

Role of cancer-associated fibroblasts in breast cancer development and prognosis

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ABSTRACT Since Paget's "Seed and Soil" hypothesis in 1889 on cancer growth and metastasis, several studies on various solid tumors have confirmed the active role of the tumor milieu on the onset, growth and spread of neoplastic cells. Fibroblasts constitute the major components of the tumor microenvironment (stroma), and are therefore the most studied cell type. Therefore, a large amount of data has emerged showing the cancer-promoting function of these cells through paracrine effects that escort tumor cells through all the carcinogenesis steps. This involves many signaling proteins that transmit the message in both directions, allowing cooperative crosstalk between cancer cells and their stroma. This prompted several researchers to investigate the potential use of the molecular and cellular features of active stromal fibroblasts to generate specific tools for prevention, prognosis and treatment of cancer. Herein, I review the cellular and molecular features of active cancer-associated fibroblasts and their origin. Additionally, I summarize our current understanding of the procarcinogenic actions of these cells and their potential prognostic value for breast cancer patients.

KEY WORDS: *Cancer-associated fibroblasts, breast cancer, paracrine signaling, prognosis*

Introduction

Breast cancer is the most frequent malignancy and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths worldwide in 2008 (Jemal *et al.*). Breast carcinomas are complex tumors that result mainly from the accumulation of genetic and epigenetic alterations in epithelial cells of the mammary gland. However, carcinoma cells, like normal epithelial cells, live in a complex microenvironment that includes the extracellular matrix (ECM) as well as cellular components such as immune and inflammatory cells, endothelial cells, adipocytes, fibroblasts and bone marrow-derived cells (Arendt *et al.*, 2009). Stromal-epithelial interactions have a fundamental role in normal mammary development such as determining normal duct formation and the initiation and maintenance of oestrogen and/or progesterone responsiveness in mammary epithelial cells (Kim *et al.*, 2005). Furthermore, normal stroma controls epithelial cell polarity, loss of which leads to an increase in cell proliferation and tumorigenesis (Polyak and Kalluri, 2010). Fibroblasts, the predominant cells of the stroma, are responsible for the elaboration of most of the components of connective tissue (Sappino *et al.*, 1990). Mammary gland fibroblasts produce ECM molecules such as fibronectin and tenascin, which influence cell adhesion

and proliferation (Schor and Schor, 2001). Thereby, modifications in stromal fibroblasts can play a significant role in overall cancer development. Indeed, several recent publications have reported genetic and epigenetic changes in stromal fibroblasts that modulate the expression of many genes encoding growth factors and cytokines (Hu and Polyak, 2008; Lin *et al.*, 2009). These soluble factors affect the microenvironment, fertilize the soil and favor the growth of the seed (tumor). To progress and spread, tumor cells keep interacting with their stromal fibroblasts through secreted molecules. This reciprocal heterotypic signaling plays a major role in the various steps of tumorigenesis. However, a key question remains: which comes first, the dysfunction of epithelial cells or the dysfunction of their microenvironment? There is so far no answer to this chicken-and-egg conundrum, but it is now becoming evident that each compartment is affecting the other one with one ultimate objective: growth and expansion of neoplastic cells. Therefore, understanding the meaning of the crosstalk between neoplastic cells and their stromal fibroblasts, one of the important hallmarks

Abbreviations used in this paper: CAF, cancer-associated fibroblast; CAV, caveolin; ECM, extracellular matrix; HGF, hepatocyte growth factor; MMP, matrix metalloprotein; PTEN, phosphatase and TENsin homolog; VEGF, vascular endothelial growth factor.

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of cancer (Pietras and Ostman, 2010), has become fundamental in improving the prognosis and therapy of various solid tumors, including mammary carcinomas.

