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Seed in soil, with an epigenetic view

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Abstract

It is becoming increasingly evident that discrete genetic alterations in neoplastic cells alone cannot explain multistep carcinogenesis whereby tumor cells are able to express diverse phenotypes during the complex phases of tumor development and progression. The epigenetic model posits that the host microenvironment exerts an initial, inhibitory constraint on tumor growth that is followed by acceleration of tumor progression through complex cell–matrix interactions. This review emphasizes the epigenetic aspects of breast cancer development in light of such interactions between epithelial cells ("seed") and the tumor microenvironment ("soil"). Our recent research findings suggest that epigenetic perturbations induced by the tumor microenvironment may play a causal role in promoting breast cancer development. It is believed that abrogation of these initiators could offer a promising therapeutic strategy.

Keywords

Breast cancer; Tumor microenvironment; Stromal fibroblast; Epigenetics; DNA methylation; Chromatin remodeling

1. Introduction

Breast cancer is a common malignancy among females in most western countries, where women have an overall lifetime risk of >10% for developing invasive breast cancer. It is not a single disease, but rather is composed of distinct subtypes associated with different clinical outcomes and is highly heterogeneous at both the molecular and clinical levels [1]. Although tumor initiation and progression are predominantly driven by acquired genetic alterations, our recent data suggest that microenvironment-mediated epigenetic perturbations may play a role in neoplasm development [2].

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The origin of breast carcinomas lies in the epithelial linings of glands or ducts, rather than in the adjacent stroma. Stroma, or the supportive platform, is composed of fibroblasts, endothelial cells, smooth muscle cells, adipocytes, inflammatory cells, and nerve cells. This complicated network of various cell types, as well as the associated factors that are secreted, is collectively known as the microenvironment. Whether and how the tumor microenvironment influences breast neoplasms by altering epigenomes is increasingly gaining the attention and interest of researchers.

Epigenetics is the study of DNA modifications and its associated histone proteins, which do not change the primary DNA sequence, but serve a critical role in regulating the dynamics of gene expression (review in [3,4]). To date, one of the best-characterized epigenetic alterations is DNA methylation, which converts the cytosine of CpG residues to 5-methylcytosine. This chemical occurrence is mediated by DNA methyltransferases and occurs in CpG-rich stretch sequences, known as CpG islands, which exist in 60–70% of the promoter or in the first exon of known genes (review in [3,4]).

Besides promoter methylation, regional modifications of chromatin also exert a similar effect of controlling gene activity in cancer cells. N-terminal tails of histones undergo post-translational modifications, including acetylation, phosphorylation, ubiquitination, and methylation [5]. With a few exceptions [6,7], acetylated lysine is commonly associated with active gene transcription, while methylation of histone H3 on lysine 27 frequently instructs the target genes to undergo silencing [8–10], an event mediated by polycomb repressors which recruit DNA methyltransferases [11]. The subsequent acquisition of DNA methylation may then warrant an irrevocable suppression of the target gene. As a result, the interplay between chromatin remodeling and DNA methylation conveys combinatorial alterations and imprints differential degrees of gene silencing. Most importantly, epigenetic markings are mitotically heritable in progeny cells [12], suggesting that they can exert long-term repression without the genetic mutations that are traditionally required.

As aberrations within the epigenome have been proven to play important roles in breast tumorigenesis, there is an urgent need for a better understanding of possible mechanistic causes leading to epigenomic shifts, particularly from the cancer-associated stroma (“soil”). This article delves into the prospect of approaching a potential treatment regimen by targeting the tumor microenvironment. Having highlighted the potential advantages of taking this approach, we believe that more efficient and valuable therapeutic strategies can be further developed along with a whole array of advancement possibilities lying ahead, in order to combat the effects of tumor microenvironment.

2. Epigenetic perturbations in breast cancer

In human cancers, aberrant epigenomes are known to contribute to various phases of neoplastic development, including initiation, invasion, metastasis, and chemotherapy resistance (review in [13,14]). Epigenome-controlled loci include repair genes (*MLH1*, *GST3*), cell cycle inhibitors ($p16^{INK4a}$, *p15*, $p14^{ARF}$), tumor suppressor genes (*VHL*, *BRCA1*), tissue remodeling enzymes and structures (*TIMP3*, *E-cadherin*), and receptors (estrogen receptor) (reviewed in [15,16]).

