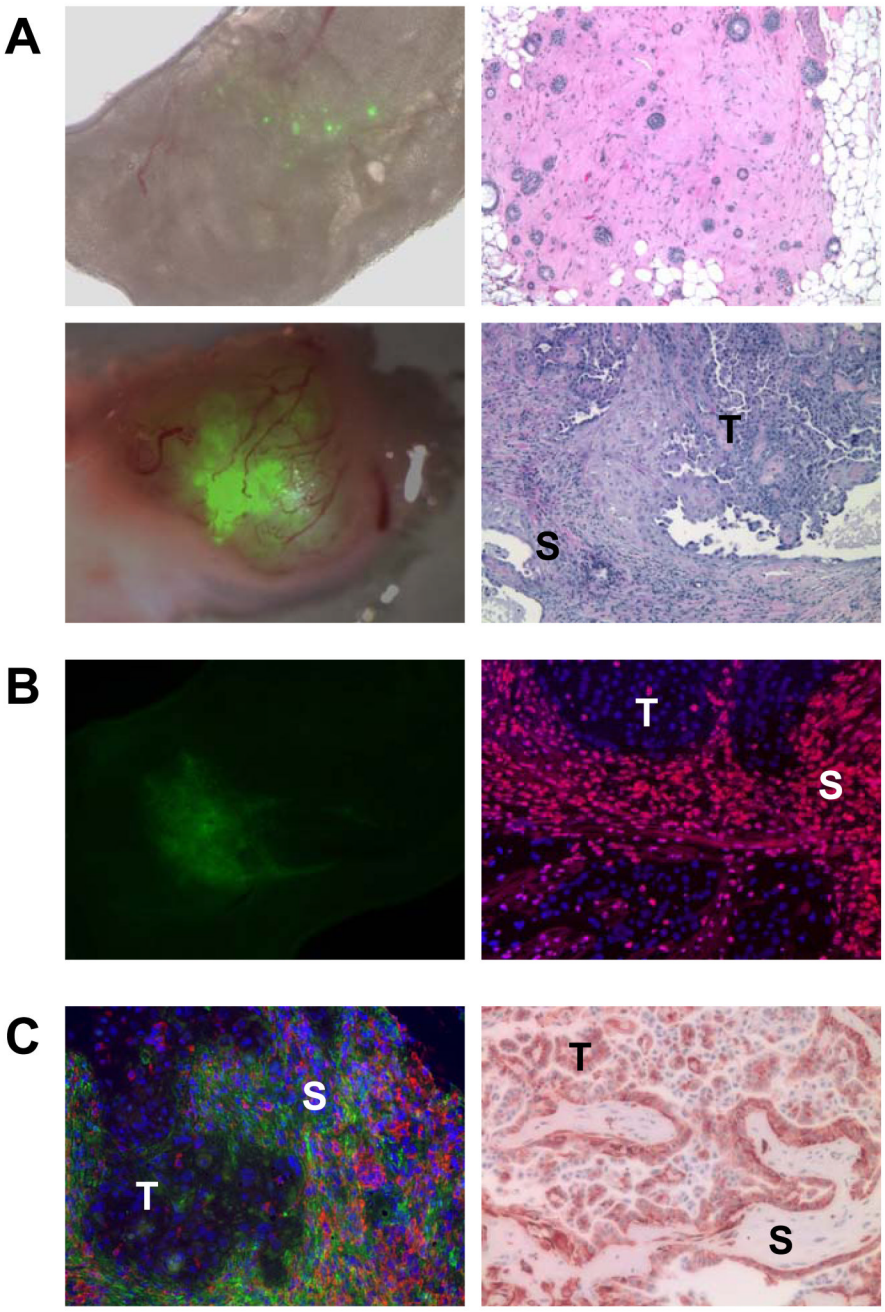

Figure 1. Stromal cells and the tumor microenvironment in the HIM model

(A) GFP-whole mount and H&E stains of humanized glands injected with GFP-lentivirus infected HMECs (top panel) or GFP + oncogene-lentivirus infected HMECs (bottom panel). Fibroblasts are present sparsely within the humanized area embedding the normal epithelial outgrowths (top panel) and as a dense stromal reaction surrounding tumor outgrowths. (B) GFP-labeled human immortalized fibroblasts used for humanizing the cleared mammary fat pads are present at 2 weeks post-humanizing (left) but are replaced by a strong recruitment of mouse-derived stromal cells (Right). Fluorescence in-situ hybridization for mouse Cot1 DNA (red) indicates that recruited stromal cells (S) are of mouse origin. Human tumor cells (T) are identified by staining for DAPI alone (blue). (C) (Left) Recruited stromal cells (S) include

αSMA-positive (green) myofibroblast-like cells and F4/80-positive (red) macrophages. Human tumor cells (T) are labeled with DAPI alone (blue). (Right) Human tumor cells stained with human-specific Vimentin antibody (red).