Cancer-associated fibroblasts

Fibroblasts are non-vascular, non-epithelial and non-inflammatory cells that form the basic cellular component of connective tissue and contribute to its structural integrity (Tarin, 1969). They play important roles in wound healing, regulation of epithelial differentiation and inflammation (Tomasek *et al.*, 2002). In healthy organs, fibroblasts have a low proliferation index and minimum metabolic capacity. By contrast, during wound healing and in cancers, fibroblasts become activated, start to proliferate, secrete higher amounts of ECM components, and acquire contractile features (Polyak and Kalluri, 2010). In tumors, these fibroblasts are known as reactive fibroblasts, peri-tumoral fibroblasts, myofibroblasts, tumor-associated or cancer-associated fibroblasts (CAFs). These cells present plump spindle-shaped mesenchymal structure with indented nuclei, peripheral myofilaments and fibronexus junctions (De Wever *et al.*, 2008). Myofibroblast cells were first identified by Gabbiani *et al.*, during wound healing (Gabbiani *et al.*, 1971) and were defined by Kalluri and Zeisberg as “activated fibroblasts within desmoplastic lesions that are associated with malignant tumors and often express α -smooth-muscle actin (α -SMA)” (Kalluri and Zeisberg, 2006). These cells are also positive for vimentin and desmin, but do not express cytokeratin, CD31 and smooth muscle myosin. These markers are often used to distinguish between myofibroblasts and normal fibroblast, epithelial, endothelial or smooth muscle cells (Beacham and Cukierman, 2005). While most CAFs are active fibroblasts, a small proportion remains inactive. Indeed, in breast tumors 80% of fibroblasts are in active form (Sappino *et al.*, 1988). Active fibroblasts play similar roles in wound healing and in cancer, which is considered as a wound that does not heal (Dvorak, 1986). However, while in normal wounds active fibroblasts are transients, CAFs are persistent in tumors. Active fibroblasts secrete high levels of various growth factors, cytokines, chemokines, and ECM degrading proteases such as the MMPs (Bhowmick *et al.*, 2004). These factors are used by activated fibroblasts to communicate with cancer cells as well as with other stromal cells. Microarray and proteomics analysis have revealed distinct mRNA and protein expression profiles in CAFs as compared to their adjacent normal counterparts (NFs) and normal fibroblasts isolated from mammary reduction surgery (NBFs), showing genetic and/or epigenetic alterations in these stromal fibroblasts (Allinen *et al.*, 2004; Bauer *et al.*, 2010; Hawsawi *et al.*, 2008; Sadlonova *et al.*, 2009). Consistent with this, CAFs exhibit some cancer-specific changes, including defective p53/p21-dependent signaling pathway in response to γ -rays, high level of survivin, increased resistance to cisplatin and UV light and strong expression of the proliferation markers Ki-67 and PCNA (Hawsawi *et al.*, 2008). However, these cells are non-neoplastic because they senesce in culture and do not grow *in vivo* in tumor xenografts (Kojima *et al.*, 2010).

Origin of mammary cancer-associated fibroblasts

In light of recent findings, breast carcinoma-associated fibroblasts are believed to have 3 major distinct origins, not necessary exclusive. The *first one* is the transformation within the tumor of

stromal resting fibroblasts into myofibroblasts. Three dimensional coculture experiments have shown that fibroblasts readily converted into a graded pattern of myogenic differentiation when confronted with tumor cells, with strong effect on cells that were in the immediate vicinity of tumor cells (Ronnov-Jessen *et al.*, 1995). Recently, conversion of resident fibroblasts into myofibroblasts during the course of tumor progression was also observed in a coimplantation breast tumor xenograft model (Kojima *et al.*, 2010). These results indicate the possible transformation of breast stromal fibroblasts to myofibroblasts under cancer-related paracrine effects. In fact, TGF- β can induce the production of α -SMA in mammary fibroblasts *in vitro* and consequently transdifferentiates fibroblasts into CAFs (Vaughan *et al.*, 2000) (Fig. 1).

The *second possible origin* of breast myofibroblasts are specialized circulating progenitor cells such as fibrocytes and bone marrow-derived mesenchymal stem cells (MSCs). Fibrocyte cells are known to express hematopoietic stem cell markers, monocyte lineage and fibroblast markers. Fibrocytes have been identified in breast carcinomas and these cells can differentiate into CAFs (Barth *et al.*, 2002; Mori *et al.*, 2005). MSCs are known to migrate to and engraft at tumor sites (El-Haibi and Karnoub, 2010; Spaeth *et al.*, 2009). MSCs can also differentiate into active fibroblasts expressing α -SMA and exhibiting myofibroblast features (Mishra *et al.*, 2008; Spaeth *et al.*, 2009). In fact, addition of lysophosphatidic acid (LPA) induces the expression of α -SMA and SDF-1 in MSCs, which stimulates the differentiation of MSCs to myofibroblast-like cells (Jeon *et al.*, 2008). Like myofibroblasts, activated MSCs by prolonged exposure to conditioned media from cancer cells ex-