Epigenetic silencing has been shown to augment various pre-neoplastic and malignant phenotypes. For example, using an unbiased global screen for aberrant CpG island methylation, Tlsty et al. have identified a non-randomized pattern of DNA hypermethylation in $p16^{INK4a}$-deprived cells [17]. Hypermethylation of a $p16^{INK4a}$ promoter was observed in a subpopulation of primary human mammary epithelial cells that would have undergone senescence after long-term cultivation [18,19]. Lack of $p16^{INK4a}$ activity, however, conveyed growth capabilities extending past the normal proliferation barriers. Furthermore, depletion of $p16^{INK4a}$ resulted

in the upregulation of polycomb repressors (EZH2 and SUZ12) which subsequently recruit DNA methyltransferases to a target gene, *HOXA9*, leading to hyper-methylation of its promoter [20]. Interestingly, *HOXA9* is expressed during normal breast development but is commonly silenced in breast cancers by an epigenetic control. Moreover, in the absence of $p16^{INK4a}$ expression but with continued proliferation, cells hold great potential for acquiring eroding telomeric sequences and centrosomal dysfunction. As a result, this subpopulation of cells is allowed to freely accumulate chromosomal abnormalities and mutations. Depletion of $p16^{INK4a}$, therefore, allows for the acquisition of multiple genetic changes necessary to oncogenic evolution and triggers the initiating steps of carcinogenesis. These results depict a causal role for $p16^{INK4a}$ disruption in modulating DNA hypermethylation, and identify a dynamic and active process whereby epigenetic modulation of gene expression appears to be an early event in breast tumor progression [17].

In addition to regional allele-specific hypermethylation, DNA in cancer cells experiences genome-wide hypomethylation. The perturbation of which is associated with gene reactivation, chromosomal instabilities, upregulation or overexpression of proto-oncogene transcription, increased recombination and mutations, loss of imprinting, and reactivation of transposable elements [21,22]. Interestingly enough, hypomethylation was observed in colorectal carcinoma cells that underwent hypoxia treatments [23]. Likewise, both hypomethylated and hypermethylated loci co-exist in colorectal and breast cancers (review in [24]). To this end, methylation-mediated E-cadherin loss in human breast cancer has been shown to be heterogeneous and unstable, characterized by coexistence of methylated and unmethylated entities at this promoter, throughout various stages of breast neoplasm [25]. Together, data indicate that epigenetic plasticity may contribute to this heterogeneity and drive metastatic progression of breast cancer in response to the dynamic tumor microenvironment.

On the other hand, a mounting body of evidence has demonstrated that histone modification alone, or its synergistic interactions with aberrant promoter methylation, can regulate gene activity. In the former occurrence, epigenomic silencing was attained solely by histone modification without detectable promoter methylation [6,7]. For example, Hinshelwood et al. observed that suppression of TGF-β-regulated downstream target genes was not associated with DNA methylation, but with chromatin remodeling involving a decrease in histone H3 lysine 27 trimethylation and an increase in histone H3 lysine 9 dimethylation and deacetylation [26]. However, in a separate incidence, Dumont et al. demonstrated that histone remodeling not only preceded but also persisted throughout the duration of promoter methylation of *E-cadherin* in breast epithelial cells undergoing epithelial to mesenchymal transition, induced by over-expression of oncogene *ras* and cultured in serum-rich media [27].

To date, the causal mechanism that initiates epigenomic alterations remains poorly understood. Yet we recently observed that epigenetic perturbations in breast epithelial cells could be induced by the surrounding tumor stromal fibroblasts, *via* an AKT1 kinase-induced mechanism ([2] and section 6 of this review).

3. Roles of tumor microenvironment in breast neoplasm

Over the past 2 decades, the majority of cancer-related studies have focused on examining the functional consequences of activating and/or inactivating mutations in critical genes involved in cell cycle control or apoptotic regulation. These studies have provided great insight into the functions of oncogenes and tumor suppressor genes and the signaling pathways that regulate cell proliferation and/or cell death. Nevertheless, they have largely ignored the fact that cancers are heterogeneous cellular entities whose growth are dependent upon reciprocal interactions between genetically altered initiated cells (“seed”) and the dynamic stromal microenvironment (“soil”) in which they reside.