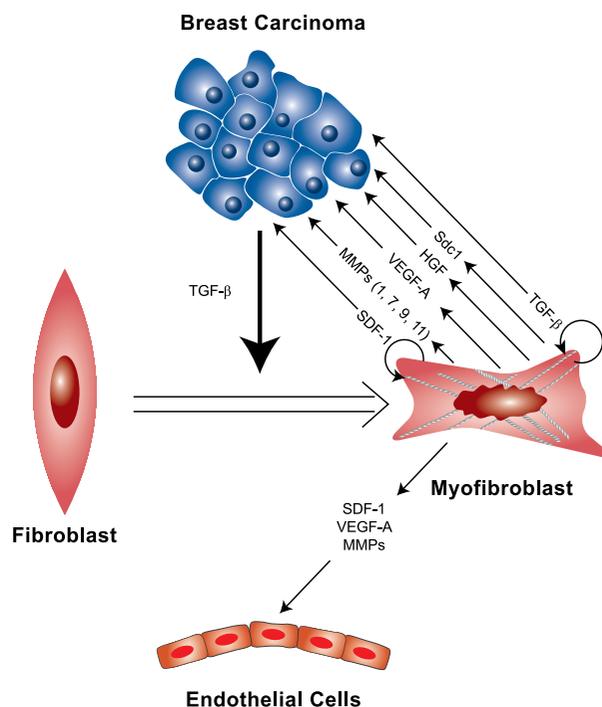


Fig. 1. Active fibroblasts promote cancer growth and angiogenesis through secreted factors. Breast carcinomas can activate stromal fibroblasts through secretion of TGF- β . Consequently, activated fibroblasts enhance tumor growth and angiogenesis via secreted mediators. SDF-1 and TGF- β cytokines maintain the active state of stromal fibroblast through autocrine signaling loops.

pressed high levels of SDF-1, a hallmark of tumor myofibroblasts, and enhanced tumor growth (Mishra *et al.*, 2008). These findings strongly suggest that MSCs constitute a major source of cancer-associated activated fibroblasts in breast tumors.

The *third source* of CAFs comes from the trans-differentiation of different cell types present in the breast such as epithelial, endothelial, adipocyte and pericyte cells into myofibroblasts. The epithelial to mesenchymal transition (EMT), an important step during cancer progression, has been shown to produce myofibroblast cells, with enhanced migratory capacity, invasiveness and increased expression of ECM proteins (Kalluri and Zeisberg, 2006; Polyak and Weinberg, 2009). Likewise, endothelial to mesenchymal transition (EndMT), characterized by the loss of endothelial markers such as CD31, and the expression of α -SMA and other mesenchymal markers, also produced myofibroblast-like cells (Potenta *et al.*, 2008; Zeisberg *et al.*, 2007). Interestingly, both EMT and EndMT transitions can take place upon exposure to TGF- β (Kalluri and Weinberg, 2009; Massague, 2008). Furthermore, studies using transgenic mice have revealed that up to 40% of CAFs arise through EndMT (Zeisberg *et al.*, 2007). Taken together, these findings suggest that breast cancer-associated fibroblasts could have different origins. However, the local cells seem to constitute the most prominent source, while other invader cells may constitute a minor pool. It is also possible that the origin of CAFs varies depending on the stage of carcinogenesis.

Role of CAFs in breast cancer development and spread

There is substantial evidence that CAFs actively contribute to the growth, expansion and dissemination of neoplastic epithelial cells. Thereby, Cheng and Weiner considered stromal fibroblasts as the "contracted farmers" that prepare a terrain for the tumor's ease and fertile growth (Cheng and Weiner, 2003). To confirm this at the cellular level, Shekhar and his collaborators used a three-dimensional cell-cell interaction model and have shown the importance of stromal fibroblasts in breast epithelial growth and differentiation *in vitro* (Shekhar *et al.*, 2001). This effect was also revealed *in vivo* by showing that human breast cell line MCF-7 cells are hardly tumorigenic in SCID mice, their tumorigenicity was dramatically increased when inoculated with fibroblasts (Trimis *et al.*, 2008). Furthermore, the coinoculation of transformed fibroblasts with breast cancer cells accelerated the growth and shortened the latency period of human tumors in athymic mice (Camps *et al.*, 1990). Loss of TGF- β type II receptor in fibroblasts cotransplanted with mammary carcinoma cells into mammary fat pad promoted tumor growth, invasion and metastasis (Cheng *et al.*, 2005). In other studies using tumor xenograft models, CAFs were shown to accelerate the growth rate of tumors and enhanced tumor angiogenesis through mobilization and recruitment of endothelial cells (Hu *et al.*, 2009; Orimo *et al.*, 2005). Together, these results provide clear evidence on the active role of myofibroblasts on various steps of breast carcinogenesis. This raises an important question on the nature of the mediators of this pro-tumorigenic factors and the mechanism of their regulation.