3.1. Disrupted tumor microenvironment promotes tumor growth

Decades ago, using skin [28] or bladder [29] tissues, investigators observed that enhanced tumor formation could be induced if carcinogen-treated stroma was heterotypically grafted with untreated epithelial cells. Likewise, in an animal model, neoplastic transformation was achieved only when stromal fibroblasts were previously exposed to the carcinogen *N*-nitrosomethylurea [30]. Furthermore, in the irradiated stroma (with cleared fat pads devoid of epithelial cells), mammary epithelial cells were able to fully develop malignant phenotypes and progressed into fast-growing tumors with a size greater than the same cells transplanted into un-irradiated counterpart stroma [31,32].

3.2. Aberrant extracellular matrix (ECM) affects breast tumorigenesis

Among microenvironment constituents, the ECM is a key component secreted by stromal cells and is situated in a position of close contact with tumor cells. It functions as a critical source for growth, survival, motility, and angiogenic factors that significantly affects tumor behavior and progression. Perturbations in the production, deposition and degradation of the ECM present during neoplastic transformation and progression have been reported to arise from alterations in the stromal response [33]. Recently, the contribution of ECM alterations to tumor development and growth was examined. Using a three-dimensional culture assay developed with reconstituted basement membrane, Weaver and colleagues [34,35] demonstrated that the malignant phenotype of human breast cancer cells could be reversed by correcting ECM-integrin signaling. When this integrin switching was reversed, proliferation was controlled, morphogenesis was restored, and tumorigenesis was dramatically reduced, despite the fact that genetic abnormalities persisted [35]. These data suggest that appropriate integrin signaling is a critical microenvironmental effector that can act dominantly by overriding genetic constraints in the epithelium, to suppress the expression of the malignant phenotype.

3.3. Stromal cells influences breast tumorigenesis

Besides the ECM, there is increasing evidence indicating that tumors actively recruit stromal cells including inflammatory cells, vascular cells, and fibroblasts [36–40], into tumor masses. This recruitment is essential for the generation of a tumor microenvironment that actively fosters tumor growth.

The fibroblast is the major cell type of the stromal compartment that is closely involved in orchestrating the stromal portion of the dialog with the epithelium in maintaining tissue homeostasis [41]. Fibroblasts are responsible for the elaboration of most connective tissue components in the ECM, including collagens and structural proteoglycans, as well as various classes of proteolytic enzymes, their inhibitors, and a variety of growth factors. As each organ has specialized requirements, fibroblasts from different organs demonstrate organ-specific variations in the classes of biologically active molecules that they express [42].

Historically, fibroblasts were thought to be passive participants in the neoplastic programming of tissues. However, alterations in stromal fibroblasts adjacent to transformed epithelial cells have been documented in several tumor systems [43–45]. These include aberrations in growth characteristics, migratory potential, and altered expression of growth factors [43–45]. Cancer associated fibroblasts (CAFs) isolated from malignant tissues exhibit altered characteristics, most notably the enhanced production of collagens, hyaluronate, epithelial growth factors, and disorganized patterns of growth as well as enhanced proliferation [46].

The tumor-supportive role that fibroblasts play has recently been proven. For instance, deletion of the type II TGF-β receptor in fibroblasts in mice [39,47] and carcinogen treatment of mammary fat pad stroma in rats promoted tumor initiation and progression [30]. Likewise, Ulrich, et al. implicated microenvironmental changes in tumorigenesis, as inflammation is

primarily a stromal reaction [48]. In support of these observations are the findings that irradiated, senescent, cancer-associated, or inflammatory fibroblasts promote tumor growth more effectively than normal fibroblasts [49–51]. Similarly, low-dose ionizing radiation-induced senescence-like fibroblasts significantly perturbed the mammary stromal microenvironment and sustained full expression of malignant potential in the resident breast carcinoma cells *in vitro* [32].

That fibroblasts exert an active role in tumorigenesis has been observed in multiple systems [52]. In combination with inflammatory cells, CAFs can promote neoplastic programming of tissues [53]. Likewise, when CAFs were grafted with immortalized (but non-tumorigenic) human prostatic epithelial cells, the interaction resulted in tumors that exceeded the weight of control grafts by five hundred-fold [36]. Interestingly enough, isolation of resultant human epithelial cell populations from these tumors (devoid of fibroblasts) and subsequent grafting into animals demonstrated that the epithelial cells were then able to form tumors in which the contributing activity from CAFs was no longer necessary [36]. In that instance, oncogenic signals from CAFs conferred nonrandom chromosomal changes and subsequently promoted nontumorigenic cells toward a malignant state.

The molecular mechanism revealing how CAFs play the tumor-promoting role has begun to be uncovered. Orimo et al. showed that CAFs promote the growth of oncogene-expressing breast cells in mice far more effectively than normal mammary fibroblasts derived from the same patients [40]. This growth-promoting effect is attributed to the secretion of stromal cell-derived factor 1 (SDF-1) that acts on the cognate receptor (CXCR4) expressed by carcinoma cells and subsequently promotes angiogenesis by recruiting endothelial progenitor cells into carcinomas [40]. Taken together, these findings suggest that oncogenic signals from CAFs can stimulate development of neoplastic properties and establish an active role in turmorigenic processes, rather than acting merely as passive participants.

4. Aberrant microenvironment influences cell plasticity and conveys epigenomic perturbations

The importance of the microenvironment and context in control of cellular differentiation and tissue polarity has been illustrated [54–56]. An *in vitro* model of differentiation that encompasses human normal mammary epithelial cells can form polarized and hollow tissue structures (acini) when cultured in the presence of basement membrane components, a technology known as a three-dimensional culture [54,57–59]. Acinar morphogenesis is accompanied by chromatin remodeling, along with an increase in expression of MeCP2 (a mediator of DNA-methylation-induced gene silencing), suggesting that DNA methylation is a mechanism by which mammary epithelial differentiation is coordinated at both the cellular and tissue levels [55]. In addition to DNA methylation, chromatin remodeling was evidenced by sensitivity to AluI digestion, in which the malignant cells resisted digestion relative to nonmalignant cells. Treatment of T4-2 breast cancer cells in a three-dimensional culture with cAMP analogs or with a phosphatidylinositol 3-kinase inhibitor not only reverted their phenotype from nonpolar to polar acinar-like structures, but also enhanced chromatin sensitivity to AluI [60]. Introduction of cAMP analogs or inhibitory antibody sequestering fibronectin resulted in phenotypic reversion, polarization, and a shift in DNA organization acting through a cAMP-dependent protein-kinase A-coupled signaling pathway.

A similar observation revealing epigenome can be influenced by microenvironment was recently reported [27]. In that report, immortalized human mammary epithelial cells with repressed *p16INK4A* but excessively expressed oncogenic *ras* (known as vHMEC-ras) was subjected to epigenomic perturbations if cells were grown in serum-rich media, a condition that can induce a gene expression pattern similar to that of a wound response. Here, the resultant

cells experienced histone modification, gained *de novo* DNA hypermethylation at targeted genes frequently silenced in basal-like breast cancer cells, became more motile, and underwent phenotypic changes indicative of epithelial to mesenchymal transition [27]. Together, these findings demonstrate that the architecture of epigenome is highly plastic and reveal the concept that modifying the tumor microenvironment, such as specified growth in serum-rich media, can alter the epigenome organization and render aberrant DNA methylation in tumor cells.

5. Epigenomic perturbations in stroma, in relation to cancer cells

How the microenvironment differs between normal and cancer tissues is attracting growing research attention. Recently, Kurose et al. observed that genetic alterations occurred in the tumor stroma without an equivalent change in cancer cells [61]. Similarly, Hu et al. showed distinct epigenetic changes in cultured epithelial and myoepithelial cells and in stromal fibroblasts from normal breast tissue and breast carcinomas [62], suggesting that aberrant epigenomes in stroma are unique and discrete from their associated carcinoma cells. However, in HER-2/neu-positive cancers, aberrant DNA methylation is found not only in the stroma, but also in the associated cancer cells [63]. Of the five genes methylated in carcinoma cells, two loci were concordantly methylated in the stroma. Both genes are involved in estrogen metabolism: (a) estrogen receptor *PGR*, and (b) 17-β-estradiol metabolizing enzymes *HSD17B4* [63]. Silencing of these two loci may account for inhibition of the anti-tumor activities intrinsic to tamoxifen. This implies that HER-2/neu cancer cells interact with the surrounding stroma and subsequently result in a “memory” by means of epigenetic imprints. However, the question of which cell type initiated the aberrant methylation remains unresolved.