CAF-derived breast cancer promoting factors

During the development of mammary carcinomas the epithelial and stromal compartments coevolve, through heterotypic interactions that do not necessitate cell-cell contact but seems rather driven

by secreted soluble effectors. Indeed, in breast cancer tissues, most of the gene expression changes that occur in the stroma take place in ductal carcinoma *in situ* (DCIS) and only few extra ones occur in the stroma of invasive tumors (Casey *et al.*, 2009; Ma *et al.*, 2009). This indicates that paracrine and endocrine effects are at the origin of these modifications since the basement membrane is largely intact in DCIS. Using a transwell system allowing diffusible factor exchange and microarray analysis, it has been shown that 160 and 178 genes were differentially expressed between MDA-MB231 and MCF-10 cells cocultured with CAFs as compared to monocultures, respectively (Rozenchan *et al.*, 2009). Moreover, co-injection of lethally irradiated fibroblasts or inclusion of fibroblast-conditioned medium with breast cancer cell grafts increased carcinogenesis and tumor growth (Camps *et al.*, 1990; Noel *et al.*, 1993). These findings show that CAFs promote tumor growth by secreting soluble factors such as proangiogenic factors, matrix metalloproteinases (MMPs), cytokines as well as chemokines and growth factors.

SDF-1

The knowledge that stromal cells have the ability to stimulate oncogenesis has been taken a step further by showing that stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1 (CXCL12) secretion (Allinen *et al.*, 2004; Orimo *et al.*, 2005). These two reports have clearly shown the important role that the SDF-1 cytokine plays in the crosstalk between myofibroblast cells and tumor cells. Allinen *et al.*, have also shown that myofibroblasts SDF-1 enhances the proliferation, migration and invasion of cancer cells (Allinen *et al.*, 2004) (Fig. 1). This effect is mediated through preferential binding of SDF-1 to the CXCR-4 receptor in cancer cells, which induces different intracellular pathways related to several aspects of tumor progression (Teicher and Fricker, 2010). Furthermore, CAFs promoted the proliferation of breast cancer CD44⁺CD24⁻ stem cells in mammosphere cells in an SDF-1-dependent manner, and enhanced their tumorigenicity in mice (Huang *et al.*, 2010). SDF-1 also enhanced angiogenesis by recruiting endothelial progenitor cells into carcinomas (Orimo *et al.*, 2005).

VEGF-A (VEGF)

The vascular endothelial growth factor A (VEGF-A) is an important mediator of angiogenesis and also has a potent tumor angiogenic effect. VEGF expression in breast cancer cells can stimulate CXCR-4, linking VEGF expression to the migratory potential of cells and to SDF-1 signaling (Bachelder *et al.*, 2002). Beside angiogenesis, VEGF released by activated stroma increased the growth of ER-positive malignant epithelial cells and of adjacent normal epithelium (Pinto *et al.*, 2010).

HGF

Another important myofibroblast mediator is the Hepatocyte Growth Factor (HGF) protein, which is primarily expressed and secreted from fibroblasts and can promote cancer development and progression (Matsumoto and Nakamura, 2006). It has been recently shown that breast cancer cells induce CAFs to secrete HGF, which in turn enhances breast tumorigenesis (Tyan *et al.*, 2011).

TGF- β

Kojima and coworkers have recently shown that autocrine TGF- β signaling drives the evolution of tumor-promoting mammary

stromal myofibroblasts (Kojima *et al.*, 2010). Furthermore, ectopic expression of HGF and/or TGF- β in mouse fibroblasts before the implantation of ostensibly normal human mammary epithelial cells resulted in the outgrowth of benign and malignant lesions in the humanized fat pads of NOD/SCID mice (Kuperwasser *et al.*, 2004; Wu *et al.*, 2009). This suggests an important role of stromal fibroblast TGF- β in promoting breast carcinogenesis (Fig. 1).

MMPs

The other major intermediate in the intercommunication between tumors and their stromal fibroblasts are Matrix metalloproteinases (MMPs). These prominent proteases that play crucial roles in various physiological processes including development, inflammation and ECM degradation, are abundantly and some time exclusively secreted by stromal cells, and play key roles in the modulation of the tumor microenvironment. They also regulate various signaling pathways that control tumor growth, angiogenesis and metastasis (Kessenbrock *et al.*, 2010). It has been shown that breast cancer cells induce stromal fibroblasts to express MMP9 via secretion of TNF- α and TGF- β , and that the level of induction depends on the degree of malignancy of cancer cells (Stuelten *et al.*, 2005). Furthermore, elevated levels of MMP9 were found associated with tumors and correlate with cancer invasion and metastasis (Nabeshima *et al.*, 2002). In addition, fibroblasts were shown to promote breast cancer cell invasion by upregulating MMP9 production (Wang *et al.*, 2002). Recently, Hu *et al.*, have shown that fibroblast MMP9 promotes breast cancer cells invasion in a coculture *in vitro* model (Hu *et al.*, 2009). This indicates that MMP9 plays an important role in the cross-talk between breast tumors and their milieu. Furthermore, MMP1 and MMP7 are mainly fibroblastic and can increase the susceptibility to breast cancer when overexpressed in transgenic mice (Lynch and Matrisian, 2002). MMP11 was identified and cloned as a gene expressed specifically in stromal cells surrounding invasive breast carcinomas (Basset *et al.*, 1990). While fibroblasts expressing normal MMP11 support *in vivo* growth of breast cancer cells, MMP11-null fibroblasts do not promote such growth, showing an important role of this protease in fibroblast-promoting breast cancer growth (Masson *et al.*, 1998) (Fig. 1).