6. Tumor stromal fibroblasts conveyed epigenetic silencing in breast epithelial cells

The initiator commanding the acquisition of promoter hyper-methylation in breast carcinoma cells remains poorly studied. Identifying these originators is critically important and should provide a better understanding of how the tumor microenvironment controls gene silencing in the surrounding pre-neoplastic and malignant cells. To this regard, our laboratory has developed a two-dimensional *in vitro* coculture system to decipher whether and how the tumor microenvironment conveys epigenetic gene silencing. We have observed that, by augmenting DNA methylation, fibroblastic signals exerted from CAFs can convey epigenetic silencing of tumor suppressor *Cystatin M* (known as *CST6*) and other genes, in the neighboring normal epithelial cells, namely MCF10A (Fig. 1) [2]. *CST6* was recently characterized as a tumor suppressor gene for breast cancer [64] and its epigenetic silencing was observed in breast cancer cell lines as well as distally metastasized lesions [65,66]. Our data, therefore, provides a proof-of-principle, depicting that CAFs induce hypermethylation of tumor suppressor loci and subsequently lead to gene silencing in the contacted breast epithelial cells. As a result, loss of *CST6* activity advances breast tumorigenesis and/or progression to metastasis.

Furthermore, we reported that the signaling pathway leading to hypermethylation of *CST6* is induced by the activated serine/threonine kinase AKT1/PKB pathway. Activation of AKT1 signaling not only conveyed DNA hypermethylation but also recruited DNA methyltransferase and repressive histone marks to the promoter of *CST6*, events which together contribute to epigenetic silencing [2]. The AKT1 kinase pathway in cocultured MCF10A cells can be aberrantly activated by being placed in contact with cancer-associated fibroblasts for a period as short as 1 week (Lin et al., unpublished data). In fact, elevated AKT1 kinase signaling pathway remarkably correlated with hypermethylation at the *CST6* promoter in primary breast tumors, demonstrating that our *in vitro* coculture model is able to closely simulate the *in vivo* occurrences observed in tumors [2].

Our findings are in agreement with those of others. It has been reported that normal fibroblasts impede or prevent tumor formation while the CAFs promote tumorigenesis [37,67]. Likewise, coculture of premalignant breast cells with normal fibroblasts resulted in only weak induction of epithelial growth and morphogenesis, but similar cocultures with benign or tumor-derived fibroblasts conveyed an induction of highly proliferative ductal-alveolar morphogenesis [68]. Interestingly, besides inhibiting morphologic transformation of pre-malignant breast cells, reduction mammoplasty-derived fibroblasts were also found to have the ability to suppress estrogen responsiveness of premalignant breast cells [68]. In that respect, breast fibroblasts derived from normal or tumor tissues have the ability to override and accentuate the genetic constraints imposed by the epithelial cells [68].

7. Future clinical applications

In conclusion, these intriguing findings not only indicate that genetic and epigenetic alterations in the stroma significantly contribute to neoplastic phenotypes, but also present the novel concept that stromal–epithelial interactions play important roles in the development and progression of breast tumorigenesis. Although the current observations merely uncover the tip of the iceberg, they hold the potential to evolve novel therapeutic regimens that antagonize the tumor-promoting effect provoked from stromal cells or from ECM. For example, as we have observed that the tumor microenvironmental niche from CAFs activates the AKT/PKB pathway and confers epigenetic imprinting within the breast epithelial cells [2], development of a therapeutic strategy by abrogating this signaling pathway may reverse hypermethylation of *CST6* and prevent metastatic spread. Clinical benefits resulting from anti-tumor microenvironment therapy may not be too far away from being a practical occurrence.

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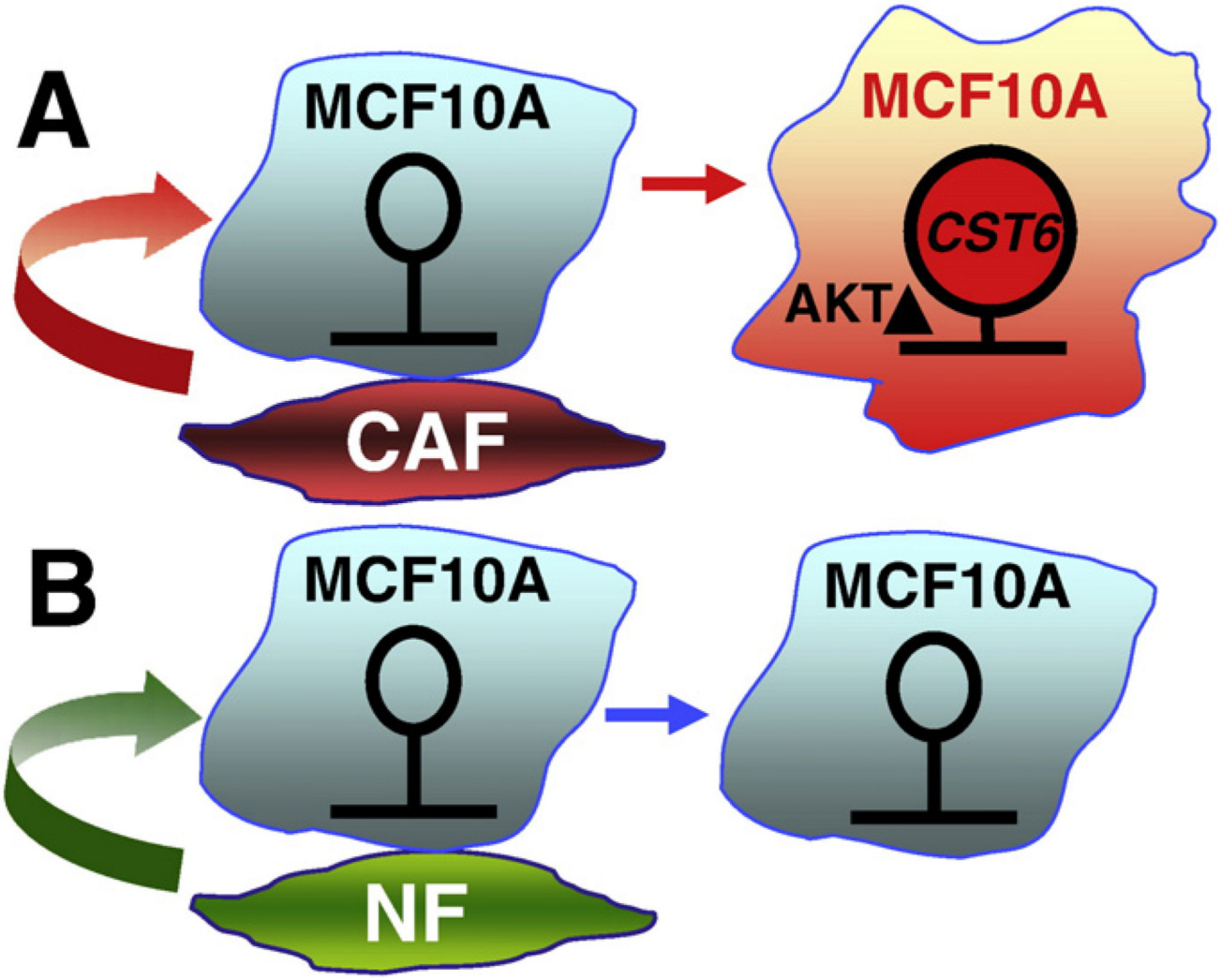

Fig. 1.

An epigenetic model depicting the influence of breast cancer-associated fibroblasts on the non-cancerous breast epithelial cells (MCF10A). (A) After cocultured with cancer-associated fibroblasts (CAF), *CST-6* (and perhaps other breast cancer-associated genes) became hypermethylated and silenced (marked in red). Epigenetic perturbation was mediated by an activation of AKT1 signaling pathway. (B) In contrast, exposure to normal fibroblasts (NF) confers negligible levels of epigenetic perturbations and AKT1 kinase activation in the same MCF10A cells (marked in light blue).