SDC1

Proteoglycans also modulate the reciprocal interactions between tumor and stromal cells through binding to ECM constituents and important growth factors (Friedl, 2009). The syndecans compose an important family of transmembrane cell surface heparan sulfate proteoglycans (HSPGs), which regulate cell-cell and cell-ECM adhesion, cell migration, and growth factor activity (Bernfield *et al.*, 1999). Among syndecan family members, Sdc1 is expressed mainly in normal epithelial cells and its induction in stromal fibroblasts is observed in more than 70% of human breast tumors. Breast carcinoma cells induce Sdc1 in fibroblasts, which reciprocally promote cancer cell growth (Maeda *et al.*, 2006; Su *et al.*, 2007). The soluble Sdc1 extracellular domain stimulates carcinoma cell growth in concert with fibroblast growth factor 2 (FGF2) and SDF-1, possibly through shuttling them from CAFs to carcinoma cells (Su *et al.*, 2007).

Altogether, these findings indicate that carcinoma-stroma cross-talk is ensured by a myriad of mediators that enable the growth and progression of tumor cells. Several other potential mediators are still awaiting identification and characterization to fully understand

this complex cellular cross-signaling and decipher its meaning.

Genetic and epigenetic changes in breast cancer-associated myofibroblasts

Several studies have reported important changes in gene expression in CAFs as compared to NFs and NBFs. Using serial analysis of gene expression, Allinen and coworkers have shown extensive gene expression changes in all cell types during cancer progression, and that a significant fraction of altered genes encode secreted proteins and receptors. In a recent study, Bauer *et al.*, used Affymetrix Human Genome U133 Plus 2.0 arrays to examine differential gene expression between CAFs and NFs from the same patients. They have identified 21 genes up-regulated and 10 genes down-regulated in CAFs, as compared with NFs. These genes are implicated in paracrine or intracellular signaling, transcriptional regulation, ECM production and cell adhesion/migration (Bauer *et al.*, 2010). In another study, 420 genes were found to be differentially expressed in CAFs versus NBFs (Sadlonova *et al.*, 2009). Furthermore, 2-Dimensional (2D) gel electrophoresis analysis revealed a great variation in global protein expression between CAFs, NFs and NBFs. Intriguingly, even fibroblasts adjacent to a tumor in a histologically normal part of the breast presented variations in protein expression as compared to NBFs (Hawsawi *et al.*, 2008). These findings clearly indicate that stromal fibroblasts both into breast tumors and in their "normal" surrounding areas are subject to variation in the expression of various genes directly or indirectly implicated in functional crosstalk between the tumor and its microenvironment.

Since many of these changes are maintained after prolonged cell culture even in absence of cancer cells, it is plausible that they result from genetic and/or epigenetic alterations. Indeed, several studies have shown alteration in DNA methylation pattern in breast CAFs. Using methylation-specific digital karyotyping, Hu *et al.*, have shown global alteration in DNA methylation pattern in both tumor epithelial cells as well as in breast cancer stromal fibroblasts as compared to their normal counterparts (Hu *et al.*, 2005). Furthermore, Fiegl and coworkers have found differential methylation status in five selected genes between normal and breast cancer-associated stroma. This methylation was HER-2/neu-dependent (Fiegl *et al.*, 2006). Together, these data indicate that breast cancer stromal fibroblasts are epigenetically abnormal, which could explain the stable gene expression variation observed in CAFs even after serial passaging *in vitro*.

On the other hand, the presence of somatic genetic alterations in active fibroblasts remains controversial (Campbell *et al.*, 2009; Eng *et al.*, 2009). While several groups reported the presence of mutations only in cancer epithelial cells, chromosomal loss of heterozygosity (LOH) and mutations have also been reported in CAFs by other groups (Campbell *et al.*, 2011; Eng *et al.*, 2009). Indeed, up to 87.5% LOH were found in CAFs microdissected from formalin-fixed paraffin-embedded breast ductal carcinoma *in situ* (DCIS) samples (Moinfar *et al.*, 2000). Another group has independently confirmed these results and has shown the presence of frequent genetic alterations in invasive human breast cancers (loss of heterozygosity and *TP53* mutations) occurring not only in the neoplastic epithelial cells, but also in the adjacent fibroblastic stroma, and that both components can share clonal features (Wernert *et al.*, 2001). Other studies have also identified

LOH in CAFs and have shown significant association between the LOH signature in stromal fibroblasts and breast tumor grade and lymph node metastasis (Fukino *et al.*, 2007; Weber *et al.*, 2006). Looking for loss of heterozygosity in the two tumor suppressor *TP53* and *PTEN* genes in epithelial and stromal tumors, Kurose and colleagues have found mutations in both compartments. However, in most cases *PTEN* and *TP53* mutations did not occur together in the same tissue compartment. Their exclusive nature and the presence of discordant mutations even within the same gene suggested that genetic alterations occur independently in the stroma and the epithelium (Kurose *et al.*, 2002). What are the reasons of this discrepancy? Several explanations have been proposed and were the topic of recent reviews (Campbell *et al.*, 2009; Eng *et al.*, 2009).

Are these genetic/epigenetic alterations the cause or the consequence of the presence of cancer cells? It has become clear that cancer cells modulate their ecosystem through paracrine signaling in order to grow and invade. However, it remains unclear whether alteration of the microenvironment is a prerequisite for tumor onset. In fact, there are indications that stromal alteration(s) precede the malignant conversion of tumor cells (Moinfar *et al.*, 2000). For instance, epigenetic modifications in stroma were shown to be unique and discrete from their associated cancer cells (Hu *et al.*, 2005; Kurose *et al.*, 2002). In addition, active fibroblasts can trigger genetic/epigenetic alterations in the epithelial cells. CAFs from breast cancer were able to trigger AKT1-mediated epigenetic silencing of the tumor suppressor cystatin M (*CST6*) and other genes in epithelial cells (Lin *et al.*, 2008). Epigenetic silencing of *CST6* has been observed in breast cancer cell lines and in metastatic lesions (Ai *et al.*, 2006; Rivenbark *et al.*, 2007). These pro-tumoral stromal modifications could be acquired or inherited. It is well known that there is a genetic component in the predisposition to breast

cancer. Indeed, skin fibroblasts derived from breast cancer patients may also be altered and exhibit characteristics associated with a transformed phenotype (Azzarone *et al.*, 1984). In another study, skin fibroblast cells derived from breast cancer patients exhibited higher sensitivity to irradiation than skin fibroblastic cells from healthy women (Hannan *et al.*, 2001). In addition, Schor and colleagues reported that skin fibroblasts obtained from cancer patients display foetal-like migratory behavior on collagen gels (Schor *et al.*, 1985). They also described a tumor-like phenotype of fibroblasts isolated from the healthy relatives of patients with familial breast disease (Haggie *et al.*, 1987). This shows that not only cells that are in proximity of the tumor are genetically altered, but also cells of the whole body. This could explain the predisposition to cancer development, and the possible inheritance of alterations, essentially epigenetic, in fibroblast cells.

Role of tumor suppressor genes in regulating epithelial-fibroblast cross signaling

Mesenchymal-epithelial cell interactions and their role in tumor growth and development are now well recognized. However, the genes and pathways controlling these tumor-promoting effects are not fully delineated. Several lines of evidence indicate the implication of tumor suppressor genes in controlling the pro-carcinogenic effects of stromal fibroblasts in a paracrine manner (Fig. 2).

TP53

The versatile p53 tumor suppressor protein was the first shown to play this role. Komarova *et al.*, have demonstrated p53-mediated growth inhibition of cancer cells *in vitro* through paracrine effect (Komarova *et al.*, 1998). Furthermore, in mice xenograft models, p53-deficient fibroblasts significantly increased the tumorigenicity of the human MCF-7 breast cancer cell line more than p53 normal counterparts (Kiaris *et al.*, 2005). In another study, p53 induction affected the secreted level of several factors implicated in the mesenchymal-epithelial crosstalk (Khwaja *et al.*, 2006). To elucidate the p53-dependent signaling pathways and mediators, Moskovits *et al.*, have shown that stromal fibroblast p53 attenuates cancer cell migration and invasion through repression of SDF-1 expression/secretion (Moskovits *et al.*, 2006). These results show cell non-autonomous tumor suppressor action of p53 in breast carcinogenesis (Bar *et al.*, 2009).

CDKN1A

One of the most important targets of p53 is the cyclin-dependent kinase inhibitor p21. p21 deficiency in stromal fibroblasts also accelerated tumor growth *in vivo* through cell non-autonomous mechanism. Indeed, transient siRNA-mediated p21 suppression in fibroblasts stimulated breast cancer growth *in vivo* (Trimis *et al.*, 2008). This suggests that the paracrine anti-tumor effect of p53 could be mediated through p21, and that this pathway may play a capital role in controlling the epithelia-stroma crosstalk (Fig. 2). The observation that p53 and p21 protein levels are down-regulated in 83% CAFs as compared to NFs, supports a major nonautonomous tumor suppressor function of these two important tumor suppressor proteins (Hawsawi *et al.*, 2008).

PTEN

PTEN, another important tumor suppressor gene, with lipid and

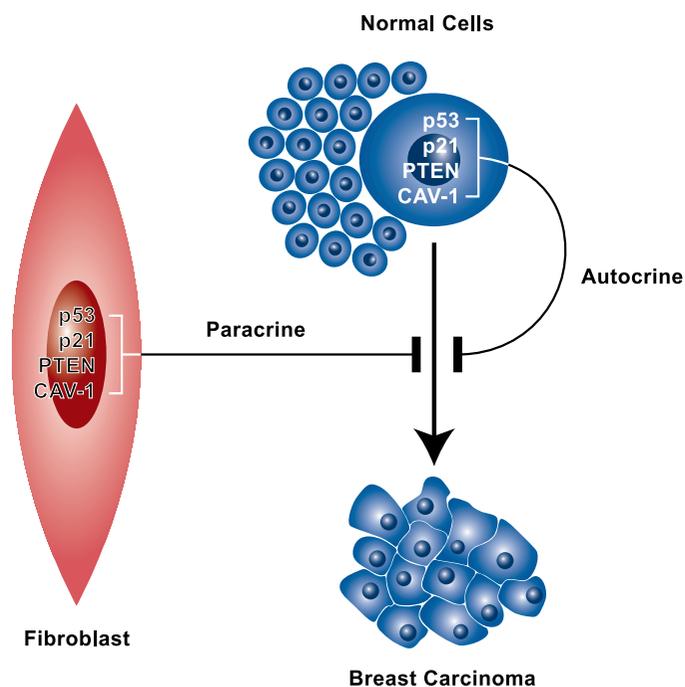


Fig. 2. Autocrine and paracrine tumor suppressor functions of p53, p21, PTEN and caveolin1 (CAV-1). These tumor suppressor proteins repress breast carcinogenesis from epithelial cells and also from stromal fibroblasts.

protein phosphatase activity, has been found mutated in various types of cancer, including breast carcinomas (Cully *et al.*, 2006). The role of PTEN in stroma has been recently revealed in an experimental mouse model by showing that inactivation of PTEN in stromal fibroblasts of mouse mammary glands accelerated the initiation, progression and malignant transformation of mammary tumors, through increase in chemokine and cytokine production in the tumor microenvironment. The authors have identified the Pten-Ets2 axis as a critical stroma-specific signaling pathway that suppresses mammary epithelial tumors (Trimboli *et al.*, 2009).

CAV-1

Caveolin-1 (*CAV-1*) codes for a principal component of caveolae membranes, and is expressed in various human cell types, including fibroblasts and mammary epithelial cells (Engelman *et al.*, 1998; Lee *et al.*, 1998). It has been reported that *CAV-1*-deficient mammary stromal fibroblasts share several features with human CAFs, including increased levels of secreted proliferative and angiogenic growth factors (Sotgia *et al.*, 2009). Moreover, co-injection of caveolin-1 deficient human fibroblasts with MDA-MB-231 cells, increased both tumor mass and tumor volume by ~4-fold, showing the paracrine tumor promoting effect of this gene (Trimmer *et al.*). Several lines of evidence indicate that *CAV-1* also acts as tumor suppressor gene in breast cancer (Bouras *et al.*, 2004). Furthermore, Williams *et al.*, have shown that the combined loss of the tumor suppressor INK4a and caveolin-1 synergistically enhances cell proliferation and oncogene-induced tumorigenesis (Williams *et al.*, 2004).

Together, these results indicate that several tumor suppressor genes act both in autocrine and paracrine fashion to stop tumor growth and spread. It is thereby possible that other important tumor suppressor genes have also this dual function (Fig. 2).

CAFs as marker of patient prognosis

The functional interplay existing between epithelial cells and their microenvironment supposes that CAFs can constitute a valuable prognostic and predictive marker for breast cancer patients. Indeed, numerous studies have shown correlation between gene expression in breast myofibroblasts and patients outcome. Finak *et al.*, have identified a new stroma-related 26-gene expression signature with strong association with breast cancer patient outcome (Finak *et al.*, 2008). In another study, a newly identified stroma-derived signature predicted resistance of breast cancer patients to neoadjuvant chemotherapy with 5-fluorouracil, epirubicin and cyclophosphamide (Farmer *et al.*, 2009). Since fibroblasts are the major cell type of breast stromal compartment, it is possible that these stroma-dependent predictive values are stromal fibroblast-related. Furthermore, the status of the tumor suppressor p53 protein in stromal fibroblasts may also be a good predictive marker for invasive breast carcinomas. Hasebe and coworkers have shown that p53 expression in tumor stromal fibroblasts, but not in tumor cells, is an independent factor associated with the number of nodal metastases and the outcome of invasive ductal carcinoma patients (Hasebe *et al.*, 2010). Additionally, the same group has recently shown a close association between p53 protein expression in tumor-stromal fibroblasts, especially in surgical specimens, and both the presence of nodal metastasis and the outcome of IDC patients who received neoadjuvant therapy (Hasebe *et al.*, 2010).

Another potentially good predictive stromal marker is the *CAV-1* gene. Indeed, caveolin-1-/- mammary stromal fibroblasts gene signature is predictive of poor clinical outcome in breast cancer patients treated with tamoxifen (Sotgia *et al.*, 2009). These findings were validated in two independent patient populations, confirming that stromal Cav-1 expression in human breast cancers is a powerful single independent predictor of early disease recurrence, metastasis, and poor clinical outcome (Sloan *et al.*, 2009; Witkiewicz *et al.*, 2009). Witkiewicz *et al.*, suggested the use of stromal Cav-1 to stratify breast cancer patients to high-risk (absence of Cav-1) and low-risk groups (Witkiewicz *et al.*, 2009). The observation that p53-deficient cells showed no Cav-1 expression suggests an association between p53 status and Cav-1 expression, (Lee *et al.*, 1998). This indicates that the loss of these two important tumor suppressor proteins in stromal fibroblasts could be of great predictive value for breast as well as other types of cancer. Moreover, high stromal fibroblast platelet-derived growth factor (PDGF) beta-receptor expression was significantly associated with high histopathological grade, estrogen receptor negativity, high HER2 expression, significantly shorter recurrence-free and breast cancer-specific survival. The prognostic significance of stromal PDGF beta-receptor expression was particularly prominent in tumors from premenopausal women (Paulsson *et al.*, 2009). Similarly, stromal CD10 expression in invasive breast carcinoma was shown to be associated with ER negativity, higher tumor grade and decreased survival, and constitutes another potential important prognostic marker (Makretsov *et al.*, 2007). In addition, multivariate analysis showed that SDF-1 status was also an independent factor related to overall survival in patients with ER-positive tumors, and therefore stromal SDF-1 status could have a significant prognostic value and may be clinically useful for assigning adjuvant therapy to patients with ER-positive invasive breast cancers (Kobayashi *et al.*, 2009). Recently Yamashita and colleagues showed that the expression of the other important hallmark of myofibroblasts, α -SMA, correlates with invasive growth and poor clinical prognosis (Yamashita *et al.*, 2010). Likewise, patients with stroma rich-tumors had a shorter relapse-free period and overall survival compared to stroma-poor tumors (de Kruijf *et al.*, 2011). These findings corroborate the previously shown association between the presence of large number of CAFs and high grade as well as poor prognosis (Cardone *et al.*, 1997). These and other findings provide clear evidence that, in the near future, stromal fibroblasts will be of great value for the diagnostic and prognostic of breast tumors and will also help for the development of individualized treatments that will take into account the status and effects of tumor microenvironment.

Conclusion

During breast carcinogenesis, activated stromal fibroblasts have active role in the initiation, progression, metastasis and recurrence of tumors, which adds new levels of complexity to cancer biology, but also brings new prognostic and therapeutic opportunities. Indeed, current cancer treatments target primarily carcinoma cells (seed), while they should also try to normalize the stroma (soil) and inhibit its supportive role. Therefore, a deep understanding of the epithelial-stromal biochemical interactions and molecular reciprocal heterotypic signaling is mandatory for preventive strategies and for the development of new therapeutic regimens that take into consideration the pro-carcinogenic actions of the microenvironment.

The fact that tumor-associated fibroblasts present some tumor features and are genetically stable and also different from normal fibroblasts make them important potential targets of anti-cancer therapy. Alexander Fleming said "If the soil causes the disease, the cure to the disease also lies in it".

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